Comprehensive utilization of microalgae (*Chlorella vulgaris*) and the application of its product

by

Yichao Ma

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2019

Keywords: microalgae, radio frequency heating, biodiesel, response surface methodology, saccharification, biodegradable packaging film

Approved by

Yifen Wang, Chair, Professor, Biosystems Engineering Department
Yi Wang, Co-Chair, Assistant professor, Biosystems Engineering Department
Timothy McDonald, Professor, Biosystems Engineering Department
Allen Davis, Professor, School of Fisheries, Aquaculture and Aquatic Sciences

Abstract

Rapid growth, fixation of carbon dioxide, and little competition with food plus some species possessing high lipid content make microalgae a suitable feedstock to produce biodiesel. However, high cost related to dehydration, lipid extraction and conversion to biodiesel hampers industrialization of biodiesel production from microalgae. The objective of this study was to develop a biodiesel production procedure directly from wet microalgae. The system was operated under atmospheric pressure without requiring any specific apparatus. Wet microalgae (*Chlorella vulgaris*) was pretreated by radio frequency (RF) heating to disrupt cell walls first, followed by esterification and transesterification with relatively small amount of methanol and catalyst (either HCL or NaOH) assisted by RF heating at 55 °C for only 20 min. The fatty acid methyl esters (FAME) yield reached as high as 79.5 ± 3.0%. The two major factors impacting biodiesel yield are catalyst and methanol. The SEM images visually verified the significant effect of cell disruption by the pretreatment. Then, the procedure for biodiesel production was optimized using response surface methodology (RSM). A three-variable, five-level central composite design (CCD) was employed to evaluate the effects of three key parameters, i.e., HCl to MeOH ratio (v/v), MeOH volume and RF heating time. An optimized point was successfully found. The best predicted FAME yield of 93.1% was obtained at HCl to MeOH ratio of 4.27 (v/v), MeOH volume of 28.5 mL and RF heating time of 19.2 min. Experiments carried out at the optimized point resulted in 92.7 ± 0.1% yield, which validated the reliability of the prediction model. All processing steps, including cell destruction, esterification and transesterification, were

carried out under temperatures below 100 °C and atmospheric pressure. Therefore, no pressure-proof nor high-temperature apparatus was required. The procedure shows great potential for industrial application because of its high FAME yield, simple operation, low chemical consumption and short processing time. The principles of the procedure can also be applied to other microalgae with high lipid contents.

Besides lipid, there was also high content of carbohydrate in *Chlorella vulgaris*. In this study, the feasibility of comprehensive recovery of lipid and carbohydrate in wet microalgae *Chlorella vulgaris* was explored. First, four sets of enzyme combinations of α-Amylase, Amyloglucosidase and CTec2 were evaluated for hydrolysis efficiency on microalgae disrupted with radio frequency heating. Then, the most suitable combination was applied to raw microalgae and microalgae residual after biodiesel production, respectively, for saccharification. Adsorption kinetics of the optimized enzyme combination on the aforementioned three samples were determined and adsorption isotherm was analyzed by Freundlich equation. Morphology of microalgae was also investigated by scanning electron microscopy. A yield of reducing sugars in microalgae residual at 53.7% was obtained after 72 hours saccharification. The results from enzyme adsorption kinetics, isotherm and SEM images were consistent with each other. This study demonstrated that the microalgae residual after biodiesel production could be used as carbohydrate feedstock for fermentable sugar production through simple enzymatic hydrolysis.

The fermentable sugar could be used for the production of Poly (hydroxybutyrate) (PHB), which is one of the biodegradable plastic material. In this study, Poly (lactic acid)-Poly (hydroxybutyrate) (PLA−PHB) based films containing bioactive elements were developed and

characterized. Seven formulations containing different contents of plasticizers (mono-caprylin glycerate (GMC) or glycerol monolaurate (GML)) were initially developed. After basic mechanical property tests, the PLA-PHB based films with 0.5% GMC or GML were selected as two formulations for further study. In the subsequent experiments, 5% cinnamaldehyde (CIN) was added into each of the two formulations selected and EVOH copolymer (widely used non-biodegradable packaging as control here) respectively. More mechanical and active properties were investigated. The results showed that PLA-PHB based films possessed some better mechanical properties than those of EVOH based film, and better active properties when applied to high lipid food simulant. In a preservative test, changes in total bacterial counts (TBC), thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N) were carried out on salmon dices packed with films and stored at 4±1 ℃. It was only 4.65 CFU/g on day 17 for the salmon packed with PLA-PHB based film with GML as plasticizer meanwhile the TBC of salmon dices sealed in other two films reached 6.65 and 6.35 CFU/g respectively on day 15. This study showed that it could be feasible to use biodegradable active packing as an alternative to replace non-biodegradable packaging for chilled salmon.

Acknowledgments

First and foremost, I would like to express my great appreciation and respect to my advisor Dr. Yifen Wang for his continuous advice, guidance, and support during my doctoral program in Auburn University and my master's study in Shanghai Ocean University. Dr. Wang helped me extend my scientific thinking, develop some important skills including critical thinking, hypothesis development, data analysis, report writing, and effective presentation.

I am also grateful to my co-advisor Dr. Yi Wang, committee members, Dr. Timothy McDonald, and Dr. Allen Davis. Their support and suggestions have greatly improved my research work and enhanced my confidence significantly. I would have never been able to finish my projects and dissertation without the guidance, encouragement and help from them. Their exemplification to scientific work will be beneficial to the rest of my career.

I would like to extend my thanks to Dr. Philippe Gaillard in the Department of Mathematics and Statistics for his agreement on being my university reader. I am also indebted to him for his support and discussions in the experimental data analysis. I would also like to show my great appreciation to Dr. Shaoyang Liu in Troy University, who helped me a lot in experimental design and chemical analysis.

I would like to thank my fellow researchers and good friends Dr. Junping Zhuo, Dr. Yuechao Ma, Dr. Haijiao Wang, Dr. Pixiang Wang, Dr. Xiaofei Wang, Qichen Wang, Shuang Wu, Wenxu Xia and many others. All of them have always be willing to assist me. I cannot

include all their names here but my research would not be completed without their friendship and support.

Last but not least, I am particularly grateful to my father, Mr. Liangcheng Ma, mother, Mrs. Yonghao Guo, my grandparents and all my family for their endless love, trust, great support and encouragement.

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List of Abbreviations

ABE	Acetone–Butanol–Ethanol
ANOVA	Analysis of Variance
AzCATI	Arizona Center for Algae Technology and Innovation
CCD	Central Composite Design
CIN	Cinnamaldehyde
EM1	Enzymatic Method One
EVOH	Ethylene Vinyl Alcohol
GMC	Mono-Caprylin Glycerate
GML	Glycerol Monolaurate
GRAS	Generally Recognized as Safe
FAMEs	Fatty Acid Methyl Esters
FAU	Fungal Amylase Unit
FFAs	Free Fatty Acids
FID	Flame Ionization Detector
HPAEC	High-performance Anion-exchange Chromatography
LB	Luria-Bertani
LPS	Lipopolysaccharides
MDA	Malondialdehyde
MW	Microwave

NREL	National Renewable Energy Laboratory
OEO	Oregano Essential Oil
OP	Oxygen Permeability
PAD	Pulsed Amperometric Detection
PBAT	Poly (Butylene Adipate Co-Terephthalate)
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
$P_{34}HB$	Poly (3-Hydoxybutyrate-Co-4-Hydroxybutyrate)
PLA	Poly Lactic Acid
QS	Quantitative Saccharification
RF	Radio Frequency
RFMA	RF Heating Disrupted Microalgae
RH	Relative Humidity
RID	Refractive Index Detector
RSM	Response Surface Methodology
RSMA	Microalgae Residual after Biodiesel Production
RWMA	Raw Microalgae
SEM	Scanning Electron Microscope
SHF	Separate Hydrolysis and Fermentation
SL	Saponifiable Lipid
SSF	Simultaneous Saccharification and Fermentation

TAGs	Triacylglycerols
TBA	Thiobarbituric Acid
TBC	Total Bacterial Counts
TCA	Trichloroacetic Acid
TGA	Thermogravimetric Analysis
TSB	Tryptic Soy Broth
TVB-N	Total Volatile Basic Nitrogen
WVTR	Water Vapor Transmission Rate

Chapter I Literature Review

1. Introduction

The limitation of fossil fuel has become a threat of energy security worldwide, and oil reserves are predicted to be exhausted by 2050. So there is an urgent need to find sustainable energy resources (Ho et al., 2013b). Biodiesel is one of the most promising alternatives to fossil fuel derived diesel fuel. It is well known by its high flash point, excellent biodegradability and renewability. Compared with petroleum diesel, biodiesel is more eco-friendly. It produces much less greenhouse gas and can be used in diesel engines without additional modification (Knothe, Sharp, & Ryan, 2006; Zabeti, Daud, & Aroua, 2009). However, the usage of terrestrial oil crops such as soy bean, oil palm etc. will compete with food, therefore, a feedstock with very little competition with food is necessary.

1.1. Algae, microalgae and genus of *Chlorella*

It's estimated there are one to ten million species of algae with most of them are microalgae in the world and fast growth, make algae a promising source of bioenergy and biomaterials (Laura Barsanti, 2014). Among them, most species are microalgae. The advantages to use microalgae are their fast growth rate, high lipid content for some species, fixation of CO_2 and no or less competition with food (S. B. Velasquez-Orta, Lee, & Harvey, 2013). Mata et al. (Mata, Martins, & Caetano, 2010) reported that most common lipid containing microalgae has 20 and 50% lipid by weight of dry mass with the maximum of 75% found in *Botryococcus braunii*. The

most common microalgae are *Chlorella, Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis, Nannochloris, Nannochloropsis, Neochloris, Nitzschia, Phaeodactylum, Porphyridium,Schizochytrium,* and *Tetraselmis.* There are few review papers discussing on either how to use microalgae as feedstock to produce biodiesel or biofuel (mostly, bioethanol). This review article will discuss on the comprehensive utilization of lipid, starch and both in microalgae with focus on *Chlorella* genus.

According to Wikipedia, *Chlorella* is a genus of single-celled green algae belonging to the division *Chlorophyta*. There are at least 13 species under the genus of *Chlorella: C. autotrophica, C. colonials, C. lewinii, C. minutissima, C. pituita, C. pulchelloides, C. pyrenoidosa, C. rotunda, C. singularis, C. sorokiniana, C. variabilis, C. volutis and C. vulgaris* (Wikipedia). *C. vulgaris* is estimated to have survived on the earth for more than 2.5 billion years with a remarkable feature of growing fast (Wiki), although it was first discovered and reported by a Dutch microbiologist and botanist, Dr. Martinus Willem Beijerinck with a color illustration in 1890 (Beijerinck, 1890).

2. Utilization of lipid

Chlorella is the most studied microalgae for its usage of production of biodiesel.

2.1. Determination of the lipid composition

The widely used method to determine lipid is based on NREL (Van Wychen, Ramirez, & Laurens, 2016). After harvest, the algae are centrifuged to remove extra water. Then, the algae are freeze dried and weighed. The lipid composition is determined as fatty acid methyl esters (FAMEs) through the direct transesterification method using a GC equipped with an FID detector.

2.2. Dried microalgae as feedstock

In the early 2000, dried microalgae were used as feedstock. There are two pathways: 1) dried microalgae—lipid extraction— transesterification and 2) dried microalgae—*in situ* transesterification including lipid extraction (S. Velasquez-Orta, Lee, & Harvey, 2012).

2.2.1. Fractional step methods

The typical steps on production of biodiesel from algae in the early 2000 are harvesting, drying, lipid extraction with solvent (normally hexane), and transesterification at the presence of alkaline (normally NaOH) (Vijayaraghavan & Hemanathan, 2009). Some studies used fermentation method to replace chemical transesterification. *Chlorella protothecoides* was vacuum dried at 40 °C before lipid extraction using Soxhlet extractor method with *n*-Hexane as the standard Soxhlet solvent (Li, Xu, & Wu, 2007). Extraction was then fermented using immobilized lipase from Candidia sp. 99–125 to produce FAME. A conversion rate of 98.15% from oil to monoalkyl esters of fatty acids was achieved at 38 °C in 12 h.

2.2.2. One step methods

In order to reduce steps (number of unit operation) and simplify production of biodiesel from microalgae, subsequently to reduce the cost, studies started to report biodiesel production from dried microalgae using *in situ*/direct transesterification. In previous study, the fatty acid methyl ester (FAME, biodiesel) production conditions from *Chlorella vulgaris* microalgae were optimized by using alkali-catalyzed *in situ* transesterification (S. Velasquez-Orta et al., 2012). The catalyst (NaOH) to lipid molar ratios was 0.15:1, and the methanol to lipid ratio was 600:1. It is also reported by using spray-dried microalgae first, then produced biodiesel via *in situ* methanolysis with a solvent to sample ratio of 204 mL: 2 g (Carvalho Júnior, Vargas, Ramos, Marino, & Torres, 2011). Although these studies have reported high FAME yields, they required dried algae and large amounts of solvent and catalyst.

2.3. Wet microalgae as feedstock

No matter what format of drying, such as hot air, spray dry, or freeze dry, the drying process is an energy intensive unit operation (Y. Ma et al., 2019). It takes about 2,260 kJ/kg of water to evaporate at 100 °C under 1 atm. In order to avoid the unite operation of drying, production direct biodiesel production from wet algae attracts more and more attention in recent years (C. L. Chen et al., 2015; Z. Chen, Wang, Qiu, & Ge, 2018; Macías-Sánchez et al., 2015; Macías-Sánchez et al., 2018).

2.3.1. Fractional step methods

Saving huge energy from drying operation is good, however, lipid extraction from wet microalgae faces new challenges that are not existing for the dried biomass. Normally, non-polar organic solvents including hexane and chloroform are used to dissolve lipids from biomass. However, water in wet algae reduces extraction yield of lipid due to the immiscibility of water and non-polar organic solvents (Sivaramakrishnan & Incharoensakdi, 2018). Another large challenge for microalgae (no matter dried or wet) is that they have a tough cell wall. For example, traditional solvent (*n*-hexane) method and supercritical CO_2 method were employed to extract lipid from enzymatically (Lysozyme and cellulose) disrupted *Scenedesmus sp*. (Taher, Al-Zuhair, Al-Marzouqi, Haik, & Farid, 2014). In previous study, five cell disruption methods on hydrated microalgae (*Botryococcus sp., Chlorella vulgaris, and Scenedesmus sp.*) were compared (J. Y. Lee, Yoo, Jun, Ahn, & Oh, 2010), namely, (1) autoclaving at 125 °C with 1.5 MPa for 5 min, (2) bead-beating using a bead beater at a high-speed of 2800 rpm for 5 min, (3) microwaves using a microwave oven at about 100 °C for 5 min, (4) sonication at a resonance of 10 kHz for 5 min, and (5) osmotic shock using a 10% NaCl solution with a vortex for 1 min and maintained for 48 h. Then, lipid extraction was conducted using a solvent mixture of chloroform–methanol (1:1 v/v). They concluded microwave heating interruption was simplest, and most efficient method for extraction of lipid from wet microalgae.

2.3.2. One step methods

In order to further reduce the number of unit operation, consequently reduce the energy consumption and production time, concurrent extraction and reaction also known as direct transesterification or *in-situ* transesterification is becoming more and more attractive. However, the presence of water has a significant negative impact on transesterification reaction (Cao, Zhang, Wu, & Miao, 2013; C. L. Chen et al., 2015). Studies have been carried out under high temperature and pressure with special container to overcome this challenge. (B. Kim, Im, & Lee, 2015; Park, Kim, Chang, & Lee, 2017; Skorupskaite, Makareviciene, & Gumbyte, 2016). Macías-Sánchez et al. (2015) investigated the production of FAME from wet microalgae (25 wt% dry biomass) in a 5 L thermostatic stirred pressure proof reactor and found the optimized reaction conditions were at 100 ºC for 105 min with a pressure of 2.5 atm. Pan et al. enhanced the lipid extraction efficiency from wet *Chlorella sp.* (33-35 wt% dry biomass) by using deep eutectic solvent in an autoclave reactor (Pan et al., 2017). In Im et al. (2014)'s study, the reactions were carried out in a 14 mL pressure proof Teflon-seal tube. But these critical conditions and special apparatus limit the upscale potential for the commercial production, especially for the continuous process.

2.3.3. Improved methods

Due to the demand of a huge amount of energy for drying microalgae, the direct processing of wet algae for value-added biofuel and biochemical production attracts more and more attention in recent years. However, the presence of water has a significant negative impact on the

conversion reaction. To obtain desirable reaction efficiency, various strategies have been employed in previous studies including the harsh pretreatment, high methanol to algae ratio, high catalyst to microalgae ratio, extreme high temperature and long time, or super critical method. In this study, they have developed a biodiesel production procedure directly from wet microalgae. The key innovations in the study are: 1) all steps involved in this study (including cell destruction, esterification and transesterification) were carried out under normal temperature and normal pressure; 2) no specific apparatus was required during the process; 3) wet algal biomass was processed directly without any drying or any other pre-processing involved; and 4) besides the biodiesel production, the carbohydrate residual of the process could be further used for production of other bio-products. This is the first report on direct biodiesel production from microalgae assisted by radio frequency (RF) heating so far.

3. Utilization of carbohydrate

There are fewer studies on utilization of carbohydrate as compared to that of lipids. Studies reporting fermentable sugars production using carbohydrate-rich microalgae have become more prevalent because microalgae is considered the third generation biomass to replace starch and lignocellulosic feedstock (normally known as the first and the second generation feedstock) for fermentable sugars (Ho et al., 2013a; K. H. Kim, Choi, Kim, Wi, & Bae, 2014). Carbohydrate exists mainly in microalgae either in the form of cellulose without lignin and hemicellulose in their cell wall, or in the form of starch in their cytoplasm. Therefore, compared with

lignocellulosic feedstock, it is much easier to convert microalgae carbohydrate into monosaccharides (Ho et al., 2013a, 2013b).

3.1. Determination of the carbohydrate content

3.1.1. Chromatographic analysis method

Recently, most of the studies adopt method on Determination of Structural Carbohydrates and Lignin in Biomass (quantitative saccharification (QS) method) (Sluiter et al., 2008) by the National Renewable Energy Laboratory (NREL) issued in 2008 and revised in 2012 with or without some minor modifications to determine carbohydrate content. The advantage of this kind of method does not need to extract carbohydrates from microalgae before hydrolysis.

In previous study, 100 mg of air-dried microalgae was used to mix with 3ml of 72% (w/w) sulfuric acid and incubated at 30 ℃ for 1 h as the primary hydrolysis. Then, the mixture was diluted to 4% (w/w) sulfuric acid and incubated at 121 ℃ for 1 h as the secondary hydrolysis. Several microalgae including *Chlorella vulgaris* UTEX 395 were measured. The carbohydrates in microalgae were determined by an HPLC, a high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and a GC respectively, and the results were compared with each other (Templeton, Quinn, Van Wychen, Hyman, & Laurens, 2012). Kim et al. (2014) incubated small amount of freezing dried *Chlorella vulgaris* with 72% (w/w) sulfuric acid for 45 min but at room temperature. GC was used to identify and quantify sugars in the microalgae. Ho et al. (2013) also used the two-stage sulfuric acid

hydrolysis, however, they decreased incubating time remarkably and maintained other conditions (temperature and sulfuric acid concentration) unchanged. In the primary hydrolysis, a small amount of dried *Chlorella vulgaris* FSP-E was incubated for 30 min vs. 1 h, and 20 min vs. 1 h in the secondary hydrolysis. The supernatant was neutralized and analyzed following a common sugar assay by an HPLC (Ho et al., 2013b). In another study (Ho et al., 2013a), the same microalgae, *Chlorella vulgaris* FSP-E, was tested for the carbohydrate content. Ho et al. reduced incubating time from 30 min to 20 min in the first stage and maintained the other conditions unchanged.

3.1.2. Spectral analysis method

Spectral analysis of starch content was rapid, simple and accurate (Fernandes et al., 2011). Before spectrophotometer measurement, three acidic and two enzymatic methods were carried out for comparison. In the Acidic Method One, small amount of freeze dried *Chlorella vulgaris* was mixed with acetone and boiling 80% (v/v) ethanol for 8 h to remove interfering compounds such as pigments, soluble sugars and lipids, and then the extracted starch was solubilized with 35% (v/v) perchloric acid for 0.5 h followed by hydrolysis in 72% sulfuric acid with 0.2% anthrone (v/v). The glucose content was measured by a spectrometer at 625 nm. In the Acidic Method Two, 80% (v/v) ethanol replaced acetone and 30% perchloric acid substitute 35% one. Total time of determination of starch was also shortened from 9 h in the Acidic Method One to 2.5 h. In the Acidic Method Three, 1.1% hydrochloric acid at 100°C for 30 min replaced perchloric acid during extraction and solubilization. Enzymatic Method One (EM1) was

designed to determine nonresistant starch. In this method, boiling 80% (v/v) ethanol was used to remove interfering compounds followed by enzymatic hydrolysis using α-amylase and amyloglucosidase. In Enzymatic Method Two which was designed to detect samples containing resistant starch, every factor remained unchanged as that in EM1except that the microalgal biomass was predissolved with 2 M KOH in an ice/water bath after removal of interfering substance and hydrolysis with enzymes. Based on results of these five methods, Fernandes et al. (2011) indicated that EM1 was the rapidest and most accurate method for determination of starch in microalgae. Spectral analysis method is relatively rapid, simple and cheap because spectrometer is used rather than using HPLC in chromatographic analysis. However, accuracy should not be as much as chromatographic analysis. In addition, only glucose content is determined by the spectral analysis method. Therefore, this type of methods were seldom adopted by researchers after 2011 since HPLC becoming more and more popular.

3.2. Saccharification and fermentation

A common method of using starch in microalgae is to first hydrolyze the microalgae from starch into reducing sugars and then ferment the sugars into biofuel such as bioethanol (Ho et al., 2013a) or simultaneously hydrolyze and ferment (Ho et al., 2013a).

3.2.1. Separate hydrolysis and fermentation (SHF)

Under the category of separate hydrolysis and fermentation, hydrolysis (it's also called pretreatment in some studies) is carried out to reduce starch into reducing sugars, followed by a

fermentation with bacteria or yeast (K. H. Kim et al., 2014). Hydrolysis of microalgae is much milder than that for the purpose of determination of the carbohydrate content. Hydrolysis of starch normally is carried out through three main methods: chemical, enzymatic, and physical hydrolysis. Some studies also combine two of these three methods. Studies on production of biofuel, especially bioethanol from microalgae, especially genius of *Chlorella* are reviewed and summarized in Table I-1.

Chemical hydrolysis including acidic and alkali methods is normally considered fast, simple and cheap, however, if conditions are too harsh (high chemical concentration, high reaction temperature and long hydrolysis time) the hydrolysis will degrade sugars too much and produce inhibitors to microorganism during fermentation stage (Harun, Danquah, & Forde, 2010).

Zhou et al. (2011) mixed small amount of wet *Chlorella sp.* TIB-A01 with 2% to 6% concentration HCl, and with or without 2.5% $MgCl_2$ as catalyst in a stainless steel cylindrical reactor with a total volume of 15 mL. Each reactor containing mixture was incubated at 120 °C for 60 min or 180 °C for 10 min followed by microbial fermentation using *Saccharomyces cerevisiae* Y01 at 30 °C to produce ethanol. A sugar recovery of about 83% was obtained at hydrolysis conditions of 180 °C for 10 min with 2% HCL + 2.5% $MgCl_2$, which was higher than that without catalyst. Ethanol yield of 0.47 g/g sugar (91% of the theoretical yield) was obtained after 48 h fermentation. One year later the same group (Zhou, Zhang, Gong, Wang, & Ma, 2012) introduced an ionic liquids (ILs)-based chemical hydrolysis to increase soluble sugar yield from

Table I-1. Comparison of glucose and ethanol yield from different studies using different methods

Species	Hydrolysis conditions	Sugar yield	Fermentation condition	Bioethanol yield	Year/reference
C. sp. TIB-A01	120 ℃ for 60 min or 180 ℃ for 10 min with 2% HCl or with 2% HCl + 2.5% $MgCl_2$	About 83% sugar recovery	*S. cerevisiae* Y01 at 30 ℃ and pH 5.5 for 48 h	0.47 g g^{-1} of sugar (91% of the theoretical yield)	2011/(Zhou, Zhang, Wu, Gong, & Wang, 2011)
C. vulgaris	Cellulase (Celluclast) and Novozyme 188 at 35−55 ℃ and pH 3.6−5.6 for 60 min	0.14 g of glucose/g of biomass	four different *E. coli* strains derived from *E. coli* W3110 strain at 37 ℃ and pH 7.0 for 24 h	0.4 g g^{-1} of biomass	2011/(S. Lee et al., 2011)
C. vulgaris	dissolution in [Emim]Cl-based ionic liquids at 105 ℃ for 3 h followed by a hydrolysis with 7 % (wt) HCl at 105 ℃ for 3 h	88.02% sugar recovery	*S. cerevisiae* Y01 at 30 ℃ for 24 h	0.49 g g^{-1} of sugar (97% of the theoretical yield)	2012/(Zhou et al., 2012)
C. vulgaris FSP-E	enzyme mixture of endoglucanase, β-glucosidase, and amylases at 45 for 72 h	0.461 g/g of biomass (90.4% of total glucose)	*Zymomonas mobilis* at 30 °C for 48 h	3.55 gL^{-1} (89.3% theoretical yield)	2013/(Ho et al., 2013a)
C. vulgaris	enzyme mixture of cellulases, amylases, and endoglucanases at 60 ℃ for 24 h	0.55 g/g of biomass	*S. cerevisiae* at 33 ℃ for 24 h	0.17 g/g of biomass	2013/(Moncada, Jaramillo, Higuita, Younes, & Cardona, 2013)
C. vulgaris	microwave assisted at 100 °C for 5 min with 1 M HCl, and autoclave at 120 ℃ for 1h with 1 M HCl	20 ± 4 wt % glucose for microwave, and 23 ± 4 wt % glucose for autoclave	*S. cerevisiae*, at 30 °C for 24 after autoclave hydrolysis	13.2 ± 0.5 wt %	2016/(Kumar, Pulidindi, Kinel-Tahan, Yehoshua, & Gedanken, 2016)
C. vulgaris FSP-E	Enzymatic (SSF), endoglucanase,β-glucosidase, and amylase at 30 ℃ for 36 h		*Z. mobilis*	4.17 gL^{-1} (87.1% theoretical yield)	2013/(Ho et al., 2013a)
C. vulgaris FSP-E	1% H_2SO_4 at 121 ℃ for 20 min	23.6 gL^{-1}	*Z. mobilis* ATCC 29191 at 30 ℃ for 24 h	11.7 gL^{-1} (87.6% theoretical yield)	2013/(Ho et al., 2013a)
C. vulgaris	cellulase (Celluclast 1.5 L), pectinase (Pectinex SP-L), Xylanase (X2629), β-glucosidase (C6105), amylase (A8220), chitinase (C6137), lysozyme (L6876), and sulfatase (S9626) at pH 4.8 and 50 ℃ for 72 h	69.9% glucose conversion yield	Saccharomyces cerevisiae (KCTC 7906) at 30 °C for 48 h	89% theoretical yield	2014/(K. H. Kim et al., 2014)
C. sp. M1	2% H_2SO_4 (v/v) at 121 ℃ for 30 min	n/a	*S. cerevisiae* at 28 ℃ and pH 5.5-6.0 for 30 h	n/a	2017/(Sanchez Rizza, Sanz Smachetti, Do Nascimento, Salerno, & Curatti, 2017)

Chlorella biomass. This type of hydrolysis consisted of dissolution in ILs including [Emim]Cl-based, imidazolium-based, pyridinium-based and quaternary ammonium-based ionic liquids and HCl catalyzed hydrolysis. Zhou et al. (2012) reported a yield of 88.02% of total sugars biomass was obtained when 15.2 mg *Chlorella* biomass was dissolved in 310 mg [Emim]Cl-based ILs at 105 °C for 3 h followed by a hydrolysis with 7% (wt) HCL (equals to 1.92 mmol HCl) at 105 °C for 3 h. The fermentable sugars was then fermented using *Saccharomyces cerevisiae* Y01at 30 °C for 24 h. An ethanol yield of 0.49 g g^{-1} of sugar (97% of the theoretical yield) was obtained. Kumar et al. (Kumar et al., 2016) put 0.2 g dried powder of *Chlorella vulgaris* and 5 mL HCl (concentration: 1−5 M) into a 30 mL big polytetrafluoroethylene (PTFE)-lined reactor. The reactor was then put into a commercial microwave oven and operated at 1200 W for 5-30 min at temperature of 80, 100, or 120 °C, respectively. In addition to microwave assisted hydrolysis, traditional autoclave hydrolysis was also carried for comparison. A fermentation to convert hydrolysate into bioethanol was carried out at 30 °C for 28 h using *S. cerevisiae*. A glucose yield of 20 ± 4 wt % and 23 ± 4 wt % was obtained in microwave assisted hydrolysis (100 °C for 5 min with 1 M HCl) and traditional autoclave hydrolysis (120 °C for 1h with 1 M HCl), respectively. A bioethanol yield of 13.2 ± 0.5 wt % was obtained using traditional autoclave hydrolysis followed by *S. cerevisiae* fermentation for 24 h. Catalysts such as $MgCl_2$ help reduce the amount of acid, incubating time and increase yield of reducing sugars but it increase the cost of catalyst. Dielectric heating including microwave (MW) heating and radio frequency (RF)

heating, and ultrasonic methods are the most popular physical assistance to thermal hydrolysis which can reduce the reaction time remarkably.

Besides HCL, H_2SO_4 was also frequently used in chemical hydrolysis. The procedure is similar to these used in determination of the carbohydrate content except for lowering the concentration remarkably (from 4% to 15%). Ho et al. (Ho et al., 2013a) hydrolyzed freeze-dried *C. vulgaris* FSP-E with 1% H_2SO_4 at 121 °C for 20 min followed by a fermentation to produce bioethanol with *Z. mobilis* ATCC 29191 at 30 °C for 24 h. A glucose concentration of 23.6 gL^{-1} and an ethanol concentration of 11.7 gL^{-1} (87.6% theoretical yield) were obtained. Rizza et al. (Sanchez Rizza et al., 2017) used 2% H_2SO_4 (v/v) to hydrolyze 5% 17 microalgae strains including *Chlorella sp.* strain MI (w/v) at 120 °C in an autoclave for 30 min followed by an ethanol formation with *Saccharomyces cerevisiae* at 28 °C for 30 h.

Harun et al. (2011) reported their work as the first study on using alkaline to hydrolyze microalgae (Harun, Jason, Cherrington, & Danquah, 2011). Although this case does not involve *Chlorella* in, another carbohydrate rich (32.52 % w/w) species, *Chlorococcum infusionum*, was used. Five grams of dried microalgae powder was mixed with 0.75% (w/v) of NaOH, and incubated at 120 °C for 30 min. The hydrolysate was then fermented using *Saccharomyces cerevisiae* at 30 °C for 72 h. Glucose yield of 0.35 g/g and ethanol yield of 0.26 g/g microalgae were obtained from hydrolysis and formation, respectively. The usage of alkaline instead of acid is in favor of lowering temperature and pressure during hydrolysis of biomass (B. Zhang, Shahbazi, Wang, Diallo, & Whitmore, 2010), and minimizing the degree of inhibition during

fermentation of ethanol thereafter, consequently to cut production cost (Rabelo, Maciel Filho, & Costa, 2009). However, we did not see any reduction of incubating temperature, pressure and time (120 °C for 30 min) during hydrolysis of carbohydrate when the alkaline was used. It might attribute to the toughness of cell wall of microalgae compared with those of sugarcane bagasse and cattail.

Compared with chemical methods, **enzymatic hydrolysis** is considered more environmentally friendly and higher glucose production from biomass without producing inhibitory products although it is not as fast as chemical ones (Ho et al., 2013a). Recently, study on enzymatic hydrolysis of microalgae for bioethanol becomes more and more attractive (Sirajunnisa & Surendhiran, 2016). However, in order to improve sugar yield, disruption of cell wall is normally needed before enzymatic hydrolysis due to the tuff structure of microalgal cell wall. Disruption of cell wall normally includes physical, chemical, enzymatic methods and sometime combination of two out of these three methods.

Ho et al. (2013) pretreated dried *C. vulgaris* FSP-E powder with an acetate buffer solution (pH = 6) by sonication for 10 min followed by an incubation using autoclave at 121 °C for 20 min. After pretreatment, an enzymatic hydrolysis with carried out at 45 °C with an enzyme mixture of endoglucanase, β-glucosidase, and amylases. A maximum glucose yield of 0.461 g/g of biomass (90.4% of total glucose) was obtained after 72 h. When fermentation was carried out, they just hydrolyzed pretreated biomass for 48 h which reduced hydrolysis time by 24 h and got pretty much similar glucose yield of 87.17% of total glucose. Fermentation was fulfilled using

Zymomonas mobilis at 30 °C for 48 h which produced an ethanol concentration of 3.55 gL^{-1} (89.3% theoretical yield). Lee et al. (2011) soaked *C. vulgaris* with citric acid, sodium citrate buffer (5% dry solid basis, w/v), and then incubated the mixture at 121 °C for 15 min to interrupt cell walls. Interrupted algae were enzymatically hydrolyzed with Cellulase (Celluclast) and Novozyme 188 at pH = 3.6 to 5.6 and 35 to 55 °C for 60 min, and a yield of 0.14 g of glucose/g of biomass was obtained. Fermentation of bioethanol was carried out using four different ethanolic *E. coli* strains (SJL25, E. coli SJL2526, E. coli SJL27 and E. coli SJL2627) in a 5 L fermentor with 2 L of Luria-Bertani (LB) broth and 5 g/L of pretreated *C. vulgaris* sugar, at pH = 7.0 and 37 °C for 24 h which produced a maximum ethanol yield of 0.4 g/g biomass. Kim et al. (2014) applied three pretreatments: autoclave, sonication, and bead-beating (milling) before enzymatic hydrolysis. Different loadings and types of enzymes including cellulase (Celluclast 1.5 L), pectinase (Pectinex SP-L), Xylanase (X2629), β-glucosidase (C6105), amylase (A8220), chitinase (C6137), lysozyme (L6876), and sulfatase (S9626) were used in enzymatic hydrolysis at pH = 4.8 and 50 °C for 72 h. After hydrolysis, continues fermentation was carried out in a 10-mL fluidized bed reactor, or a batch fermentation was performed for comparison, with both using *Saccharomyces cerevisiae* (KCTC 7906) at 30 °C for 48 h. The maximum glucose yield of 69.9% was obtained from the hydrolysis with bead-beating pretreatment. The ethanol conversion yield reached 89% at 18 h for the continuous fermentation and at 24 h for the batch formation. Moncada et al. (2013) conducted an enzymatic hydrolysis using an enzyme cocktail of cellulases, amylases, and endoglucanases directly without pretreatment/interruption of cell wall

at 60 °C for 24 h. After the enzymatic hydrolysis, the hydrolysate was fermented with *Saccharomyces cerevisiae* at 33 °C for about 26 h. The yields of glucose and bioethanol were 0.55 g/g biomass and 0.17 g/g of biomass, respectively.

3.2.2. Simultaneous saccharification and fermentation (SSF)

Besides SHF, Ho et al. (2013) tried SSF operation as a comparison. They mixed sonication-pretreated *Chlorella vulgaris* with acetate buffer solution (pH 6.0) at a biomass concentration of 20 g/L. Then, the mixture was autoclaved and the solution was inoculated with *Z. mobilis* at an inoculum size of 10% (or optical density, OD 600 = 2.0). Meanwhile a filter-sterilized enzyme solution (consisting of: endoglucanase, 0.61 UmL 1; b-glucosidase, 0.30 U mL 1; amylase, 0.75 UmL 1) was added into the mixture. The SSF was then conducted at a temperature 30 °C in a desktop fermenter for 36 h. An ethanol yield of 4.17 gL^{-1} (87.1% theoretical yield) was obtained.

4. Comprehensive utilization of lipid and starch

In order to make production of biodiesel from microalgae economically feasible, an integrated biorefinery concept of microalgae was introduced in 2010 (René H Wijffels & Barbosa, 2010; Rene H Wijffels, Barbosa, & Eppink, 2010). In short, the concept requires mild cell disruption, extraction and separation technologies on microalgae to reduce production cost meanwhile encourage utilization of all compounds in microalgae including ω-3-fatty acids, carbohydrates, pigments, vitamins, and proteins to increase product monetary value. In essence, the concept is actually the ‘comprehensive utilization’ that we usually refer to as, although it is

defined as a fancy term of biorefinery by Wijffels et al. (Rene H Wijffels et al., 2010). All aforementioned studies focus on either production of biodiesel from lipid in microalgae or saccharification of starch into reducing sugars, and consequently, fermentation of reducing sugars into biofuel (mainly bioethanol) separately. There are few reports discussing about comprehensive utilization of lipid and starch, i.e., using both compositions of lipid and starch in microalgae to lower costs of raw materials.

A study was reported on bioethanol production from residual of *Chlorella sp.* KR-1after lipid was extracted (O. K. Lee, Oh, & Lee, 2015). A small amount of freeze-dried microalgae was mixed with dimethyl carbonate and methanol (7:3, v/v), and the total lipid extraction was carried out at 60 °C for 12 h. After lipid extraction, the residual was subject to either enzymatic or diluted acidic hydrolysis. In enzymatic hydrolysis, 5% (w/v) of the microalgae residual was mixed with enzyme cocktail (Pectinex Ultra SP-L with 9500 polygalacturonase units (PGU)/mL), amyloglucosidase (AMG 300L with 300 amyloglucosidase units (AGU)/mL), cellulase (Celluclast 1.5L with 700 endoglucanase units (EGU)/mL) and Viscozyme L (with 100 fungal β-glucanase units (FBG)/g)). The mixture was then incubated at various temperatures (35–55 °C) and pH values (3.5–6.5). In diluted acidic hydrolysis, 5% (w/v) of the microalgae residual was mixed with either HCl or H_2SO_4 at various concentrations (0.1, 0.3, 0.5, 0.7, 1 N) and autoclaved at 121 °C for 15 min. Sugar content after each hydrolysis was determined using dinitrosalicylic acid (DNS) method. A fermentation using *S. cerevisiae* KCTC 7931 (ATCC 20626) was carried out after hydrolysis to complete the separate hydrolysis and fermentation

(SHF) processes. SSF was also carried out by mixing 5% of the residual with enzyme cocktail and *S. cerevisiae* and sodium acetate buffer for comparison purpose. The ethanol yield via SSF was 82.3% vs. 79.3% through SHF. Lee et al. (2015) concluded that compared with SHF process, SSF method was more suitable for residual of *Chlorella sp.* KR-1after lipid extraction due to simpler reaction, less reaction time and a higher ethanol yield. We think during the lipid extraction process, cell walls of microalgae, *Chlorella sp.* KR-1, has already been well disrupted which leads to a good access for enzyme cocktail to starch in microalgae cells. Therefore, lipid extraction from microalgae rich in both lipid and starch followed by either a hydrolysis or a SSF saves a cell wall disruption, consequently, lowers production cost.

Although this study is not about *Chlorella vulgaris*, it is about another microalga, *Scenedesmus sp.*, which is rich in both lipid and starch. Based on the integrated biorefinery concept of microalgae, an integrated production of methyl ester and bioethanol was carried out following either 1) an order of direct transesterification first followed by starch hydrolysis, or 2) an order of starch hydrolysis first followed by direct transesterification (Sivaramakrishnan & Incharoensakdi, 2018). In starch hydrolysis, 1 g of ultrasound pretreated freeze-dried microalgae was mixed with 0.3 N H_2SO_4 and autoclaved at 120 °C for 30 min. In direct transesterification, 1 g of ultrasound pretreated freeze-dried biomass was mixed with dimethyl carbonate (DMC), lipase immobilized on Celite material with the enzyme activity of 182 U/g and distilled water. In bioethanol fermentation, a mixture of 20 mL hydrolysate from starch hydrolysis, 5 g/L yeast extract, 10 g/L peptone and 47.5 mL distilled water in a 125 mL conical flask was autoclaved.

Then, 2.5 mL of Saccharomyces cerevisiae was added into the mixture and the mixture was incubated at 30 °C for 72 in an incubating shaker with 180 rpm. For the first combined order, direct transesterification was carried out first. The residual after transesterification was washed, ready for the starch hydrolysis, and subsequent bioethanol fermentation. For the second combined order, the starch hydrolysis was carried out first followed by separation of supernatant and solid biomass using centrifugal. The supernatant from starch hydrolysis was used for bioethanol fermentation, and the solid residual was washed and dried for direct transesterification. The results indicated combined method with an order of direct transesterification first followed by ethanol fermentation yielded 92 and 93% of methyl ester and ethanol, respectively which were higher than those by the other combined order (starch hydrolysis first followed by direct transesterification). Authors attributed this observation to a reduction of some phenolic compounds occurred in direct transesterification which act as an inhibitor in the fermentation process.

Ma et al. (2019) also explored feasibility of comprehensive recovery of lipid and carbohydrate in wet microalgae *Chlorella vulgaris* which is the first report on a comprehensive usage directly from wet microalgae. Due to the demand of a huge amount of energy for drying microalgae, the direct processing of wet algae for value-added biofuel and biochemical production attracts more and more attention in recent years. However, when there are both free fatty acids (FFAs) and triacylglycerols (TAGs), plus a high concentration of water at the same time, the conversion process becomes very complicated. To obtain desirable reaction efficiency,

various strategies have been employed in previous studies including the harsh pretreatment, high methanol to algae ratio, high catalyst to microalgae ratio, extreme high temperature and long time, or super critical method. In this study, they have developed a biodiesel production procedure directly from wet microalgae. Wet microalgae (*Chlorella vulgaris*) was pretreated by radio frequency (RF) heating to disrupt cell walls first, followed by esterification and transesterification with relatively small amount of methanol and catalyst (either HCL or NaOH) assisted by RF heating at 55 °C for only 20 min. The fatty acid methyl esters (FAME) yield reached as high as 79.5 ± 3.0%. Many major microalgal species used for biodiesel production have high content of lipid as well as carbohydrate. In this study, the feasibility of comprehensive recovery of both lipid and carbohydrate in wet microalgae *Chlorella vulgaris* was explored. The key innovations in their study are: 1) there is no report so far on comprehensive utilization of carbohydrates in microalgae residual after lipid extraction or biodiesel production directly from wet algae; 2) all steps involved in this study (including cell destruction and in situ transesterification for biodiesel production) were carried out under normal temperature and normal pressure and no specific apparatus was required during the process; 3) the combination of RF pretreatment and CTec2 could maximized cell wall disruption; and 4) adsorption kinetics of the optimized enzyme combination on raw microalgae (negative control), RF heating disrupted microalgae (positive control) and microalgae residual were determined and adsorption isotherm was analyzed with Freundlich equation. In addition, scanning electron microscope (SEM) was

used to investigate morphology of the microalgae before and after enzymatic saccharification, which further explained their saccharification and enzyme adsorption results.

5. Conclusion

Taken together, the results showed that it is feasible to use microalgae residual after biodiesel production as carbohydrate feedstock to be hydrolyzed for fermentable sugar production, which can be further used as the substrate for valuable biofuel and biochemical production. This represents an effective approach for comprehensive utilization of wet microalgae. Although the review is focusing on the species of *Chlorella vulgaris*, the technologies of mild cell disruption, extraction and separation can be used on any other microalgae rich in both lipid and starch.

Chapter II Direct biodiesel production from wet microalgae assisted by radio frequency heating

Abstract

Rapid growth, fixation of carbon dioxide, high lipid content and limited competition as a human food make microalgae a suitable feedstock to produce biodiesel. However, high costs related to dehydration, lipid extraction and conversion to biodiesel hampers industrialization of biodiesel production from microalgae. The objective of this study was to develop a biodiesel production procedure directly from wet microalgae. The system was operated under atmospheric pressure without requiring any specific apparatus. Wet microalgae (*Chlorella vulgaris*) was pretreated by radio frequency (RF) heating to disrupt cell walls first, followed by esterification and transesterification with relatively small amount of methanol and catalyst (either HCL or NaOH) assisted by RF heating at 55 ℃ for only 20 min. The fatty acid methyl esters (FAME) yield reached as high as 79.5 ± 3.0%. The two major factors impacting biodiesel yield are catalyst and methanol. Also, scanning electron microscope (SEM) was used to investigate morphology of the algae before and after the RF heating pretreatment. The SEM images visually verified the significant effect of cell disruption by the pretreatment. Although this pathway was developed for *Chlorella vulgaris*, it can be applied to any other microalgae which are rich in both free fatty acids and triglycerides.

1. Introduction

The limitation of fossil fuel has become a threat of energy security worldwide, and oil reserves are predicted to be exhausted by 2050. So there is an urgent need to find sustainable energy resources (Ho et al., 2013b). Biodiesel is one of the most promising alternatives to fossils derived diesel fuel. It is well known by its high flash point, excellent biodegradability and renewability. Compared with petroleum diesel, biodiesel is more eco-friendly. It produces much less greenhouse gas and can be used in diesel engines without additional modification (Knothe et al., 2006; Zabeti et al., 2009).

There is a great variety of algae, which accounts for 40% of global photosynthesis (Andersen, 1992). Algae grows rapidly under a range of environmental conditions. Some species have high lipid content and unique lipid composition (Guschina & Harwood, 2006). Thus, they have been recognized as promising feedstocks for biodiesel production (Yao, Gerde, Lee, Wang, & Harrata, 2015). In early 2010's, studies started to report biodiesel production from dried microalgae using *in situ*/direct transesterification. It is reported that the fatty acid methyl ester (FAME, biodiesel) production conditions from *Chlorella vulgaris* microalgae were optimized by using alkali-catalyzed *in situ* transesterification (S. Velasquez-Orta et al., 2012). The catalyst (NaOH) to lipid molar ratios was 0.15:1, and the methanol to lipid ratio was 600:1. In previous study, microalgae was spray-dried first, then biodiesel was produced via *in situ* methanolysis with a solvent to sample ratio of 204 mL: 2 g (Carvalho Júnior et al., 2011). Although these

studies have reported high FAME yields, they required dried algae and large amounts of solvent and catalyst.

Regardless of the drying methods, such as hot air, spray dry, or freeze dry, the drying process consumes a huge amount of energy. Therefore, direct biodiesel production from wet algae has received more attention in recent years (C. L. Chen et al., 2015; Z. Chen et al., 2018; Macías-Sánchez et al., 2015; Macías-Sánchez et al., 2018). The presence of water has a significant negative impact on transesterification reaction (Cao et al., 2013; C. L. Chen et al., 2015). Studies have been carried out under high temperature and pressure with special container to overcome this challenge. (B. Kim et al., 2015; Park et al., 2017; Skorupskaite et al., 2016). For example, the production of FAME from wet microalgae (25 wt% dry biomass) was investigated in a 5 L thermostatic stirred pressure proof reactor and found the optimized reaction conditions were at 100 ºC for 105 min with a pressure of 2.5 atm (Macías-Sánchez et al., 2015). Research also reported to enhance the lipid extraction efficiency from wet *Chlorella sp.* (33-35 wt% dry biomass) by using deep eutectic solvent in an autoclave reactor (Pan et al., 2017). It is also reported that the reactions were carried out in a 14 mL pressure proof Teflon-seal tube (Im, Lee, Park, Yang, & Lee, 2014). But these critical conditions and special apparatus limit the upscale potential for the commercial production, especially for the continuous process.

Electromagnetic radiation could assist a variety of chemical and physical processes. To improve production efficiency of biodiesel from wet algae, microwave heating has been investigated. It has been found to be effective on pretreatment/disruption of cell (J. Kim et al.,

2013), lipid extraction and transesterification (Ali & Watson, 2015; Wahidin, Idris, & Shaleh, 2014), and could accelerate direct/one-step biodiesel production (C. L. Chen et al., 2015; Cheng, Yu, Li, Zhou, & Cen, 2013; G. Ma et al., 2015; Wahidin, Idris, Yusof, Kamis, & Shaleh, 2018).

Radio frequency (RF) heating is another popular technique utilizing electromagnetic radiation. Both RF and microwave heating are categorized as dielectric heating which can efficiently heat up liquid, solid and semi-solid materials with ions and/or dipoles (Y. Wang, and Wang, J, 2008). Although they have similar mechanism, RF heating overmatches microwave heating on the following four aspects: RF has greater penetration depth because of its longer wavelength than microwave, which results more uniform heating and is more suitable for large amount of material. The configuration of RF heater is relatively simple and loading cavity is big, so it is easy to operate and can process objects with large size (Liu, Wang, McDonald, & Taylor, 2008; Y. Wang, T. Wig, J. Tang, & L. Hallberg, 2003; Y. Wang, T. D. Wig, J. Tang, & L. M. Hallberg, 2003). RF heater also has a higher efficiency on converting electricity to electromagnetic power, and metal can be used in it. Since its first application by Cathcart and Park to thawing frozen food in 1946, RF heating has been wildly used in various industries for heating and drying. Recently, its usage has been expanded to assist turning sustainable biomass and industrial, agricultural and forestry by-products into biofuels and biomaterials (Y. Wang, and Liu, S., 2014). Our group has successfully used RF heating to accelerate biodiesel production from canola oil, beef tallow and waste cooking oil. It has also been used to pretreat various biomaterials including switchgrass and sweetgum to enhance productions of glucan, acetone–

butanol–ethanol (ABE) and polyhydroxybutyrate (PHB) (X. Wang, Taylor, & Wang, 2016; X. Wang, Zhang, Wang, & Wang, 2016a, 2016b).

In the current work, biodiesel production directly from wet algae assisted by RF heating was investigated. *Chlorella vulgaris* microalgae was chosen as the feedstock because of its high lipid and carbohydrate contents (Ho et al., 2013b; Shakya et al., 2017). RF heating was used for both cell wall destruction and reaction acceleration. Acid or alkali-catalyzed esterification and alkali-catalyzed transesterification were carried out with various RF heating time and methanol/hexane/catalyst ratios. All steps involved in this study including cell destruction, esterification and transesterification were carried out under normal temperature and pressure without any specific apparatus, which makes continuous and large-scale production possible in the future. The objectives of this study were to develop a biodiesel production procedure directly from wet microalgae, and investigate the effects of different reaction factors including catalyst, methanol, hexane and RF heating time.

2. Materials and methods

2.1. Biomass and chemicals

Chlorella *vulgaris* was cultivated at Arizona State University's Arizona Center for Algae Technology and Innovation (AzCATI) in a 640 L acrylic vertical flat panel with a 2 inch light path. The PBR was grown in BG-11 culture medium and maintained at a pH value of 8.08. All culture medium chemicals were analytical grade and purchased from VWR International. The

PBR was inoculated on April 3, 2015 at a density of 0.57 g/L with 67.8 mg/L NO3. The biomass was harvested on April 13, 2015 at a density of 2.96 g/L upon NO3 depletion (0.01 mg/L NO3). Harvested biomass was dewatered in a Lavine centrifuge to approx. 20% solids, immediately placed into airtight bags and frozen at -10 ºC.

2.2. Determination of moisture and neutral lipid contents in algae

The algae moisture content was measured by a moisture analyzer (MF-50, A&D Company, Limited, Japan) in triplicates. The neutral lipid content of 7.50 ± 0.20 wt% (based on dry mass) was determined with Nile Red assay described by Wang et al. (Q. Wang, Peng, & Higgins, 2019). Briefly, 1.5 mL of Folch solvent (2:1 chloroform/methanol) was added into 20 mg of freeze-dried algal biomass to extract lipid. Then, 80 μL of the lipid extracts, 30 μL of isopropyl alcohol, 200 μL of Nile Red solution (1 μg/mL) and 20 μL of 50% bleach solution (mixing 6% hypochlorite with deionized water) were added into a microplate well. Fluorescence of the samples at 530 nm excitation/575 nm emission with auto cutoff of 570 nm was measured by using a SpectraMax Seried microplate reader (Model M2, Molecular Devices, LLC, CA, USA) and was used for quantification. The measurement was carried out in triplicates.

2.3. Scanning electron microscope (SEM)

In order to investigate morphology of the algae before and after pretreatment, SEM was used. Scanning electron images were obtained using a Zeiss EVO 50VP SEM (Carl Zeiss AG,

Jena, Germany). The wet algae samples were placed on aluminum support stubs. The osmium tetroxide was used to fix liquid for 6 hours. Then the samples were air dried in fume hood overnight. After gold coating, the SEM images of samples were taken at 20.00 kV at a magnification of 12.00 k×.

2.4. Experimental design of biodiesel conversion

The biodiesel production through esterification and transesterification with methanol is based on simple chemical principles. However, in practice, the conversion was significantly affected by multiple factors, including moisture content of algae, type of catalysts and solvents, order of reactions, reaction time and temperature, mixing ratio of biomass (algae to methanol in this study), etc.

According to literature review, our past studies (Liu, McDonald, & Wang, 2010; Liu et al., 2008; Liu, Wang, Oh, & Herring, 2011) and preliminary experiments, a processing flow chart was composed (Fig. II-1). Wet algae were first pretreated by RF heating to disrupt cell walls (details in Section 2.3.1). Then, a first stage of conversion was carried out, in which the wet algae was directly mixed with methanol and catalyst to react (details in Section 2.3.2). It is referred as "direct transesterification" (Cao et al., 2013; C. L. Chen et al., 2015; Macías-Sánchez et al., 2015; Patil et al., 2012) or "*in situ* transesterification" (Im et al., 2014; B. Kim et al., 2015; G. Ma et al., 2015; Park et al., 2017) in some previous studies. Immediately after that, a second

stage of conversion was carried out to mainly transesterify remained triacylglycerols (TAGs, details in Section 2.3.3).

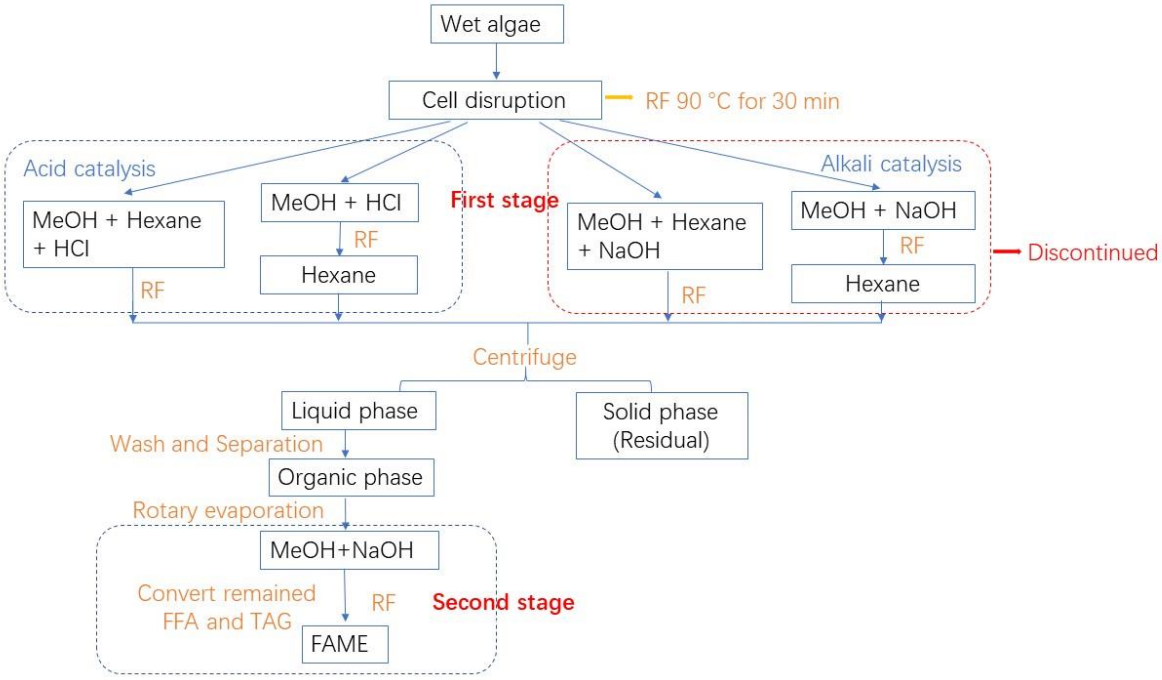

Fig II-1. Flow chart of experimental design

2.4.1. Cell disruption with RF heating

An aliquot of 120 g of wet algae was placed in a 150 ml glass beaker and heated in a RF heater (SO6B; Strayfield, Berkshire, England) at 90 ºC for 30 min. The RF heater has a frequency of 27.12 MHz and maximum power output of 6 kW. The beaker with algae was covered with a plastic sheet to reduce water loss. A fiber-optic sensor (Neoptix, Inc., Quebec City, Quebec, Canada) were employed to monitor the temperature during the heating process. After reaching 90 ºC, the RF heater was switched on and off to maintain the temperature.

2.4.2. First stage of conversion

In this stage of the conversion, there were two sets of experiments: alkali catalyzed (up right of Fig. II-1) and acid catalyzed conversions (up left of Fig. II-1). Under each set, mixture of wet algae, methanol, catalyst with or without the presence of hexane was heated by the RF heater. So, there were four tests in the first stage of conversion. In the alkali catalyzed set, 4.0 mL of NaOH-MeOH solution (NaOH to MeOH, w/v, 0.5:100), and 6 mL of MeOH were added into 5.0 g of pretreated algal sample in a 125 mL Erlenmeyer flask with or without addition of 10 mL of hexane. The mixture was stirred for 5 min with a magnetic stirring bar, and then heated with RF for 20 min. A condenser was used to reduce evaporation of MeOH during the RF heating. The schematic diagram of the RF heating apparatus is shown in Fig. II-2. The mixture with/without hexane reached ca. 50 ºC and 55 ºC, respectively, during the RF heating. After the RF heating stopped, the stir continued for additional 5 min to fully utilize the heat. For the mixture without addition of hexane, 10 mL of hexane was added into the flask. The flask was sealed with parafilm and shaken at 200 rpm for 1 hour to extract FAMEs, TAGs and remained free fatty acids (FFAs). In the acid catalyzed set, 4 mL of HCl-MeOH solution (36% HCl to MeOH, v/v, 5:95) replaced the 4 mL of NaOH-MeOH solution while keeping other conditions unchanged.

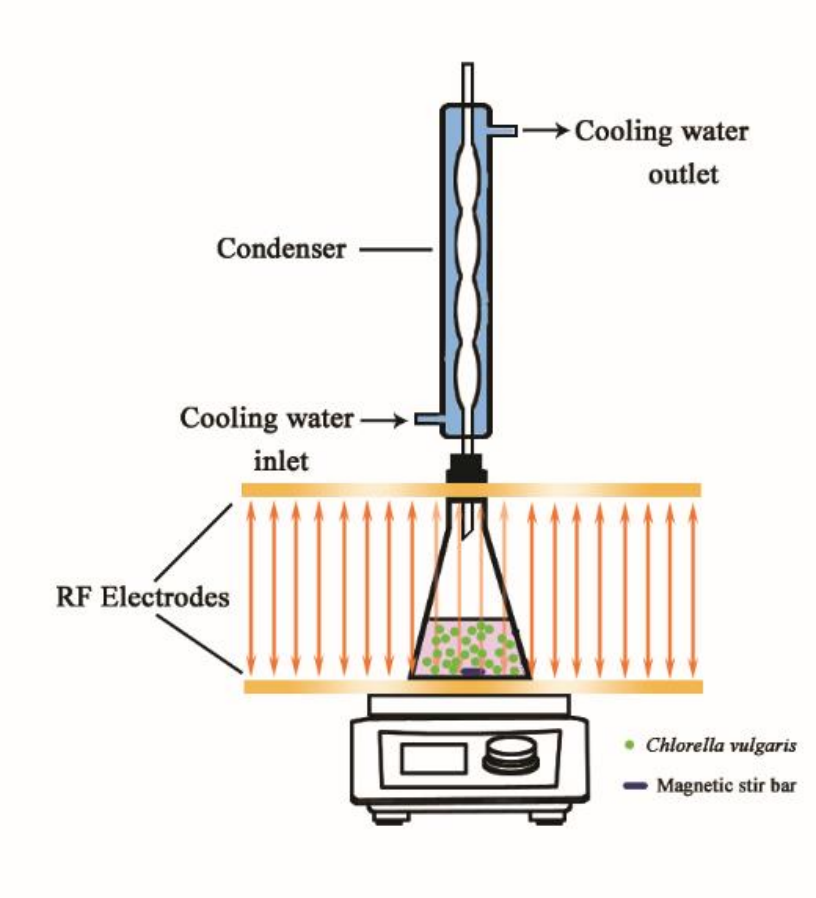

Fig II-2. Schematic diagram of RF heating apparatus

After the extraction, 10 μL of internal standard (methyl tridecanoate) and 30 mL deionized water were added to the mixture. Then, the sample was centrifuged at 9900 rpm for 10 min. The upper liquid phase was separated and washed with deionized water twice using a separatory funnel. The liquid phase was used for FAME analysis and the second stage conversion. The algal residuals (solid phase in Fig. II-1) could be used as carbohydrate sources for hydrolysis and fermentation to produce other bio-products such as bioethanol and PHB.

2.4.3. Second stage of conversion

In this stage of conversion, the upper liquid phase obtained from the first conversion was evaporated with a rotary evaporator at 45 ºC to recovery the hexane. Then, 10 mL of NaOH-MeOH solution (NaOH to MeOH, w/v, 0.5:100) was added into the flask, and followed by the RF heating at 55 ºC for 20 min. Finally, another 10 mL of hexane was added to extract the FAMEs. The upper hexane phase was sampled for FAME analysis.

2.5. Effects of major factors on conversion rate

Based on the results of the four types of experiments described above (alkali catalyzed with or without hexane during RF heating, and acid catalyzed with or without hexane during RF heating), the acid catalyzed conversion without hexane was selected as the base trials. Then, four more trials were performed to investigate the effects of four major factors (i.e., catalyst, methanol, hexane and RF heating time) by doubling the amount of each factor, respectively, in the first stage. The second stage were kept unchanged. The reaction conditions of these trials are summarized in Table II-1.

Table II-1. Detailed reaction parameters in the first stage of conversion

Trial		HCL-MeOH (mL)	MeOH (mL)	Hexane (mL)	RF time (min)
Base	A	4	6	10	20
Double catalyst	B	8	2	10	20
Double MeOH	C	4	16	10	20
Double hexane	D	4	6	20	20
Double RF time	E	4	6	10	40

2.6. FAME analysis

The FAME content was analyzed based on the method described by Van et al. (Van Wychen et al., 2016) with minor modification. An Agilent 7890 GC (Santa Clara, CA, USA), equipped with a flame ionization detector (FID) and a DB-WAXetr capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, Santa Clara, CA) was employed. The flow rate of helium was 1 mL/min. The temperature of FID detector and injector was 260 ºC and the oven temperature was programmed from 100 ºC (held for 1 min) to 240 ºC (held for 15 min) at a rate of 4 ºC/min. The inlet split ratio was 5:1. Both of the hexane phases after the first and the second stages were analyzed. The FAME yield was calculated by Eq. (1):

$$\text{FAME yield (\%)} = \frac{FAME\ (g)}{Total\ Neutral\ lipid\ (g)} \times 100\% \quad (1)$$

3. Results and discussion

3.1. Moisture contents

The algae moisture content was 80.3 ± 0.4 wt% before pretreatment, and 78.5 ± 0.7 wt% after Cell disruption with RF heating. It indicated that there was little water loss during the RF heating pretreatment.

3.2. Effect of RF pretreatment

As shown in Fig. II-3, the changes of *Chlorella vulgaris* cells before and after the pretreatment by RF heating was illustrated. Before the pretreatment, the cells were clear and intact in general (Fig. II-3(a)). After RF pretreatment, the cells became very blurred and a lot of holes were observed on the cell walls, indicating damage. The results visually verified the cell disruption effect of RF pretreatment, which indicate that the pretreatment broke the cell walls and exposed the substances originally inside the cells. With the breakage of algae cell wall, lipids inside algae cells could be more easily reached by solvent and catalyst, thus increasing FAME conversion rate.

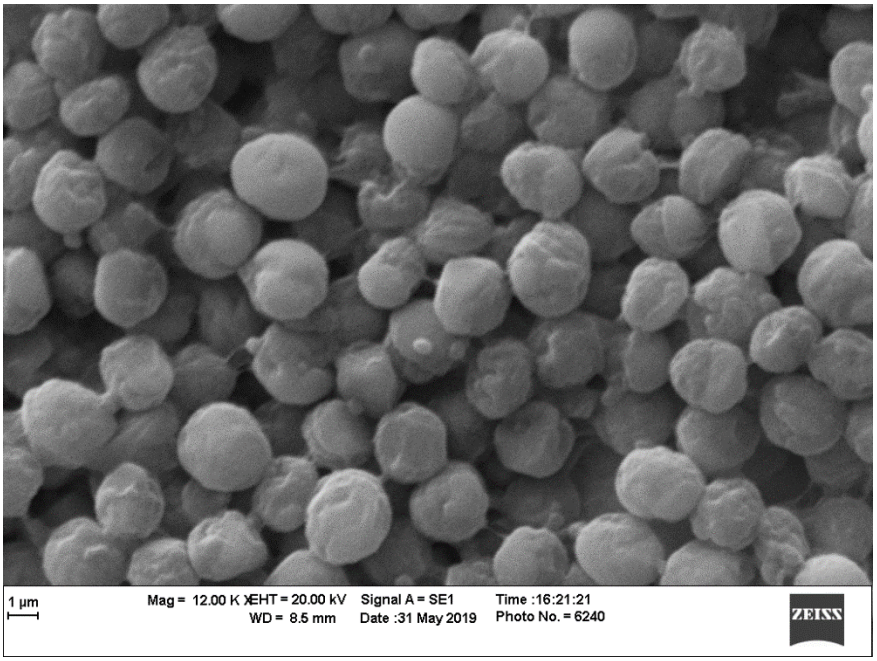

Fig II-3 (a). SEM image of algae raw material (*Chlorella vulgaris*)

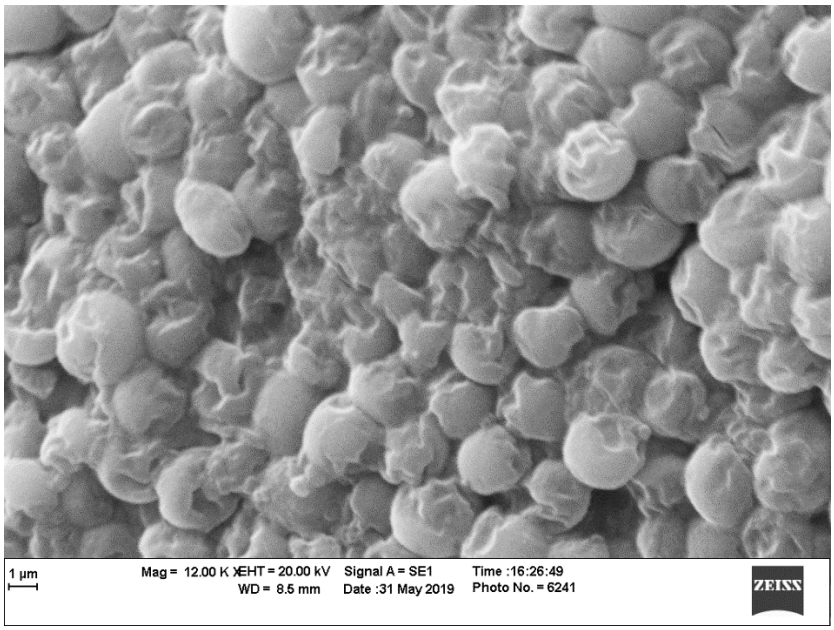

Fig II-3 (b). SEM image of algae after RF pretreatment

3.3. The first stage conversion

3.3.1. Alkali catalyzed first stage conversion

Alkali has been widely used as an effective catalyst to accelerate the transesterification reaction between TAGs and methanol to produce biodiesel (Baskar & Aiswarya, 2016; Liu et al., 2010; Liu et al., 2008; Veljković et al., 2018). However, the total FAME yield of the alkali catalyzed conversion (without hexane during RF heating) was only 19.0% after the second stage, and was even much lower for the one with hexane during RF heating. There are possibly two major reasons causing such low FAME yields. Firstly, there was a large amount of water present in the reactants. The moisture content of the *Chlorella vulgaris* after the RF pretreatment was 78.5 ± 0.7%, which means there was 30% of water in the reactants including wet algae and methanol. With alkali catalyst, water would significantly accelerate the saponification reaction and, subsequently, encourage FAME hydrolysis, resulting in low FAME yield. Secondly, the FFA content of *Chlorella vulgaris* was high. Neutral lipids are the major feedstock in algae to produce biodiesel (FAMEs). Within the neutral lipid class, FFAs account for about 53.5%, while TAGs only account for about 46.5% in *Chlorella vulgaris* (Yao et al., 2015). The FFAs would react with alkali catalyst to generate soap (saponification) and water, which would further compromise the yield of FAME (Wright et al., 1944). Our previous study has found that notable saponification would occur with alkaline catalyst if FFA accounted for more than 2% in waster cooking oil (from fast food restaurants), which would result in very low conversion rate or even failure of the reaction (Liu et al., 2010). In another study, similar observation was reported on

biodiesel production from high free fatty acid Karanja oil (Naik, Meher, Naik, & Das, 2008): once the FFAs content increased from 0.3 wt% to 5.3 wt% in the oil, the yield of biodiesel decreased dramatically from 97% to 6% with alkali catalyst. Due to the very low FAME yield, further study on alkali catalyzed first stage conversion was discontinued.

3.3.2. Acid catalyzed first stage conversion

Acid can catalyze esterification between FFAs and methanol as well as transesterification between TAGs and methanol to produce biodiesel. Since it does not react with FFAs to form soap, acid catalyst is much less affected by FFAs and/or water, which is acid catalyst's major advantage over alkali catalyst, although it catalyzes transesterification evidently slower than alkali one does. So, acid catalyst is often used to convert FFAs to FAMEs for biodiesel production (Dong, Wang, Miao, Zheng, & Chen, 2013), (Thoai, Tongurai, Prasertsit, & Kumar, 2019). The FAME yield for the acid catalyzed conversion (without hexane during RF heating) after the first stage was 30.0 ± 0.7% (Sample A in Fig. II-4), which was already higher than that of the alkali catalyzed one (19.0%) after two stages. The results confirm that acid catalyst (HCl in this study) was less suppressed by water and FFAs in the reactants and was more suitable for the first stage. In this stage, HCl was mainly catalyzed the conversion of FFAs and might help TAGs to convert, if any. Lotero et al. also reported that acid catalyst showed much higher efficiency in converting FFAs through esterification (Lotero et al., 2005).

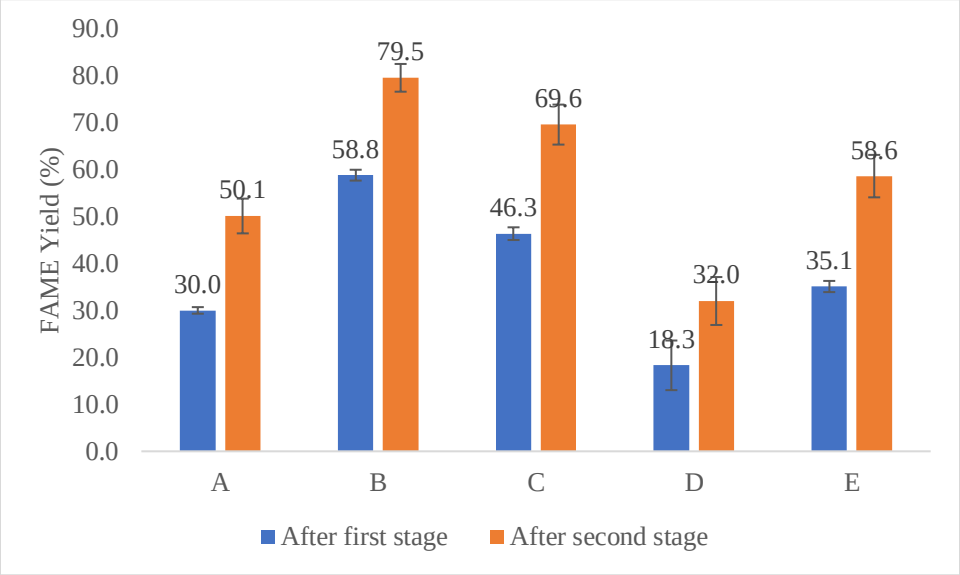

Fig II-4. FAME Yields after two stages under different reaction conditions

It has been reported that hexane could be added to perform an *in situ* extraction of FAMEs and/or TAGs during the reaction and improve the conversion rate (Cao et al., 2013; B. Kim et al., 2015). However, the FAME yield with hexane during the RF heating was only 2.4 ± 0.1% for the acid catalyst, which were significantly lower than the one without hexane during RF heating. Similar result was also found for the alkali catalyzed ones. The results show that the presence of hexane during RF heating hampered biodiesel production in this study. A slower increase in temperature during the RF heating was observed with the presence of hexane, which suggests that hexane probably lowered the RF heating efficiency and, subsequently, reduced the FAME yield. Moreover, the hexane created a new phase in the reactant mixture, which might reduce the mixing efficiency and cause a lower reaction rate. Since the addition of hexane during the RF heating has evident negative effect, no additional tests were further attempted.

Since FFAs possess about 53.5% of the neutral lipids in *Chlorella vulgaris* (Yao et al., 2015), the conversion rate of the first stage could reach 53.5% or even higher if all the FFAs could be converted. The actual FAME yield of 30.0 ± 0.7% (without hexane during RF heating) was still notably lower than that. This might be attributed to the following reasons: 1) the pretreatment (cell wall disruption) assisted with RF heating was not strong enough; 2) the reaction conditions were not sufficient to achieve complete conversion; and 3) although the same species of microalgae (*Chlorella vulgaris*) was used in this study, its FFA content might be lower than that was reported (Yao et al., 2015).

It has been reported that moisture content of algae has significant negative effect on FAME yield during direct biodiesel production from wet microalgae. Cao et al. reported a sharp decrease of the yield from 91.4% to 10.3% if the moisture content of algea increased from 10% to 90% when reacting at 90 °C with 0.5 M H_2SO_4 for 120 min (Cao et al., 2013). Kim et al. also reported negative impact of moisture content on biodiesel production on *in situ* transesterification from high moisture content algae using HCl as acid catalyst (B. Kim et al., 2015). In a study of direct biodiesel production from *Chlorella* with H_2SO_4 as catalyst and reacting at 60 °C for up to 6 h, Ehimen et al. observed that the reaction was almost stagnant when moisture content of algae reached 72.5% (Ehimen, Sun, & Carrington, 2010). In contrary, several other studies reported high conversion rates even when the moisture content of wet microalgae reached around 80%. However, all of these studies employed either harsh pretreatment (mixture of microalgae sludge with methanol was disrupted by microwave heating

followed by adding double amount of methanol and being dried out), or high methanol to algae ratio (171.1 mL/g), or high catalyst to microalgae ratio (1.5 mL of 95% H_2SO_4/g), or extreme high temperature and long time (150 ℃ for 2 h), or super critical method (240 to 260 ℃) to lower the effect of water in the reactant mixtures (Cao et al., 2013; C. L. Chen et al., 2015; Im et al., 2014; Macías-Sánchez et al., 2015; Patil et al., 2012). Using too much methanol and/or catalyst, or performing the reaction above the boiling point of methanol (65 ℃) with sealed pressure-proof reactor would significantly increase the operation and equipment costs, which is not environment-friendly and limits their industry scale applications. The process proposed in this study might have better economic feasibility, and be easier and safer to scale up.

3.4. The second stage conversion

The upper liquid phase gained from the first stage by centrifuge and separatory funnel contained hexane, FAMEs, TAGs and remained FFAs. Before the second stage of the conversion, the hexane in the upper liquid phase was recovered by a rotatory evaporator. Only FAMEs, TAGs and a small amount of FFAs remained. This step can save solvent, especially when it is scaled up. It also reduces the amounts of methanol and catalyst needed in the second stage.

To achieve high conversion rate of biodiesel production from algae, both FFAs and TAGs need to be turned into FAMEs. In the first stage, acid (HCl) mainly catalyzed esterification of FFAs and further broke down the algal cells. But, the TAGs in the algae probably had not be

effectively converted. So, a second stage of conversion is desired. In the second stage, water had been removed after a hexane extraction and centrifuging. Therefore, alkali catalyst (NaOH) could efficiently convert remained TAGs to FAMEs through transesterification. As shown in Fig. II-4 (Sample A), the total FAME yields significantly increased to 50.1 ± 3.7% after the second stage, indicating the second stage contributes 40.1% of the overall conversion rate. Therefore, the optimum pathway for direct biodiesel production from wet microalgae in this study was as follows: cell disruption with RF heating, first stage of conversion with acidic catalyze, centrifuge, separation and evaporation, and second stage of conversion (Fig. II-5).

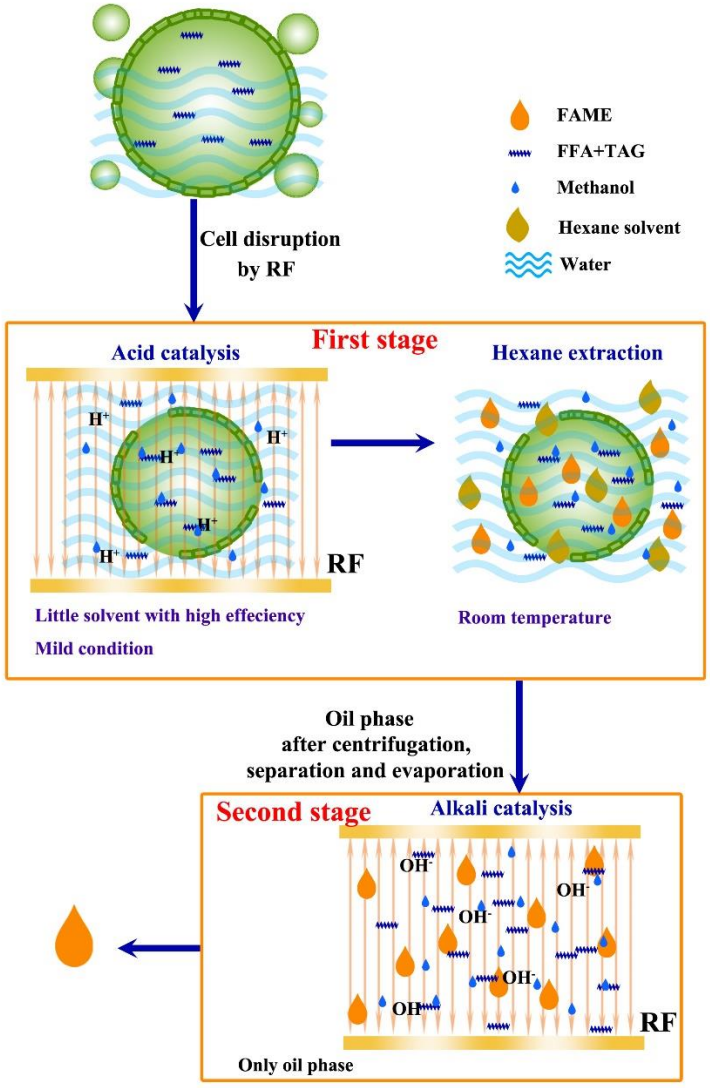

Fig II-5. The optimum pathway for direct biodiesel production from wet microalgae

Dong et al. (Dong et al., 2013) reported a similar observation on a two-step production of biodiesel from freeze dried microalgae (*Chlorella sorokiniana*, moisture content: 1.73 wt%). The yield of the pre-esterification (the first step) was 60.89 ± 2.37% (reaction conditions: H_2SO_4 of 6.9 wt%, methanol to biomass ratio of 4 mL/g, reacting at 90 °C for 70 min) and the yield of the

base-catalyzed transesterification (the second step) was 82.60 ± 2.19% (reaction conditions KOH of 1.8 wt%, methanol to biomass ratio of 10 mL/g, reacting at 90 °C for 10 min).

3.5. Effects of major factors on conversion rate

Since only 50.1% of the neutral lipids in algae was converted to biodiesel through the above two stages, there is a large potential to improve the FAME yield by optimizing reaction conditions. Four major factors, i.e., the amounts of catalyst, methanol and hexane, and RF heating time, were investigated in this work (Table II-1). Compared with the above mentioned base trial (Sample A, Fig. II-4), doubling the catalyst amount (Sample B) showed significant positive effect. The FAME yields reached 58.8 ± 1.2% and 79.5 ± 3.0% after the first and second stages, respectively, which account for 96% increase for the first stage and 59% increase for the overall conversion rate. Doubling the amount of methanol (Sample C) also evidently improved the yields to 46.3 ± 1.3% and 69.6 ± 4.3% after the two stages, respectively. Those are 55% improvement for the first stage, and 39% improvement for the overall conversion rate. The results suggest that the increase in catalyst or methanol amount could considerably promote the biodiesel production, and higher conversion rate might be achieved by further increasing them.

When the amount of hexane was doubled (Sample D), the FAME yields decreased to 18.3 ± 5.3% and 32.0 ± 5.1% after the two stages, respectively. It was expected to improve the extraction efficiency with the extra hexane, but the results suggest evident negative effect. After the hexane was added, the sample in 125 ml Erlenmeyer flask was shaken at 200 rpm for 1 hour

to perform the extraction. The additional hexane notably increased the volume of the sample during the extraction, which might lower the mixing efficiency and, subsequently, lower the extraction efficiency. The FAME concentration of Sample D in the recovered hexane was only about 40% of that of the Sample A. Furthermore, based on the concentrations of the internal standard after the centrifugation, Sample D's hexane recovery rate was about 23% lower than that of the Sample A. A larger portion of hexane would stay with the algal residue in Sample D and was discarded, causing additional loss of lipids.

Doubling the RF heating time (Sample E) showed moderate positive effect on the biodiesel production. The yields were 35.1 ± 1.2% and 58.6 ± 4.5% after the two stages, respectively, which account for 17% increase for both the first stage and overall conversion rates. With the prolonged heating and reaction time, more FFAs and TAGs were able to be converted. However, the moderate increase suggests that it was difficult to convert more with the base trial's reactant composition (Sample A). So, the increases in catalyst and/or methanol amounts were more effective.

In our future work, further optimization of the reaction parameters will be carried out to achieve higher conversion rate.

3.6. FAME composition

The composition of the resulted FAMEs was determined by the GC analysis. The major components were C16:0, C18:1, C18:2 and C18:3 for all the samples, although the relative

amounts varied slightly from sample to sample. The FAME composition of the sample with the highest yield (sample B, Table II-1) is shown in Fig. II-6 as a representative, which accounted for 28.08%, 26.26%, 25.82% and 11.96% of C16:0, C18:1, C18:2 and C18:3, respectively. The profiles were mainly composed of saturated and unsaturated fatty acids with 16 or 18 carbons, which is in accordance with previous studies (Ehimen et al., 2010; S. Velasquez-Orta et al., 2012; Yao et al., 2015).

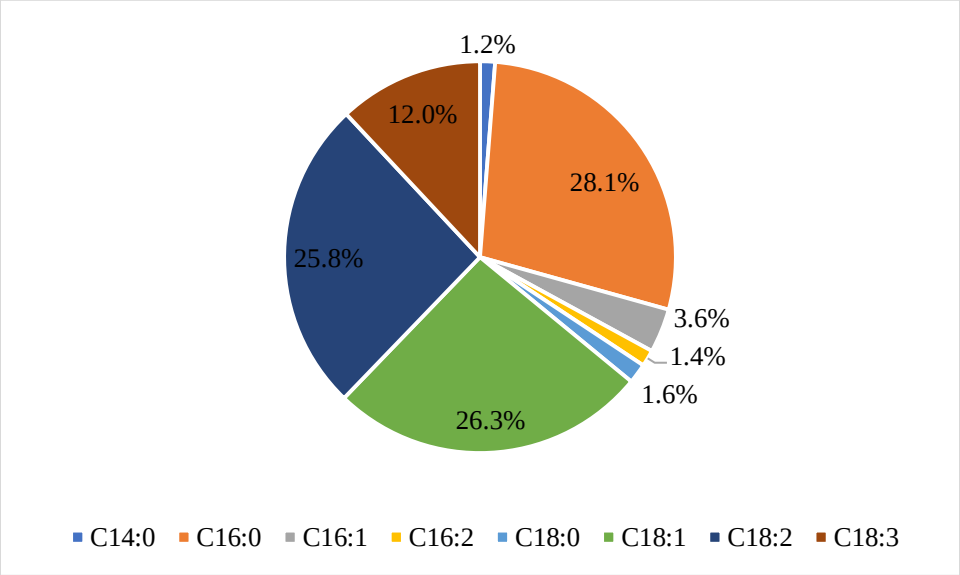

Fig II-6. FAME composition (relative weight percent)

4. Conclusions

This study provided an effective biodiesel production procedure directly from wet microalgae. With the assistant of RF heating, the cell wall of algae was disrupted, and esterification/transesterification reactions were promoted. Compared with other studies, the

whole process in this work avoided high temperature or pressure, saved large quantities of solvents and eliminated the process of drying algae. It is safer and more economically feasible for industrial application. The highest FAME yield was 79.5 ± 3.0% in this study, which could be possibly further improved by increasing the amounts of catalyst and/or methanol. In addition, the carbohydrate residual could be used for further production of bio-products. Although this pathway was developed on *Chlorella vulgaris*, it can be applied to any other microalgae which are rich in FFAs and TAGs.

Chapter III Application of response surface method for optimization of biodiesel production directly from wet microalgae assisted by radio frequency heating

Abstract

The procedure for biodiesel production directly from wet microalgae (*Chlorella vulgaris*) assisted by radio frequency (RF) heating was optimized in this study using response surface methodology (RSM). A three-variable, five-level central composite design (CCD) was employed to evaluate the effects of three key parameters, i.e., HCl to MeOH ratio (v/v), MeOH volume and RF heating time. An optimized point was successfully found. The best predicted FAME yield of 93.1% was obtained at HCl to MeOH ratio of 4.27 (v/v), MeOH volume of 28.5 mL and RF heating time of 19.2 min. Experiments carried out at the optimized point resulted in 92.7 ± 0.1% yield, which validated the reliability of the prediction model. All processing steps, including cell destruction, esterification and transesterification, were carried out under temperatures below 100 °C and atmospheric pressure. Therefore, no pressure-proof nor high-temperature apparatus was required. The procedure shows great potential for industrial application because of its high FAME yield, simple operation, low chemical consumption and short processing time. The principles of the procedure can also be applied to other microalgae with high lipid contents.

1. Introduction

The great variety of algae accounts for 40% of global photosynthesis (Andersen, 1992). The advantages of rapid growth, short life cycle, little competition with food and use of marginal or non-arable land make algae a promising feedstock for bio-products (Yao et al., 2015; Zhou et al., 2011). Microalgae has been recognized as a third-generation feedstock for biofuel production due to high lipid contents of some specific species (Ho et al., 2013a). The production of biodiesel from wet microalgae is usually conducted through a series of steps including drying algae, extracting algal oil by organic solvents, and converting algal oil to biodiesel with catalyst (acid or base) (Cao et al., 2013). The drying process after harvesting is highly energy consuming, and accounts for 20-30% of the total cost of the biodiesel production (Macías-Sánchez et al., 2018).

To avoid the high cost of the drying process, simplify operational procedures and increase efficiency, research pertaining to biodiesel production directly from wet microalgae has increased (C. L. Chen et al., 2015; Z. Chen et al., 2018; Macías-Sánchez et al., 2015). However, high moisture content in microalgae makes the conversion process from lipid to biodiesel much more complicated (Cao et al., 2013; C. L. Chen et al., 2015). To achieve desirable reaction efficiency and conversion rate, various strategies have been employed in previous studies including harsh conditions for pretreatment, high methanol (MeOH) to algae ratio, high catalyst to microalgae ratio, extremely high temperature and pressure, long reaction time, and super critical conditions (Im et al., 2014; B. Kim et al., 2015; Pan et al., 2017; Park et al., 2017;

Skorupskaite et al., 2016). The high operation cost associated with these harsh reaction conditions becomes a major obstacle for their commercial applications.

Microalgae cell walls are chemically complex and structurally robust, which is a significant obstacle to the utilization of algal oil (Gerken, Donohoe, & Knoshaug, 2013). Suitable pretreatment is one of the most critical steps to destruct the cell walls for biodiesel production (Choi, Nguyen, & Sim, 2010). Pretreatment can break cell walls, increase accessibilities of solvents and chemicals, promote the conversion rate of algal oil, and subsequently improve production efficiency and reduce cost. Among the different pretreatment methods, the costs from low to high are physical, chemical and enzymatic methods (Hernández, Riaño, Coca, & García-González, 2015; Talebnia, Karakashev, & Angelidaki, 2010; Tao et al., 2011). However, due to the enormous diversity of microalgae, no standard pretreatment method has yet been developed that can be applied for pretreating most microalgae species efficiently.

Microwave heating is a widely used physical pretreatment method. As a dielectric heating method, the advantages of microwave heating include effective cell wall disruption, excellent recovery of bioactive components, relatively low energy input, fast heating, short treatment time, and reduced solvent usage (Al Hattab, Ghaly, & Hammoud, 2015; J. Kim et al., 2013). In addition, microwave heating shows great accelerative effect on biodiesel conversion and has been employed in studies of direct/one-step biodiesel production (Barnard, Leadbeater, Boucher, Stencel, & Wilhite, 2007; C. L. Chen et al., 2015; Leadbeater & Stencel, 2006; G. Ma et al., 2015; Wahidin et al., 2018). Radio frequency (RF) heating is another dielectric heating method

with a mechanism similar to microwave heating. Compared to microwave, RF has greater penetration depth because of its longer wavelength, which is more suitable for destructing complicated cell walls. Moreover, RF heating equipment has simpler configuration, bigger loading cavity, higher efficiency on converting electricity to electromagnetic power, and compatibility with metal (Liu et al., 2008; Y Wang et al., 2003; Yifen Wang et al., 2003). RF heating has been successfully used to pretreat various biomaterials or accelerate biodiesel production in our previous studies (Liu et al., 2010; Liu et al., 2011; X. Wang, Taylor, et al., 2016; X. Wang, Zhang, et al., 2016a, 2016b).

Recently, a procedure of biodiesel production directly from wet microalgae assisted by RF heating was established by our group (Y. Ma et al., 2019). Wet microalgae were first pretreated by RF heating. Then, a two-stage biodiesel conversion was carried out. In the first stage, HCl was used to catalyze largely esterification which converts free fatty acids to fatty acid methyl esters (FAME, biodiesel). After extracting with hexane, the mixture of microalgae sample was centrifuged. The hexane phase was separated, and the solvent was evaporated. After that, NaOH was used in the second stage to catalyze transesterification. RF heating was used in both stages to accelerate the reactions. A good conversion rate was achieved, albeit optimization of the operational parameters have yet to be optimized. Hence, the goal of this work was use response surface methodology (RSM) to evaluate the effects of HCl to MeOH ratio (v/v), MeOH volume and RF heating time in the first stage of conversion. A three-variable, five-level central composite design (CCD) was employed to investigate the FAME yield.

2. Materials and methods

2.1. Chemicals and microalgae

Chlorella vulgaris was cultivated in Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University's. After harvesting, the biomass was dewatered in a Lavine centrifuge to approximately 20% solids and was immediately frozen at -10 ºC in airtight bags until use. All chemicals were of either analytical grade or GC grade, and were purchased from VWR International (Radnor, PA, USA).

2.2. Determination of moisture and total lipid contents in the microalgae

The moisture content of microalgae was measured by a moisture analyzer (MF-50, A&D Company, Limited, Japan) in duplicate. The total lipid was analyzed based on the Folch method (Folch, Lees, & Stanley, 1957) with minor modification. A 20 mg of lyophilized algae biomass was mixed with 1.5 mL of Folch solvent (2:1 chloroform/MeOH). After bead beating for 100 s at a speed of 6.5 m/s, another 4.5 mL of Folch solvent and 1.2 mL of 0.9% (w/v) NaCl solution was added for phase separation. The lower phase was recovered and evaporated until constant weight reached. Then the moisture was calculated as wet base moisture content.

2.3. Experimental design and statistical analysis

The production of biodiesel by esterification and transesterification seems simple chemical reactions, though there are multiple factors affecting the yield of biodiesel, such as moisture content of algae, reaction time and temperature, type of catalysts and solvents, mixing ratio among biomass (algae in this study), catalyst and solvent, etc. According to our previous study, catalyst (HCl) and MeOH amounts and RF heating time were the three most important factors affecting the yield (Y. Ma et al., 2019).

Experimental design is an effective way to have logical and systematic data collection and analysis in research (S. Velasquez-Orta et al., 2012). RSM is a widely used experimental design method which can effectively optimize processes affected several factors with possible interactions (İbanoğlu & Ainsworth, 2004; Varnalis, Brennan, MacDougall, & Gilmour, 2004). To reduce the number of experimental trails, CCD is often used when the number of factors is high (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008; Sun, Liu, & Kennedy, 2010). In this work, a three-variable, five-level CCD was employed to optimize the biodiesel production directly from wet algae (Liu et al., 2011; Xu & Ting, 2004). Table III-1 lists the three variables and five levels. For statistical calculation, the variables were coded based on Eq. (1):

$$x_i = \frac{X_i - X_i^*}{\Delta X_i} \quad (1)$$

where x_i is the coded value of the *i*th variable, X_i is the actual value of the *i*th variable, X_i^* is the actual center value of X_i, and ΔX_i is the step size.

Experiment results were analyzed by linear multiple regression, which were fitted using the following quadratic polynomial model (Liu et al., 2011):

$$Y = A_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} C_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} D_{ij} x_i x_j \quad (2)$$

where Y represents the response function, x_i are input variables, and A_0, B_i, C_{ii} and D_{ij} are regression coefficients. Microsoft Excel for Office 365 was used for regression analysis. Data analysis software, Origin 9.0 (OriginLab Corporation, Northampton, MA 01060) was used to draw response surface plots.

Table III-1. Levels of variables used in the experimental design

Independent variables	Range and levels				
	-2	-1	0	1	2
HCl to MeOH ratio (v/v)	2	2.75	3.5	4.25	5
MeOH (mL)	10	15	20	25	30
RF heating time (min)	10	15	20	25	30

2.4. Biodiesel production

2.4.1. Microalgae cell disruption with RF heating

Based on the method described in our previous study (Y. Ma et al., 2019) with minor modification, 140 g of wet microalgae was mixed with 2.5% (w/w dry biomass) phosphoric acid (85%) in a 250 mL glass beaker. Then, the beaker was covered with a plastic wrap to minimize water loss. A RF heater (SO6B; Strayfield, Berkshire, England) operated at a frequency of 27.12 MHz was used to heat up the mixture. The maximum output power of the RF heater was 6 kW. A fiber-optic sensor (Neoptix, Inc., Quebec City, Quebec, Canada) was used to monitor the

temperature during the heating process. After reaching 85 ºC, the RF heater was switched on and off to keep the sample temperature at 85 ± 2 ºC for 20 min.

2.4.2. First stage of conversion

Based on CCD, various aliquots of HCl (36%) and MeOH was added into 5.0 g of pretreated algal sample in a 125 mL Erlenmeyer flask, respectively. Then, the sample was stirred for 5 min with a magnetic stirring bar and heated to 55 ± 2 ºC with RF heater for predetermined time. As shown in the schematic diagram (Fig. III-1), a condenser was used to reduce the evaporation of MeOH during the RF heating. After the first stage of conversion, an extraction was carried out with 10 mL of hexane. The flask was sealed with parafilm and shaken at 200 rpm under room temperature for 1 hour. After the extraction, 30 mL of deionized water and 10 μL of internal standard (methyl tridecanoate) were added to the mixture. Then, the sample was centrifuged at 9,900 rpm for 10 min (Thermo Scientific Sorvall Legend RT+, Waltham, MA, USA). The upper hexane phase was collected for FAME analysis and the second stage conversion.

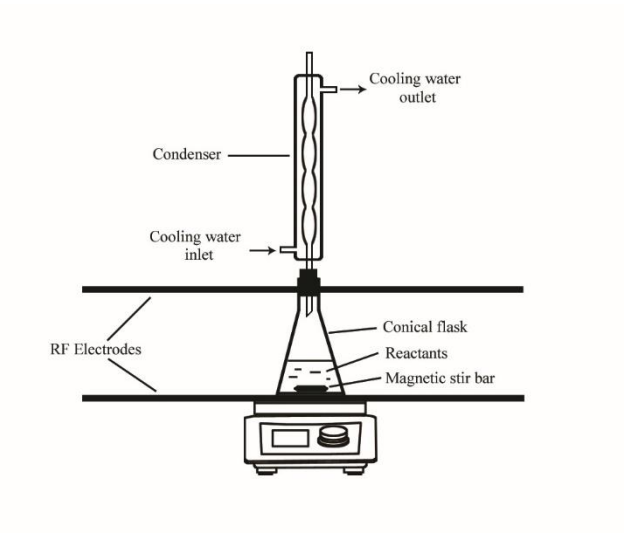

Fig III-1. Schematic diagram of RF heating apparatus

2.4.3. Second stage of conversion

According to preliminary experiments, the conditions used in our previous study (Y. Ma et al., 2019) was sufficient enough for complete biodiesel conversion; hence it was adopted for this work. Before the conversion, the hexane in the liquid phase obtained from the first stage was recovered through evaporating with a rotary evaporator at 45 ^{o}C. In the second stage of conversion, 10 mL of NaOH-MeOH solution (NaOH to MeOH, w/v, 0.5:100) was added. The mixture in an Erlenmeyer flask was heated with RF to 55 ^{o}C for 10 min. Then another 10 mL of hexane was added to extract FAME for conversion rate analysis.

2.5. FAME analysis

The FAME content was analyzed according to NERL method (Van Wychen et al., 2016) with minor modification. An Agilent 7890 GC (Santa Clara, CA, USA), equipped with a DB-WAXetr capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, Santa Clara, CA) and a flame ionization detector (FID) was used. The oven temperature was programmed from 100 ºC (held for 1 min) to 240 ºC (held for 15 min) at a rate of 4 ºC/min. The temperature of FID detector and injector was 260 ºC. The flow rate of helium was 1 mL/min and the inlet split ratio was 5:1. The hexane phase after first and second stage of conversion were analyzed, respectively. The FAME yield was calculated by Eq. (3):

$$\text{FAME yield (\%)} = \frac{FAME\ (g)}{Total\ lipid\ (g)} \times 100\% \quad (3)$$

3. Results and discussion

3.1. Microalgae moisture, lipid content and FAME composition

The original moisture content of *Chlorella vulgaris* microalgae was 77.0 ± 0.2%. After RF heating for cell disruption, the moisture content decreased to 74.2 ± 0.1%. The RF heating pretreatment only evaporated limited amount of water and slightly reduced the moisture content, which avoids the intensive energy consumption of drying algae. The total lipid of the microalgae was 23.4 ± 0.7% based on dry biomass.

The FAME composition was determined by the GC analysis and the main components were saturated and unsaturated fatty acids with 16 or 18 carbons. The composition of FAME after the

second stage of conversion of the sample at CCD central point (code value 0, 0, 0) is shown in Fig. III-2 as a representative. The major components were C16:0, C18:1, C18:2 and C18:3, which accounted for 32.0%, 25.9%, 24.4% and 11.0%, respectively.

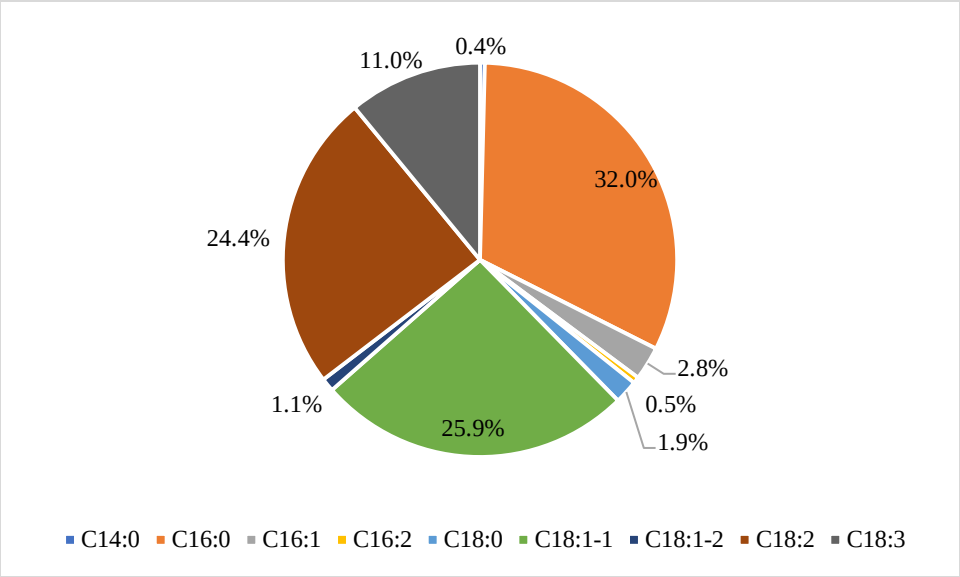

Fig III-2. FAME composition of sample at central point (relative weight percent)

3.2. Statistical analysis and the model fitting

A total of 20 runs were carried out in the first stage of conversion for optimizing the FAME yields. The FAME yields of samples after both of the stages of conversion were determined respectively and the predict value was obtained according to the quadratic polynomial model (Eq. (2)). The CCD matrix and experimental results are shown in Table III-2.

Table III-2. Response surface central composite design matrix and FAME yields

Run	Natural value[a]			Code value			FAME yield (%) after the first stage		FAME yield (%) after the second stage	
	X_1	X_2	X_3	x_1	x_2	x_3	Observed	Predicted	Observed	Predicted
1	2.75	25	15	-1	1	-1	61.3	64.2	74.3	77.4
2	4.25	15	25	1	-1	1	61.4	58.2	61.7	61.2
3	3.5	20	20	0	0	0	63.0	66.1	72.7	75.7
4	2.75	25	25	-1	1	1	74.7	73.9	79.5	81.0
5	2.75	15	25	-1	-1	1	44.3	44.5	46.9	47.2
6	3.5	20	20	0	0	0	63.4	66.1	74.4	75.7
7	2.75	15	15	-1	-1	-1	36.2	31.8	41.2	36.3
8	4.25	25	15	1	1	-1	76.8	76.4	85.2	87.5
9	3.5	20	20	0	0	0	64.7	66.1	76.3	75.7
10	4.25	25	25	1	1	1	78.7	82.9	80.8	88.2
11	4.25	15	15	1	-1	-1	48.1	48.6	52.3	53.3
12	3.5	20	20	0	0	0	69.6	66.1	81.0	75.7
13	5	20	20	2	0	0	73.3	72.7	85.3	81.4
14	3.5	20	30	0	0	2	73.3	73.0	72.3	69.2
15	3.5	20	20	0	0	0	67.1	66.1	77.5	75.7
16	3.5	20	20	0	0	0	68.6	66.1	74.8	75.7
17	3.5	10	20	0	-2	0	19.2	22.5	20.2	23.5
18	3.5	20	10	0	0	-2	53.2	53.7	57.1	57.6
19	3.5	30	20	0	2	0	82.6	79.6	97.5	91.6
20	2	20	20	-2	0	0	45.8	46.8	56.0	57.3

X_1 = HCl to MeOH ratio (v/v), X_2 = MeOH (mL), X_3 = RF heating time (min).

3.2.1. FAME yield after first stage of conversion

In the first stage of conversion, RSM was performed to investigate the effects of three independent variables, i.e., HCl to MeOH ratio (v/v), MeOH volume and RF heating time, using a three-variable, five-level CCD. The response variable and the test variables were related by applying multiple linear regression analysis on the experimental data, and the regression result is shown below:

$$Y = 66.11 + 6.47x_1 + 14.27x_2 + 4.81x_3 - 1.60x_1^2 - 3.76x_2^2 - 0.69x_3^2 - 1.18x_1x_2 - 0.79x_1x_3 - 0.76x_2x_3 \quad (4)$$

where Y represents the FAME yield, x_1, x_2 and x_3 are the code values of HCl to MeOH ratio (v/v), MeOH volume and RF heating time, respectively.

The analysis of variance (ANOVA) results of the model were shown in Table III-3. The whole regression model was significant ($P << 0.05$). The goodness of fit of the model was examined by determination coefficient R^2 and adjusted R^2. The R^2 represents a proportion of total variation in the response predicted by the model, and the adjusted R^2 corrects the R^2 value for sample size and number for terms in the model (Macías-Sánchez et al., 2018). The R^2 indicated that 98.8% of the total variations were explained by the model. The value of the adjusted R^2 was 0.954, which also confirmed that the model was highly significant and indicated a high correlation between the observed and predicted values.

Table III-3. ANOVA analysis for Eq. (4)

	df	SS	MS	F	p-value
Regression	9	4694.33	521.59	44.63	<0.01
Residual	10	116.86	11.69		
Total	19	4811.19			
R^2=0.988	Adjusted R^2 =0.954				

The regression coefficient results of Eq. (4) were listed in Table III-4. The significance of each coefficient was tested by Student's t-test. A larger magnitude of t-test and smaller P-value suggest a more significant corresponding coefficient (Zheng et al., 2008). The results confirmed that all the three independent variables chosen in this study, i.e., HCl to MeOH ratio (v/v), MeOH volume and RF heating time, had a significant influence on the FAME yield after the first stage of conversion ($P << 0.05$). Also, the quadric effects of HCl to MeOH ratio (v/v) and MeOH volume were significant.

Table III-4. Estimated regression coefficients for Eq. (4)

	Coefficients	Standard Error	t-value	P-value	Lower 95%	Upper 95%
Intercept	66.11	1.36	48.48	<0.01	63.07	69.15
x1	6.47	0.85	7.57	<0.01	4.56	8.37
x2	14.27	0.85	16.70	<0.01	12.37	16.18
x3	4.81	0.85	5.63	<0.01	2.90	6.71
x1*x1	-1.60	0.68	-2.34	<0.01	-3.12	-0.08
x2*x2	-3.76	0.68	-5.52	<0.01	-5.28	-2.24
x3*x3	-0.69	0.68	-1.01	0.34	-2.21	0.83
x1*x2	-1.18	1.21	-0.97	0.35	-3.87	1.52
x1*x3	-0.79	1.21	-0.65	0.53	-3.48	1.91
x2*x3	-0.76	1.21	-0.63	0.54	-3.45	1.93

3.2.2. FAME yield after second stage of conversion

After the second stage of conversion, FAME yields of the 20 runs of samples were also regressed. The correlation is given by the following equation:

$$Y = 75.71 + 6.04x_1 + 17.02x_2 + 2.88x_3 - 1.58x_1^2 - 4.55x_2^2 - 3.08x_3^2 - 1.70x_1x_2 - 0.74x_1x_3 - 1.79x_2x_3 \quad (5)$$

where Y represents the FAME yield, x_1, x_2 and x_3 are the code values of HCl to MeOH ratio (v/v), MeOH volume and RF heating time, respectively. The coefficient of determination value indicated that only 3.4% of the total variations were not explained by the model (Table III-5) and the model fitted well ($P << 0.05$). The regression coefficient of the three variables, and the quadric effects of MeOH volume and RF heating time were significant (Table III-6). No evident interaction was observed among the three investigated variables.

Table III-5. ANOVA analysis for Eq. (5)

	df	SS	MS	F	p-value
Regression	9	6040.59	671.18	31.48	<0.01
Residual	10	213.21	21.32		
Total	19	6253.80			
R^2=0.966	Adjusted R^2 =0.935				

Table III-6. Estimated regression coefficients for Eq. (5)

	Coefficients	Standard Error	t-value	P-value	Lower 95%	Upper 95%
Intercept	75.71	1.84	41.11	<0.01	71.60	79.81
x1	6.04	1.15	5.23	<0.01	3.46	8.61
x2	17.02	1.15	14.74	<0.01	14.45	19.59
x3	2.88	1.15	2.50	0.03	0.31	5.45
x1*x1	-1.58	0.92	-1.72	0.12	-3.64	0.47
x2*x2	-4.55	0.92	-4.94	<0.01	-6.60	-2.50
x3*x3	-3.08	0.92	-3.34	<0.01	-5.13	-1.03
x1*x2	-1.70	1.63	-1.04	0.32	-5.34	1.93
x1*x3	-0.74	1.63	-0.45	0.66	-4.38	2.90
x2*x3	-1.79	1.63	-1.10	0.30	-5.43	1.85

3.3. Effects of independent variables in the first stage of conversion

A set of three-dimensional response surface plots was presented in Fig. III-3 to illustrate the effects of the three independent variables in the first stage of conversion. According to Eq. (4), one of the three variables was fixed at the central point of the CCD and the other two were varied within their ranges. Fig. III-3A shows the effects of MeOH volume and RF heating time at a fixed HCl to MeOH ratio of 3.5 (v/v), while Fig. III-3B and C illustrate the response surfaces where the MeOH volume was kept at 20 mL and RF heating time was kept at 20 min, respectively. In the lower ranges of the variable values, all of the three independent variables showed strong positive effects on FAME yield, led by the volume of MeOH. When the variables approach to their high ends, their effects gradually fade out and a plateau is observed.

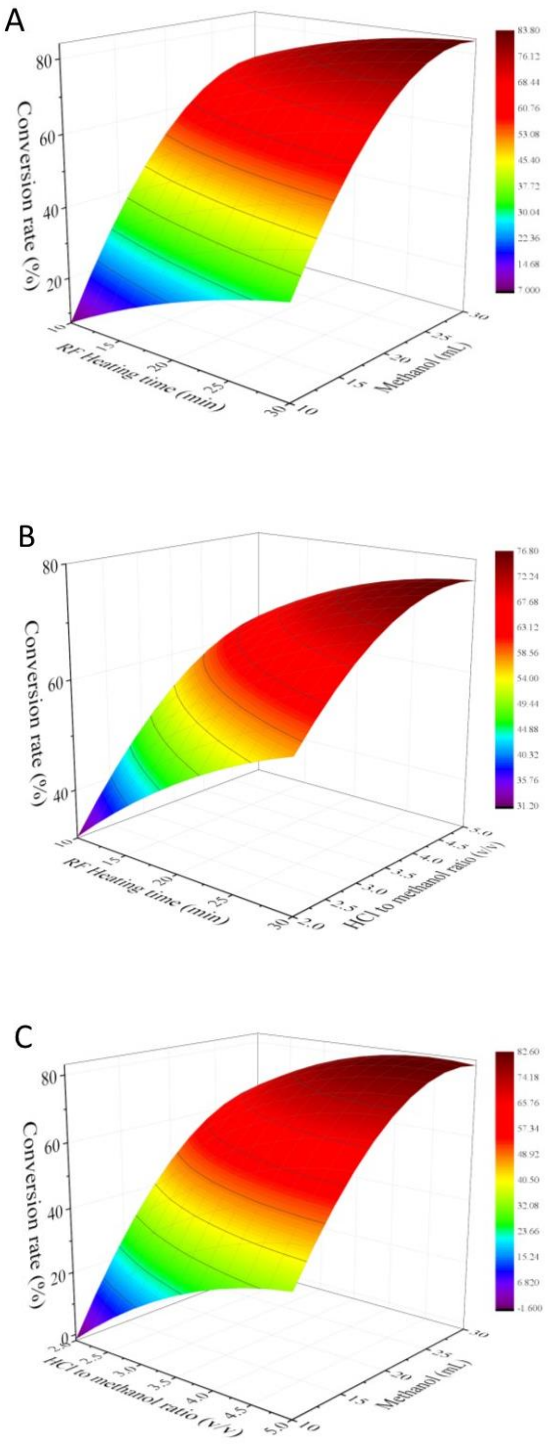

Fig III-3. Response surface plots of FAME yield at the central point of the CCD: (A) fixing HCl to MeOH ratio at 3.5 (v/v); (B) fixing MeOH at 20 mL; (C) fixing RF heating time at 20 min.

3.4. Maximum FAME yields and Model Validation

In the first stage of conversion, a maximum FAME yield of 82.9% was achieved with the HCl to MeOH ratio (v/v) of 4.45, MeOH volume of 27.5 mL and RF heating time of 24.2 min according to Eq. (4). The final conversion rate after the second stage can reach as high as 93.1% at the point of HCl to MeOH ratio 4.27 (v/v), MeOH 28.5 mL and RF heating time 19.2 min based on Eq. (5). The optimal conditions for the two stages were slightly different.

According to Yao et al. (2015), free fatty acids (FFAs) roughly account for 50% of the total saponifiable lipids in *Chlorella* (Yao et al., 2015). The maximum FAME yield of the first stage could reach much higher than 50%, indicating that the acid (HCl) might be able to catalyze not only esterification of FFAs but also transesterification of TAGs under suitable conditions in the first stage. The predicted maximum FAME yield after the second stage was notably improved compared to that after the first stage, suggesting that the alkali-catalyzed (NaOH) transesterification in the second stage efficiently converted remained lipids, like triacylglycerols (TAGs), to FAME. To obtain the highest possible conversion, the two stages of conversion were indispensable. The algal biomass contained a number of unsaponifiable lipids, such as hydrocarbons, sterols, waxes and pigments. They could not be converted into FAME (Macías-Sánchez et al., 2018) and prevent the FAME yield to reach 100% in this work.

Experiments at the optimal conditions of the overall conversion (HCl to MeOH ratio 4.27 (v/v), MeOH 28.5 mL and RF heating time 19.2 min) were carried out to validate the statistical

model. A very close FAME yield of 92.7 ± 0.1% was obtained, which confirmed that the RSM model was adequate to find the optimized conditions in the experimental range.

In previous study, several recent studies of direct biodiesel production from wet microalgae were summarized and compared (Macías-Sánchez et al., 2018). There was a trend that the FAME yield tended to increase considerably with the increase of MeOH concentration, and the MeOH volume required was depended on the water content of the reaction medium (T.-H. Kim et al., 2015; Wahlen, Willis, & Seefeldt, 2011). The results were consistent with this study that MeOH showed the greatest impact on the FAME yield. They also optimized the direct transesterification from wet Eustigmatophyceae *Nannochloropsis gaditana*. The best FAME yield of 100% of the saponifiable lipid (SL) (95.6% of total lipids) was obtained with MeOH/SL ratio of 254 mL/g, a sulfuric acid concentration of 3.7% (v/v) and a hexane/SL ratio of 107.7 mL/g in a thermostatic stirred tank reactor at 100 ^{o}C for 105 min with a pressure of 2.5 atm (Macías-Sánchez et al., 2015; Macías-Sánchez et al., 2018). In our study, the FAME yield of 92.7 ± 0.1% was obtained by using 127.5 mL of MeOH per gram of total lipid (total amount of the two stages of conversion), which was only about half of the amount used in Macías-Sánchez et al.'s study. The total hexane amount we used was 66.3 mL per gram of total lipid, and most of it could be recovered. Most importantly, the whole conversion was carried out at 55 ^{o}C under normal pressure for less than 30 min (total reaction time for two stages of conversion) and no special container was required, which would significantly lower the cost for commercial

application. The optimized method could save large amounts of reagents and reaction time, which provides a potential for commercial production, especially for continuous process.

4. Conclusions

This study evaluated the essential parameters for the biodiesel production directly from wet microalgae assisted by RF heating using RSM. The production procedure consisted of two stages of conversion: acid-catalyzed first stage and base-catalyzed second stage. A three-variable, five-level CCD was employed to optimize the parameters of HCl to MeOH ratio (v/v), MeOH volume and RF heating time. The results showed that both of the two conversion stages were indispensable. The response surface plots illustrated that the MeOH volume had the largest impact on FAME yield. The maximum predicted FAME yield of 93.1% was obtained at HCl to MeOH ratio 4.27 (v/v), MeOH 28.5 mL and RF heating time 19.2 min. The validation experiments confirmed that the RSM model was adequate. Although this biodiesel production procedure was developed on *Chlorella vulgaris*, it can be applied to other microalgae.

Chapter IV Fermentable sugar production from wet microalgae residual after biodiesel production assisted by radio frequency heating

Abstract

In this study, the feasibility of comprehensive recovery of lipid and carbohydrate in wet microalgae *Chlorella vulgaris* was explored. First, four sets of enzyme combinations of α-Amylase, Amyloglucosidase and CTec2 were evaluated for hydrolysis efficiency on microalgae disrupted with radio frequency heating. Then, the most suitable combination was applied to raw microalgae and microalgae residual after biodiesel production, respectively, for saccharification. Adsorption kinetics of the optimized enzyme combination on the aforementioned three samples were determined and adsorption isotherm was analyzed by Freundlich equation. Morphology of microalgae was also investigated by scanning electron microscopy. A yield of reducing sugars in microalgae residual at 53.7% was obtained after 72 hours saccharification. The results from enzyme adsorption kinetics, isotherm and SEM images were consistent with each other. This study demonstrated that the microalgae residual after biodiesel production could be used as carbohydrate feedstock for fermentable sugar production through simple enzymatic hydrolysis.

1. Introduction

Microalgae grows almost everywhere in the earth from marine water to brackish water to fresh water (ponds, puddles, canals, and lakes) as well as terrestrial ecosystem due to its simple structure (unicellular or simple multicellular), rapid growth rate, and huge variety (more than 50000 species) (Hamed, 2016). The major biochemical composition of microalgae are proteins, lipids and carbohydrates (Du et al., 2013). As early as 1950s, protein-rich microalgae was initiatively studied and considered as an alternative resource to close the "protein gap" for animal feed and human food (Becker, 2007). However, it is food and feed related resource rather than bioenergy and biomaterial related one. Lipids and carbohydrates, as the other two major compositions, are the products of fixation of carbon dioxide by microalgae (Y. Ma et al., 2019).

With the growth of population and the development of economy, people's demand for energy, majorly fossil fuel suppliers, is growing. However, fossil fuel is not a sustainable feedstock and it causes more greenhouse effect than biofuel does (Rajvanshi & Sharma, 2012). Compared with other biofuels, such as biogas, ethanol, butanol etc., biodiesel is an immediately applicable replacement for fossil-based diesel (Liu et al., 2008). Previously, biodiesel was generally produced by transesterification of triglyceride of virgin oil, waste cooking oil and animal fat, with methanol or ethanol using either alkaline or acidic catalyst (Liu et al., 2010; Liu et al., 2011). Since early 2010s, studies started to report producing biodiesel from dried microalgae through transesterification (Carvalho Júnior et al., 2011; Velasquez-Orta et al., 2012). In recent years, biodiesel production directly from wet microalgae has become a research hotspot

due to the advantage of avoiding high-energy consumption of drying process (Chen et al., 2015; Chen et al., 2018; Macías-Sánchez et al., 2015; Macías-Sánchez et al., 2018).

In the meantime, studies on fermentable sugars production using carbohydrate-rich microalgae becomes more and more popular because microalgae is considered to be the third generation biomass to replace starch and lignocellulosic feedstock (normally known as the first and the second generation feedstock) for fermentable sugars (Ho et al., 2013a; K. H. Kim et al., 2014). Carbohydrate exists mainly in microalgae either in the form of cellulose without lignin and hemicellulose in their cell wall, or in the form of starch in their cytoplasm. As compared with lignocellulosic feedstock, it is much easier to convert microalgae carbohydrate into monosaccharides (Ho et al., 2013a, 2013b).

In the nature, several major microalgal species used for biodiesel production have high content of both lipid and carbohydrate. Yao et al. (Yao et al., 2015) reported that *Chlorella vulgaris* has 43.4% carbohydrate (dry base), *Nannochloropsis sp.* has 37.3%, *Chlamydomonas reinhardtii* has 35.5%, *Scenedesmus sp.* has 35.4%, and *Schizochytrium limacinum* SR-21 has 25.3 %, respectively. Shakya et al. (Shakya et al., 2017) reported there are 30.28 % lipids and 49.70% carbohydrates (dry base) in *Chlorella* (C-2), and 35.66 % lipids and 50.40% carbohydrates in *Scenedesmus* (S-1). In order to make the best use of microalgae and lower the costs of biodiesel production and fermentable sugars production, both lipid and carbohydrate in microalgae should be completely utilized. However, there is no report so far on comprehensive

utilization of carbohydrates in microalgae residual after lipid extraction or biodiesel production directly from wet algae.

In our previous study, wet microalgae *Chlorella vulgaris* was disrupted by radio frequency (RF) heating first, followed by *in situ* transesterification for biodiesel production (Y. Ma et al., 2019). Then, the mixture of wet microalgae, methanol, HCl, hexane and water was centrifuged to separate liquid and solid phases. Due to its uniform heating pattern, easy to operate, high efficiency on converting electricity to electromagnetic power, and possibility of using metal (Liu et al., 2008; Y Wang et al., 2003; Yifen Wang et al., 2003), RF heating has recently been used to pretreat various biomass including switchgrass and sweetgum for enhanced production of glucan, acetone–butanol–ethanol (ABE) and polyhydroxybutyrate (PHB) (X. Wang, Taylor, et al., 2016; X. Wang, Zhang, et al., 2016a, 2016b).

In the present study, enzymatic saccharification of the carbohydrates left in microalgae residual (solid phase from previous study) after *in situ* biodiesel production directly from wet *C. vulgaris* was explored, so that the whole microalgal biomass could be comprehensively utilized. Four sets of enzyme combinations (α-Amylase, Amyloglucosidase and CTec2) were tested, and the most suitable one was selected and applied to hydrolyze microalgae residual after biodiesel production, with raw microalgae as negative control, RF heating disrupted microalgae as positive control. Adsorption kinetics of the optimized enzyme combination on the aforementioned three samples were determined and adsorption isotherm was analyzed by Freundlich equation. Morphology of microalgae was also investigated by a scanning electron microscope (SEM).

2. Materials and methods

2.1. Microalgae, chemicals and enzymes

Microalgae biomass of *Chlorella vulgaris* was provided by Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University. Three types of microalgal samples subject to saccharification were raw microalgae (referred as 'RWMA' hereafter), RF heating disrupted microalgae (referred as 'RFMA' hereafter) and microalgae residual after biodiesel production (referred as 'RSMA' hereafter). All chemicals were of analytical grade and purchased from VWR International. The commercial enzyme cocktail Cellic CTec2 was obtained from Novozymes (Franklinton, NC, USA) with enzyme activity of 94.37 FPU/mL determined by Whatman #1 filter paper as the substrate. The α-Amylase from *Aspergillus oryzae* and Amyloglucosidase from *Aspergillus* were obtained from Sigma-Aldrich (St. Louis, MO, USA). The enzyme activity of α-Amylase and Amyloglucosidase was 1130 FAU/mL (FAU: fungal amylase unit) and 260 U/mL, respectively. More detailed information about these enzymes is shown in Table IV-1.

Table IV-1. Detailed characteristics of the enzymes used in this study*

	Source	Optimum temperature	Optimum pH	Substrate specificity
α-Amylase	*Aspergillus oryzae*	50-55 ^{o}C	5.0	α-1,4 glycosidic bonds in soluble starches and related substrates
Amyloglucosidase	*Aspergillus niger*	60 ^{o}C	4.5	1,4- as well as 1,6-alpha-linkages in liquefied starch
CTec2	A blend of aggressive cellulases, high level of β-glucosidases and hemicellulase	45-50 ^{o}C	5.0-5.5	Cellulose, hemicellulose, and lignin

*Based on the information provided by the manufacturers/vendors.

2.2. RF heating disrupted microalgae (RFMA)

Two hundred grams of wet microalgae was placed in a 250 ml glass beaker and 2.5% (w/w dry biomass) phosphoric acid (85%) was added to promote cell wall disruption. The inorganic phosphate is a constituent of nucleic acids, nucleotides, phospholipids, lipopolysaccharides (LPS), teichoic acids in microorganisms, which can also be used as nutrient sources during subsequent fermentation (Boonmanumsin et al., 2012; Nieves, Geddes, Miller, et al., 2011; Nieves, Geddes, Mullinnix, et al., 2011). The beaker with algae was heated in an RF heater (SO6B; Strayfield, Berkshire, England) at a frequency of 27.12 MHz with maximum output power of 6 kW. To make the heating uniform, a magnetic bar was used to stir the wet microalgae. A fiber-optic sensor (Neoptix, Inc., Quebec City, Quebec, Canada) was used to monitor the temperature during heating process. The beaker was covered with a plastic sheet to

reduce water loss. After reaching 90 ºC, the RF heater was switched on and off to keep the sample temperature at 90 ± 2 ºC for 20 min (Y. Ma et al., 2019).

2.3. Microalgae residual after *in situ* transesterification for biodiesel production (RSMA)

As described earlier (Y. Ma et al., 2019), 120 g of RFMA was used for RSMA preparation. The 192.0 mL of HCl-MeOH mixed solution (36% HCl to MeOH, v/v, 5:95) and 48 mL of MeOH were added into 5 RF-heating-disrupted microalgal samples. The mixture was stirred and heated in RF heater at 55 ºC for 20 min. Most of the free fatty acids (FFAs) and a small portion of other lipids in the algae were converted to FAME (biodiesel) during this acid catalyzed esterification assisted with RF heating. Then, 240 mL of hexane was added into the flask and shaked for 1 hour to extract FAMEs, other lipids and remained FFAs. One hundred and fifty mL of deionized water was added after the extraction and the mixture was centrifuged at 9900 rpm for 10 min (Thermo Scientific Sorvall Legend RT+, Waltham, MA, USA). The liquid phase was used for further conversion to biodiesel through transesterification. The solid phase, RSMA, was air dried in a fume hood for 48 hours and used for the following saccharification in this study.

2.4. Enzymatic saccharification

2.4.1. Optimization of enzyme combination

The RFMA was utilized to optimize the enzyme combination for two reasons. First, it was used as a positive control which could act as a link to the untreated sample (RWMA) and the

RSMA, the response to the treatment of which was unknown. Secondly and more importantly, the pretreated RFMA did not contain organic solvents or at least was not treated by any organic solvent. Therefore, it could exclude the potential inhibition of organic solvents to enzymes as possibly seen in RSMA.

Before hydrolysis, the pH value of the RFMA was adjusted using NaOH. Enzymatic hydrolysis was performed by mixing 10 g of RFMA with concentrated sodium citrate buffer to reach a total volume of 50 mL (pH value of 5.0) and the final sodium citrate concentration of 50 mM. To test the effect of the various enzymes on saccharification, different enzyme combinations with various dosages were added into different samples (Table IV-2), respectively. The enzymatic saccharification of RFMA was carried out in an incubator shaker at 50 ºC and 200 rpm for 72 h. Samples were taken periodically and the reducing sugar concentration was determined with HPLC. Each enzymatic saccharification was carried out in triplicate. The best enzyme combination was selected based on the analytical results and applied to RWMA and RSMA.

Table IV-2. The various combinations of enzymes used in this study

Combination	α-Amylase (FAU/g glucan)	Amyloglucosidase (U/g glucan)	CTec2 (FPU/g glucan)
A	1500	100	-
B	1500	100	20
C	1500	300	20
D	1500	100	40

2.4.2. Enzymatic saccharification on RWMA and RSMA

The amount of RSMA used for each saccharification was calculated to make the glucan content consistent with all the other hydrolysis reaction systems (RWMA or RFMA). The best enzyme combination obtained from the previous step was added to RSMA and RWMA (negative control) respectively with other reaction conditions remaining unchanged.

2.5. Analytical methods

2.5.1. Chemical composition of algae samples

The algae samples including RWMA, RFMA and RSMA were analyzed for their moisture content and ash following the methods described by Shakya et al. (Shakya et al., 2017). The total lipid was performed based on Bligh & Dyer (Bligh & Dyer, 1959).

The carbohydrate in RWMA and RSMA was analyzed according to NREL protocol (Sluiter et al., 2008) with minor modification. The freeze-dried microalgae sample was primarily hydrolyzed with 72% sulfuric acid for an hour at 30 ºC in water bath. The hydrolysate was then diluted with distilled water to let the level of sulfuric acid reach 4%, and then autoclaved at 121 ºC for 1 hour. The supernatant was neutralized and used for sugar analysis by HPLC.

2.5.2. HPLC analysis of sugars

The sugars in hydrolysate were analyzed based on the method described by Wang et al. (P. Wang et al., 2019). An Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a refractive index detector (RID), an Aminex HPX-87P column (300 mm ×

7.8 mm (i.d.) × 9 μm; Bio-Rad, Hercules, CA) and a guard column (30 mm × 4.6 mm (i.d.), Bio-Rad, Hercules, CA) was employed. The mobile phase was Nano-pure water at an isocratic flow rate of 0.6 mL/min. During the 35 min elution, the temperature was maintained at 85 ºC. The glucose (or galactose) yield was calculated by Eqs. (1-3):

$$\text{Glucose yield of RFMA/RWMA (\%)} = \frac{Mass\ Glucan \text{ in hydrolysate}}{Mass\ Glucan\ in\ RF/raw\ material} \times 100\% \quad (1)$$

$$\text{Galactose yield of RFMA/RWMA (\%)} = \frac{Mass\ Galactose \text{ in hydrolysate}}{Mass\ Galactose\ in\ RF/raw\ material} \times 100\% \quad (2)$$

$$\text{Glucose yield of RSMA (\%)} = \frac{Mass\ Glucan \text{ in hydrolysate}}{Mass\ Glucan\ in\ residual} \times 100\% \quad (3)$$

2.6. Adsorption of enzymes on algae samples

2.6.1. Adsorption kinetics

The enzyme adsorption kinetics was investigated based on the method described by Tu et al. (Tu, Pan, & Saddler, 2009) with minor modification. The RWMA, RFMA or RSMA (0.5 g dry weight for each) was soaked in 10 mL of sodium citrate buffer (50 mM, pH 5.0) respectively with protein content at ~0.15 mg/mL of the enzyme combination (the proportion of enzyme mixture was based on the best combination selected in Section 2.4.1). The reactions were incubated in a shaker at 25 ºC and 200 rpm for 6 hours. Samples were taken periodically and the protein content was determined using Coomassie (Bradford) assay (Kruger, 2009).

2.6.2. Adsorption isotherm

The enzyme adsorption isotherm was determined according to Tu et al. (Tu et al., 2009) with minor modification. Enzyme combination with different concentrations (0.05-0.4 mg/mL)

was added to RWMA, RFMA or RSMA (0.3 g dry weight for each) respectively in 5 mL of sodium citrate buffer (50 mM, pH 5.0). The reactions were incubated at 25 ºC for 3 hours to reach equilibrium. The protein content in supernatant was determined as unabsorbed enzyme. The difference between initial enzyme dosage and unabsorbed enzyme was calculated as adsorbed enzymes. Enzyme adsorption on microalgae samples was characterized by Freundlich adsorption isotherm (Shafqat & Pierzynski, 2014).

2.7. Scanning electron microscope (SEM)

A Zeiss EVO 50VP SEM (Carl Zeiss AG, Jena, Germany) was used to exam the morphology of microalgae. Seven samples: RWMA and RFMA before enzymatic saccharification, RFMA after enzymatic saccharification by three different enzyme combinations, RWMA and RSMA after enzymatic saccharification by the best enzyme combination were placed on aluminum support stubs respectively. The osmium tetroxide was used for sample liquid fixation and the samples were then air-dried in fume hood. After drying, the samples were gold coated and the SEM images were taken at 20.00 kV at a magnification of 12.00 k×.

3. Results and discussion

3.1. The chemical composition of the algae samples

The chemical compositions including moisture content, ash, total lipid, and carbohydrate are shown in Table IV-3. The moisture content of RWMA, RFMA and RSMA was 77.0 ± 0.2%, 74.2 ± 0.1% and 12.5 ± 0.1% respectively, which indicated there was little water loss during RF pretreatment.

Table IV-3. The Chemical composition of various algae samples*****

	RWMA	RFMA	RSMA
Moisture (%)	77.0 ± 0.2	74.2 ± 0.1	12.5 ± 0.1
Ash (%)	2.9 ± 0.1	5.7 ± 0.6	2.5 ± 0.4
Total lipid (%)	23.4 ± 0.7	23.4 ± 0.7	ND
Total carbohydrates (%)	41.7 ± 0.2	41.7 ± 0.2	23.0 ± 0.7
Glucose (%)	31.5 ± 0.4	31.5 ± 0.4	23.0 ± 0.7
Galactose (%)	10.2 ± 0.2	10.2 ± 0.2	ND

*Percentages calculated from values based on dry biomass. Data are averages of duplicates.

The carbohydrates contained in the algae samples including cellobiose, glucose, xylose, galactose, arabinose and mannose were all determined. The total carbohydrates content in *C. vulgaris* was 41.7 ± 0.2%, which consisted of 31.5 ± 0.4 % glucose and 10.2 ± 0.2 % galactose. In the study by Templeton et al., silimar results were reportedthat the two major sugars in *C. vulgaris* were glucose and galactose (Templeton et al., 2012). While in RSMA, only 23.0 ± 0.7

% glucose was detected. The monosaccharide galactose mostly comes from the large fraction of galactolipids in *C. vulgaris*, which play an important role in making up the photosynthetic membranes in the growing algae cells (Siegenthaler & Murata, 2006). During biodiesel production stage, the process of lipid extraction may also extract galactolipids, so there was little galactose left in the hydrolysate of RSMA. However, the 23.0 ± 0.7 % glucose content in RSMA was still very high, even higher than that in the raw material of most microalgae species. Thus, the RSMA is of great value for further utilization.

3.2. Enzymatic saccharification of RFMA

In the cytoplasm of *C. vulgaris*, starch is the main source of carbohydrates (Subhadra & Edwards, 2010). So α-amylase should be the most important enzyme for hydrolysis. Amyloglucosidase was also added to decompose disaccharides into monosaccharides during starch hydrolysis. Besides the starch in cytoplasm, some carbohydrates are entrapped within cell wall of the algae as cellulose and hemicellulose (Hernández et al., 2015; Hu, Kurano, Kawachi, Iwasaki, & Miyachi, 1998). The enzyme cocktail CTec2 was added to hydrolyze cellulose and hemicellulose, if any, and exacerbate the disruption of cell wall to release starch from the cell.

As shown in Table IV-2, different enzyme combinations with various dosages were evaluated for microalgae saccharification. The glucose yield dynamics of RFMA over time is shown in Fig. IV-1. Through the saccharification of starch in microalgae by amylase and glucosidase, the glucose yield of RFMA by enzyme Combination A was 33.26 ± 0.09 % after 72

hours. With the addition of CTec2, the glucose yield by Combination B reached as high as 52.23 ± 0.85 %, which suggested that CTec2 likely saccharifies cellulose and hemicellulose in cell wall and effectively improves the saccharification rate of starch. In Combination C, the load of amyloglucosidase was tripled and the yield by Combination C increased to 54.59 ± 1.57 %. In the first 36 hours, the saccharification rate of RFMA by Combination C was significantly higher than that by Combination B. The large amount of amyloglucosidase in Combination C increased the hydrolysis rate of disaccharides decomposing into monosaccharides. However, the increase in glucose yield by Combination C was not significant after 72 hours, which demonstrated that the load of amyloglucosidase in Combination B was sufficient. The saccharification rate was also increased at the early stage by doubling CTec2 in Combination D. Also, the glucose yield was as high as 59.66 ± 3.00 % after 72 hours, which was significantly improved compared to that by Combination B.

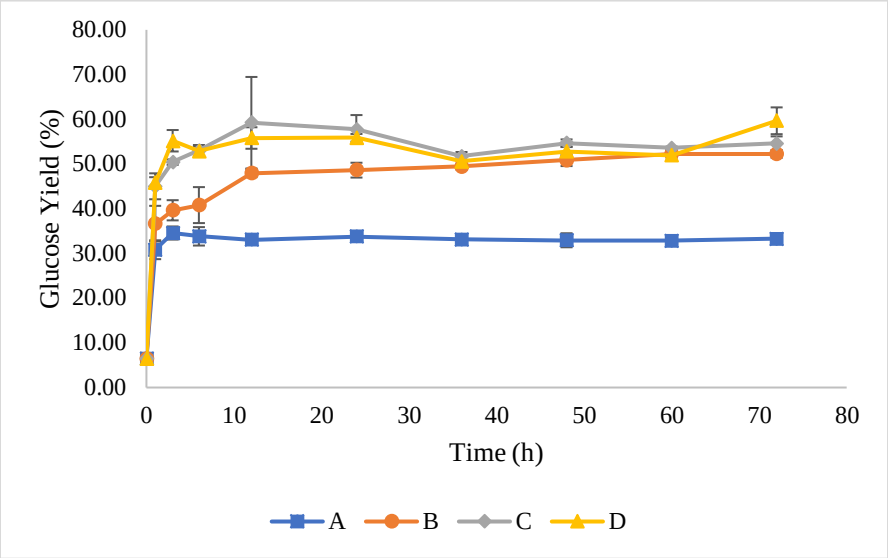

Fig IV-1. Glucose yield of RF pretreated algae saccharified by different enzyme combinations. A/B/C/D: The enzyme Combination A, B, C or D as defined in Table IV-2.

The galactose yield profile of the RFMA samples saccharified with four different sets of enzyme combination are shown in Fig. IV-2. The yield was slightly increased by adding CTec2. However, the galactose yield trends of the four samples were quite closed and had relatively large standard deviation. This was probably because the proportion of galactose in total carbohydrates of *C. vulgaris* was low and was even not presented in RSMA. Therefore, based on such saccharification results, the enzyme Combination D was selected as the best one for the following steps.

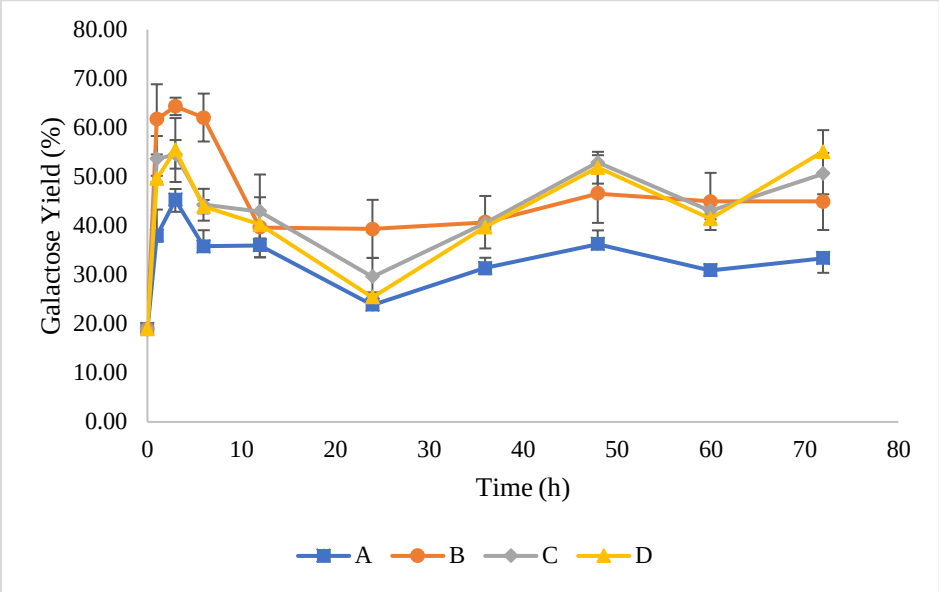

Fig IV-2. Galactose yield of RF pretreated algae saccharified by different enzyme combinations. A/B/C/D: The enzyme Combination A, B, C or D as defined in Table IV-2.

3.3. Saccharification of RWMA and RSMA

The enzyme Combination D was applied to RSMA and RWMA for saccharification. As shown in Fig. IV-3, the glucose yield of RWMA was only 32.23 ± 0.95 %, which was much lower than that of the other two samples. This proved that RF heating disruption could effectively improve the efficiency of saccharification. The glucose yield trends of RSMA and RFMA were quite similar during the saccharification. The final yield of reducing sugars in RSMA after 72 hours was 53.74 ± 1.17 %, which was very close to that of RFMA (59.66 ± 3.00 %). The solvents applied during biodiesel production did not show significant inhibitory effects on saccharification. It might be because that solvents like MeOH and hexane were volatilized during the air drying process due to their low boiling point and high volatility. The results

showed that it was feasible to use RSMA as carbohydrate feedstock for fermentable sugar production through simple enzymatic hydrolysis.

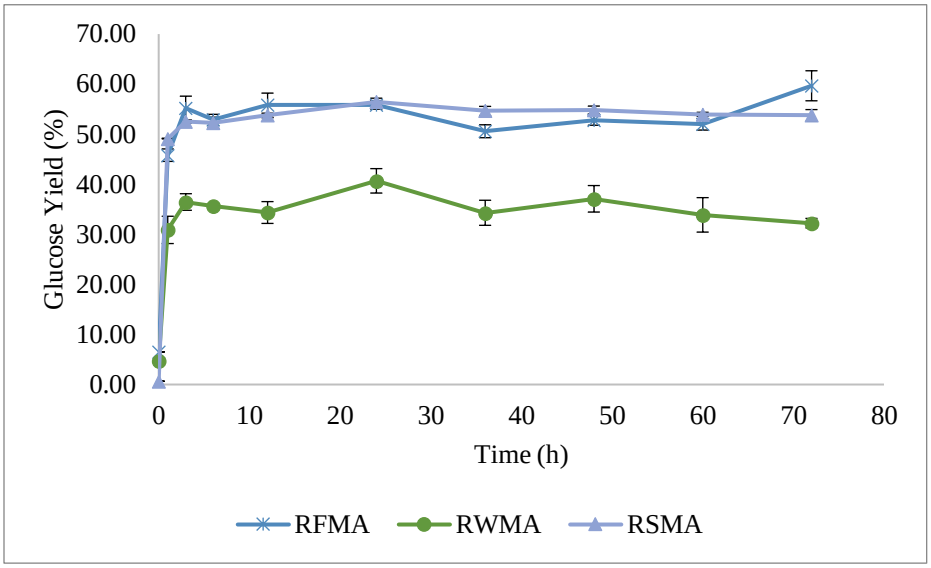

Fig IV-3. Glucose yield of RFMA/RWMA/RSMA saccharified by enzyme Combination D

3.4. Adsorption of enzymes on algae samples

We further carried out experiments of enzyme adsorption onto algae samples, which could be helpful to explain the different saccharification efficiencies for different microalgae samples.

3.4.1. Enzyme adsorption kinetics

The enzyme adsorption kinetics onto algae samples are shown in Fig. IV-4. The adsorption of enzyme onto RWMA reached equilibrium as soon as the enzyme was added and the adsorption curve showed almost no downward trend. It indicated that the raw algae had poor capability for enzyme adsorption. The adsorption curve of both RFMA and RSMA showed obvious downtrends at the initial stage of the adsorption experiment, which was due to the

damage of microalgal cell walls. RF heating caused destruction on the surface of cells making it more accessible for enzyme adsorption. The adsorption curve indicated that the protein content decreased more in RSMA than that in RFMA. This was probably due to the further destruction of microalgae cell walls during biodiesel production. The equilibrium time of RFMA was about 45 min while the RSMA required 60 min to reach equilibrium. The enzyme adsorption onto RFMA was faster than that of RSMA.

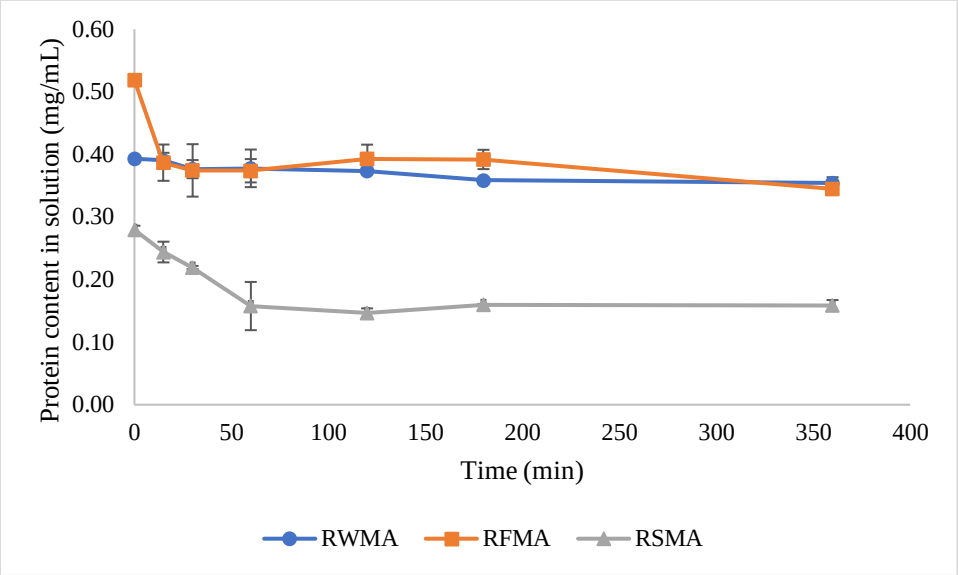

Fig IV-4. Enzyme adsorption kinetics on RWMA/RFMA/RSMA at 25 ºC

3.4.2. Enzyme adsorption isotherm on algae samples

The protein adsorption onto various particle surfaces can be performed by Freundlich isotherm (Palonen, Tjerneld, Zacchi, & Tenkanen, 2004). The Freundlich isotherm is a nonlinear model, which can be expressed with the following equation (Tu et al., 2009):

$$\Gamma = K_F C^n \quad (4)$$

Γ is the adsorbed protein (mg/g of dry algae), C is the concentration of unadsorbed protein in bulk solution (mg/mL), K_F is the Freundlich constant, and *n* is the heterogeneity factor.

The linear model of Freundlich isotherm is:

$$\text{Ln } \Gamma = \ln K_F + n \ln C \quad (5)$$

After incubating at 25 ºC for 3 hours, the adsorption of enzyme reached equilibrium and the parameters of the Freundlich isotherm are calculated and shown in Table IV-4. The data fitted the isotherm equation well. The RWMA had the lowest Freundlich constant K_F and heterogeneity factor *n*. Lager K_F and *n* suggest greater sorbent capacity and affinity (Shafqat & Pierzynski, 2014). The RF heating significantly increased the capacity and affinity of enzyme on microalgae samples. This was due to the damage to the algal cell walls from RF treatment, which provided more binding sites for enzymes.

Table IV-4. Freundlich adsorption isotherm parameters of enzyme adsorption on algae samples at 25 ºC

	Slope	Intercept	*n*	K_F	R^2
RWMA	1.80	4.16	1.80	64.35	0.94
RFMA	2.05	4.40	2.05	81.27	0.85
RSMA	2.08	4.23	2.08	67.99	0.96

3.5. SEM analysis

We further investigated the morphology of the microalgae samples with SEM, as it could closely relate to the enzyme adsorption to microalgae surface. The SEM images of *C. vulgaris* before and after RF heating treatment are shown in Figs. IV-5 (a) and (b). Although the cells looked a little wrinkled due to the air drying for SEM imaging, they were intact and free of extracellular substance in general. After the RF heating, a large amount of extracellular substances appeared on the surface and damaged cell walls were observed. RF pretreatment broke the robust structure of the algae cell wall and exposed the substances inside the cells, thereby making cellulose and hemicelluloses in the cell walls and starch within cells easier to be accessed by enzymes.

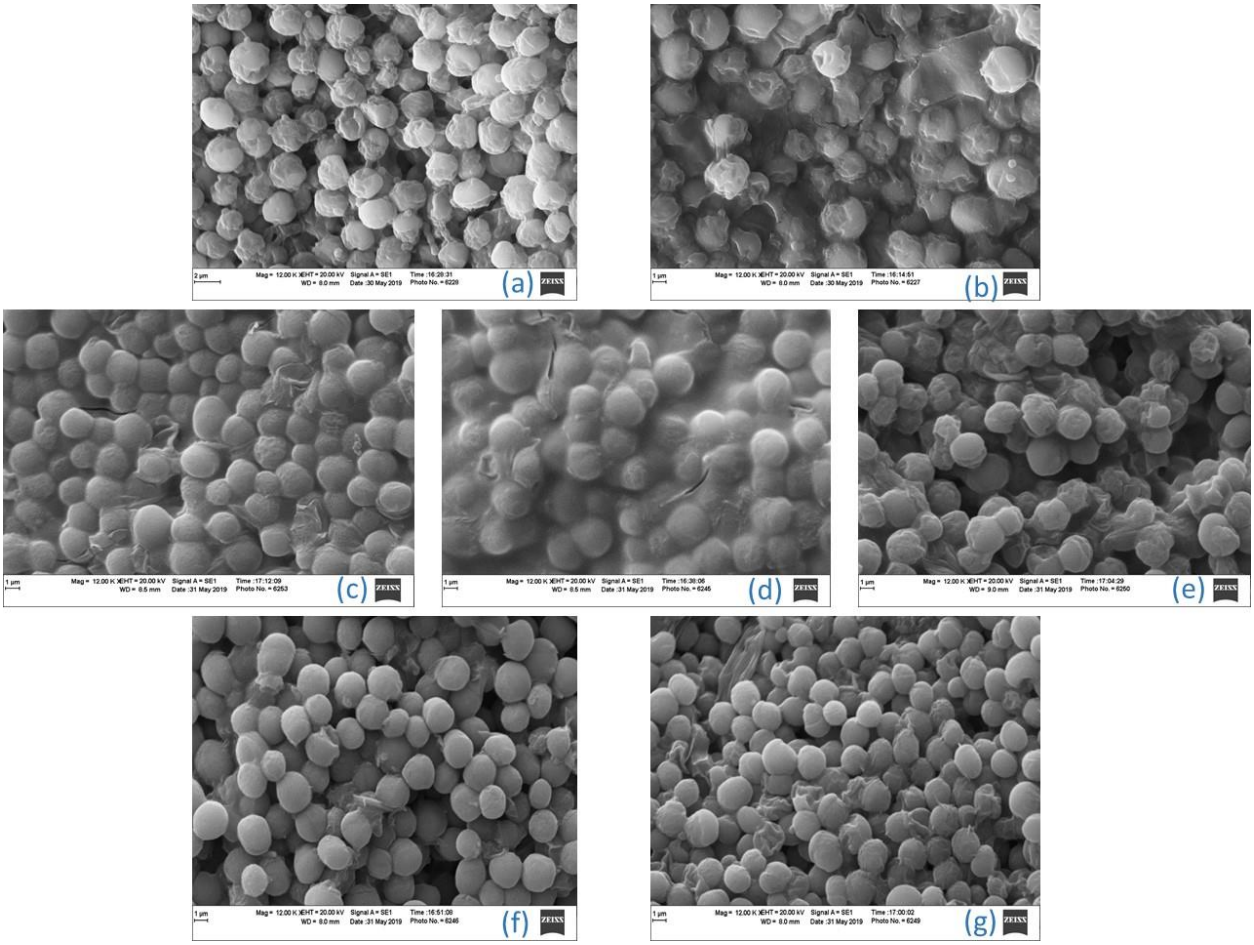

Fig IV-5. SEM image of microalgae samples. (a). RWMA (*Chlorella vulgaris*). (b). RFMA. (c). RFMA after saccharification by Combination A. (d). RFMA after saccharification by Combination C. (e). RFMA after saccharification by Combination D. (f). RWMA (negative control) after saccharification by Combination D. (g). RSMA after saccharification by Combination D

Figs. IV-5 (c) and (e) show the cell morphology of RFMA saccharified by enzyme Combination A and D, respectively. It can be clearly noticed that with the addition of CTec2, the cells of RFMA by Combination D were damaged more severely and hydrolyzed more

completely compared to that by Combination A. After RF treatment, cellulose in the cell wall was more easily hydrolyzed by CTec2, which further destroyed cell wall and also promoted starch hydrolysis. In RFMA by Combination A, although there was effect of RF pretreatment, the cell wall was not further hydrolyzed due to the absence of CTec2. Therefore, the binding of starch to amylase was not as sufficient as that by Combination D. Thus, although the amount of α-Amylase and Amyloglucosidase in Combinations A and D were the same, there was a thin film covered on the surface of RFMA by Combination A, which was small amount of enzyme residue. The surface by Combination C was covered with a thicker film (Fig. IV-5 (d)) than that by Combination A. Although the sugar yield by Combination B was improved slightly, it was obvious that the Amyloglucosidase was excessive. The SEM images visually verified that the enzyme Combination D was the best formulation. The effects by RF pretreatment and CTec2 together could maximize cell wall destruction and thus increased sugar yields. The cell morphology of RWMA after hydrolysis (control) is shown in Fig. IV-5 (f). Comparing to algae cell without any treatment (Fig. IV-5 (a)), the cell of control samples looked smoother. This was because that the cells adsorbed some enzymes. Although hydrolysis was not sufficient, the cell surface of control sample did not show any “enzyme film” like that seen in samples treated by Combination A and C (Fig. IV-5 (c) and (d)). This was probably due to the poor affinity of RWMA to be attached to enzyme protein. The morphology revealed by SEM photographs was consistent with the results from enzyme adsorption experiments.

4. Conclusions

RF heating disruption could break the recalcitrant structure of microalgae cell walls and exposed the substances inside the cells. The total carbohydrates in *C. vulgaris* consist of 31.5 ± 0.4% glucose and 10.2 ± 0.2% galactose (based on dry biomass). After biodiesel production, the glucose was the dominant reducing sugar in RSMA with content of 23.0 ± 0.7% (based on dry biomass). The enzyme Combination D was selected as the best formulation with glucose yield of 59.66 ± 3.00 % (RFMA) after 72 hours. After applying the same enzyme combination to RSMA and RWMA, the glucose yield was 53.74 ± 1.17 % and 32.23 ± 0.95 %, respectively. The combination of RF pretreatment and CTec2 could maximized cell wall disruption, thus made cellulose and hemicelluloses in the cell walls and starch within cells easier to interact with enzymes, and consequently increased sugar yield. The solvents applied during biodiesel production did not show significant inhibitory effect on saccharification of RSMA. The enzyme adsorption for RFMA and RSMA were more efficient than that for RWMA. RF pretreatment could significantly increase the affinity of enzyme to be attached onto microalgae samples. The SEM images visually presented the cell morphology of microalgae samples after RF pretreatment and saccharification, which verified the results of saccharification and enzyme adsorption. Taken together, the results showed that it is feasible to use microalgae residual after biodiesel production as carbohydrate feedstock to be hydrolyzed for fermentable sugar production, which can be further used as the substrate for valuable biofuel and biochemical

production. This represents an effective approach for comprehensive utilization of wet microalgae.

Chapter V Development of PLA-PHB Based Biodegradable Active Packaging and Its Application to Salmon

Abstract

In this study, Poly (lactic acid)-Poly (hydroxybutyrate) (PLA−PHB) based films containing bioactive elements were developed and characterized. Seven formulations containing different contents of plasticizers (mono-caprylin glycerate (GMC) or glycerol monolaurate (GML)) were initially developed. After basic mechanical property tests, the PLA-PHB based films with 0.5% GMC or GML were selected as two formulations for further study. In the subsequent experiments, 5% cinnamaldehyde (CIN) was added into each of the two formulations selected and EVOH copolymer (widely used non-biodegradable packaging as control here) respectively. More mechanical and active properties were investigated. The results showed that PLA-PHB based films possessed some better mechanical properties than those of EVOH based film, and better active properties when applied to high lipid food simulant. In a preservative test, changes in total bacterial counts (TBC), thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N) were carried out on salmon dices packed with films and stored at 4±1 °C. It was only 4.65 CFU/g on day 17 for the salmon packed with PLA-PHB based film with GML as plasticizer meanwhile the TBC of salmon dices sealed in other two films reached 6.65 and 6.35 CFU/g respectively on day 15. This study showed that it could be feasible to use biodegradable active packing as an alternative to replace non-biodegradable packaging for chilled salmon.

1. Introduction

Biodegradable packaging is attracting more and more attention as an alternative to films that made from non-renewable sources. Poly (lactic acid) (PLA) is one of the most promising biodegradable materials. It has advantages of relatively high mechanical strength, transparency and biocompatibility, while brittleness, slow crystallization rate and low heat deflection temperature have limited its practical application (Nagarajan, Zhang, Misra, & Mohanty, 2015).

Poly (hydroxyalkanoate) (PHA) is a biopolymer that can be synthesized and accumulated by many types of bacteria as intracellular carbon and energy storage material (R. Zhang et al., 2013). Poly (hydroxybutyrate) (PHB) is a major member of PHA family with good biodegradability and biocompatibility (Wei, McDonald, & Stark, 2015). Moreover, PHB are produced from biomaterials including corn, switch grass etc (X. Wang, Zhang, et al., 2016b). Among PHBs, the copolymer Poly (3-hydoxybutyrate-co-4-hydroxybutyrate) ($P_{34}HB$) has relatively good toughness and processing properties due to presence of 4HB linear chain, which reduces the crystallinity and lowers melting temperature. High molar fraction of 4HB monomer makes $P_{34}HB$ more flexible. It can be used to improve mechanical properties of brittle thermoplastic polymers (Han, Han, Zhang, Chen, & Dong, 2012).

PLA can be melted with PHB due to their similar melting temperatures. It has been reported that PLA-PHB (75:25) blends have best mechanical properties and optimal miscibility (Arrieta et al., 2014). PHB acts as nucleating agent and enhances the crystallinity of PLA. At the same time,

their processability could be improved by adding plasticizers to produce more flexible materials (Han et al., 2012).

Mono-caprylin glycerate (GMC) and glycerol monolaurate (GML) are two kinds of food preservatives as well as plasticizers. GMC is an intermediate metabolite of fat and GML is a derivative extracted from coconut oil. GML has been granted Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration (Yu, Jiang, Xu, & Xia, 2017). Meanwhile GMC has similar metabolic functions in animal body. In this study, they were used as plasticizers and consequently were incorporated at low levels of inclusion.

Ethylene vinyl alcohol copolymer (EVOH) is a non-biodegradable packing material. It is well known for its excellent oxygen barrier property, while it is hydrophilic and its oxygen barrier properties are only good when the film is kept dry (Muriel-Galet, López-Carballo, Gavara, & Hernández-Muñoz, 2012). In our previous work, the incorporation of EVOH copolymer with 5% (w/w) cinnamaldehyde (CIN), an essential oil, has been proved to have considerable changes in mechanical properties and positive preservative effects on prolonging shelf life of aquatic products compared to EVOH copolymer without CIN. The shelf life of aquatic food could be extended for 3-4 days (Y. Ma, Li, & Wang, 2017).

All packaging films developed in this study can be used as independent packaging materials, unlike edible films. Thus, the objective was to develop biodegradable and active films that could match non-biodegradable films both in mechanical and active properties. To fulfill this objective, biodegradable packaging films based on PLA-PHB containing 5% CIN were

developed with two steps. Then, two best formulations were compared with EVOH based film also containing 5% CIN by characterizing film properties and preservative effects on salmon fillet.

2. Materials and methods

2.1. Materials

Chemicals including ethanol, CIN, TBA, trichloroacetic acid (TCA) etc. were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). EVOH Resins (with 44mol% ethylene content, melt flow rate 13g/10min, E105B grade) were purchased from Kuraray Technology Co., Ltd. (Shanghai, China), PLA (2003D) was provided by Dongguan Yue Fa Co., Ltd. and PHB ($P_{34}HB$) supplied by Tianjin GreenBio Material Co., Ltd.. Fresh salmon fillets were airlifted from Norway within three days of being caught through a seafood import and export company named Haizhixin (Minhang District, Shanghai, China).

2.2. Two-step development of PLA-PHB based active film

2.2.1. Formulation determination

There were two steps to develop PLA-PHB based biodegradable active films. In step 1, the proportions of the plasticizer were optimized. The 0.5%, 1%, 2% of GMC or GML each, or combination of 1% GMC and 1% GML was added into PLA-PHB (3:1) respectively. The PLA-PHB film (3:1) was prepared as control. Basic mechanical properties including tensile strength,

water vapor transmission rate (WVTR), oxygen permeability (OP) were tested. After analyzing, the best formulations were selected for further study.

In our previous study, 5% had been proved to be the maximum concentration of cinnamaldehyde (CIN) that can avoid slipperiness of the single screw extruder. Also, in previous work, the addition of 5% CIN in EVOH copolymer showed great significance for prolonging shelf life of aquatic food (Y. Ma et al., 2017). Thus in step 2, 5% CIN was added into the two formulations selected in the first step respectively to obtain two PLA-PHB based active films, and then compare with the EVOH based film (widely used non-biodegradable packaging) with 5% CIN.

2.2.2. Casting of films

Raw materials including EVOH and PLA in particle form, and PHB and plasticizer powders were individually dried in a vacuum oven at 80 ℃ for 2 hours to remove moisture. When preparing PLA-PHB based film, a twin-screw extruder (Kechuan, Shanghai, China) was employed to melt and blend three components (PLA, PHB and GMC, or PLA, PHB and GML respectively). A thread of plastic-like mixture was produced and cut into particles to make two kinds of modified resins. Heating temperature of seven sections on the extruder ranged from 125 ℃ to 155 ℃.Then all films were prepared by casting method to make them smooth and uniform. Two kinds of modified resins mentioned above and EVOH particles were mixed with 5% CIN respectively by a single screw extruder which was connected to a casting machine. The casting

temperature ranged from 135 ℃ to 170 ℃ for PLA-PHB film and 165 ℃ to 210 ℃ for EVOH film.

2.3. Characterization of films

In step 1, only basic mechanical properties including tensile strength, WVTR and OP, were determined to screen two PLA-PHB based active films from seven formulations. While in step 2, the two PLA-PHB based active films were compared with EVOH film containing 5% CIN through more detailed properties. Firstly, physical and mechanical properties including tensile strength, elongation at break, WVTR, OP, optical properties (haze and transmittance), SEM image and thermogravimetric analysis (TGA) were analyzed. Then, active properties including release of bioactive element (HPLC), antioxidant (DPPH method) and antibacterial ability were also tested (EN, 2002; Y. Ma et al., 2017; Muriel-Galet et al., 2012; Shalaby, Salama, Abou-Raya, Emam, & Mehaya, 2011).

2.3.1. Physical and mechanical properties

After cutting the films into 1.5 × 15 cm strips, a digital micrometer (Mitutoyo, Osaka, Japan) was used to measure the thickness of films. Tensile properties were tested by an Instron universal testing machine (Model 1130) based on ASTM method (ASTM, 2010).

Based on the method of ASTM method (Testing & Materials, 1998), OP test was carried out with a Gas Permeability Tester G2/132 (Labthink Instruments Co., Ltd., Jinan, China). Each film sample was tested in the tester for 10 hours at 23 ℃ and under 50% relative humidity (RH).

The WVTR of film was determined according to ASTM method (E. ASTM, 2003) by using a PERMATRAN-W, model 1/50G (Mocon, Minneapolis, USA). The permeant and dry side RH was 50% and 10% respectively. The temperature was set at 37.8℃.

Optical properties including haze and transmittance were measured based on ASTM method (Geometries & Abrasion, 2012) by a Light Transmittance Rate and Haze Tester, model WGT/S (Precision Instrument Company, Shanghai, China).

SEM images were used to observe the tensile fracture surface of films. The images were taken at a scale of 1:3000 by a QUANTA 200 FEG scanning electron microscope (FEI, Netherlands) (Ghasemlou, Khodaiyan, & Oromiehie, 2011).

According to Marina et al (Arrieta et al., 2014) with little modification, film samples of 11.5 ± 0.5 mg was heated from 30 to 800°C at a rate of 15 °C /min under nitrogen atmosphere by a NETZSCH TG209/F3 thermal analyser (Bavaria, Germany)

2.3.2. Release of bioactive element (HPLC)

According to European law, the neutral, acid, alcoholic and fatty food could be simulated by distilled water, 3% acetic acid solution (v/v), 10% ethanol solution (v/v) and 65% ethanol solution (v/v) respectively (EN, 2002). These four simulations were used to determine the migration of CIN from films to food.

Each film (2.00 g) was soaked in these four simulated solution respectively, which was then extracted periodically and determined by HPLC. To determine CIN in simulation solution, a Waters e2695 HPLC equipped with a 2998 photodiode array detector (Model 2998, SpectraLab

Scientific Inc., Toronto, Canada), and a ZORBAX Eclipse Plus C18 column (150 mm × 4.6 mm, 5 μm) (ZORBAX, USA) were used (Y. Ma et al., 2017; Shalaby et al., 2011).

Release processes of CIN from films to simulations can be described by kinetics of migrant diffusion and expressed by diffusion coefficient (D value). The D value was calculated according to Fick's 2nd law by using Equation (Y. Ma et al., 2017; Poças, Oliveira, Brandsch, & Hogg, 2012):

$$\frac{M_{F,t}}{M_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2_{\pi^2}} \exp\left[-\frac{(2n+1)^2\pi^2}{4d_p^2} Dt\right] \qquad (1)$$

Where $M_{F,t}$is the amount of migrant in the food simulant at particular time t (s), $M_{F,\infty}$ is the amount of migrant in the food simulant at equilibrium, d_p (cm) is film thickness, D (cm^2 s^{-1}) is the diffusion coefficient of migrant in the film and t (s) is time. The initial diffuse period of migration can be simplified into Equation 2 (S. Zhang & Zhao, 2014):

$$\frac{M_{F,t}}{M_{F,\infty}} = \frac{4}{d_p}\left[\frac{Dt}{\pi}\right]^{0.5} \qquad (2)$$

2.3.3. Aantioxidant (DPPH method)

The 0.1 mol/L DPPH solution was prepared. Three pieces of films (12 cm^2 each) were placed into 50 mL of 95% ethanol solution, and then reacted in an oven at 65℃ for three hours to obtain extracted solution. Antioxidant property of films could be evaluated in terms of radical scavenging ability of the extracted solution. The absorbance of solution was measured by a UV spectrophotometer at 517 nm (López-de-Dicastillo, Gómez-Estaca, Catalá, Gavara, & Hernández-Muñoz, 2012; Y. Ma et al., 2017).

2.3.4. Antibacterial ability

Based on Ma et al (Y. Ma et al., 2017), 100 μL of 10^5 CFU/mL bacteria (E. coli, Salmonella and Lester) were inoculated into tryptic soy broth (TSB) solutions in which 0.5 g of the films had been soaked for five days. After cultivating for 12 hours and spreading plates, colonies were counted.

2.4. Application of films to salmon fillets

Salmon fillets were selected as the aquatic product for preservation test according to the properties of the PLA-PHB based films according to the tests above.

2.4.1. Preparation of salmon dices.

Salmon fillets were kept at 0℃ throughout transportation to the laboratory to ensure freshness. Then, the fillets were cut into dices of 10 to 15 grams (about 30×40×10 mm). The salmon dices were packed individually with the three kinds of films obtained from step 2. Each pack carried one piece of salmon dice and the size of each pack was about 8×10 cm. Then the packs were sealed at atmosphere and stored in a refrigerator at 4 ± 1℃. The preservative effects of films were tested and verified by determining total bacterial counts (TBC), total volatile basic nitrogen (TVB-N) and thiobarbituric acid (TBA) of fish dices periodically.

2.4.2. Preservative measurements on salmon dices

TBC of salmon was carried out based on the methods described by AOAC (William, 2000). Salmon samples (10 g) were homogenized with 0.85% NaCl solution. The mixture was made

into ten dilution gradients. Appropriate gradients were pipetted onto the surface of the count agar plates, and then were incubated at 37 for 48 hours.

A Kjeltec™ 2300 Analyzer (FOSS Tecator AB, Hillerød, Denmark) was used for TVB-N determination of salmon sample (5.00 g each) (Goulas & Kontominas, 2007).

TBA was determined as described by Song et al (Song, Liu, Shen, You, & Luo, 2011) with minor modification. Fish sample (5 g each) was homogenized for 1 min with 15mL of 20% TCA solution and 10mL of distilled water. After settling down for 1 hour, the supernatant was filtered and diluted to 50mL with distilled water. Then 5 mL of the solution was mixed with 5mL of 0.02 M TBA and heated in boiling water for 20 min. The absorbance of the solution was measured at 532 nm with a UV2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The standard curve was established using malondialdehyde (MDA) and TBA value was expressed as mg MDA/kg sample.

2.5. Statistical analysis

All data analysis was performed by SPSS 20.0 (IBM Corp. NY, USA). A CRD model was used to analyze the data set. The significant differences among mean values were tested by one-way ANOVA. The Ryan-Einot-Gabriel-Welsh F (REGWF) method was selected for the test.

3. Results and discussion

3.1. Basic mechanical properties of films in step 1

The basic mechanical properties including tensile strength, WVTR and OP in step 1, were tested and are shown in Fig. V1-3. The best formulations were selected based on a criterion of high tensile strength, low WVTR and low OP. PLA-PHB film is easy to be broken during storage and transportation, which limits its application in packaging industry. So tensile strength is an important mechanical property to be considered. In Fig.V-1, with the addition of plasticizer, tensile strengths were significantly improved compared to PLA-PHB film. Low WVTR and OP indicate excellent barrier property of film, which is helpful to prolong the shelf life of food packed with the film. PLA-PHB film with 0.5% GMC / GML shows lowest WVTR in Fig. V-2. In Fig. V-3, it shows that OP value increases gradually with the increase of GMC. While in films containing different amounts of GML, the OP values changed little over the change of GML amount. Thus, PLA-PHB film with 0.5% GML / GMC were selected as the best two formulations for the further tests since they had the lowest WVTR and OP. Although these two films did not have highest tensile strength, they showed statistically significant improvements compared to PLA-PHB film without any plasticizer (α=0.05). However, OP changes over temperature and relative humidity. Zhang et al (Z. Zhang, Britt, & Tung, 2001) reported that OP for EVOH with 44 mol% ethylene decreased from 4.5 mL m^{-2} day^{-1} at 35 ℃, to 2.5 at 25 ℃, to 0.9 at15 ℃ when RH was fixed at 50%; The OP were 12 mL m^{-2} day^{-1} at 35 ℃, to 3.2 at 25 ℃, to 1.2 at15 ℃ when RH was fixed at 80%. The OP of EVOH with 44mol% ethylene decreased

from 2.4 cm^3.20 µm/m^2.day.atm to 1.2 to 0.8 to 0.3 as temperature decreasing from 35 ℃ to 23 to 20 to 5, when the RH was set at 0%. Kuraray Technology also reported in the same technical brochure that the OP of EVOH with 44mol% ethylene increased from 0.9 cm^3.20 µm/m^2.day.atm to 1.1 to 2.2 to 4.2 with increasing RF from 0 to 50 to 80 to 90% when the temperature was fixed at 20 ℃. The net effect of decreasing temperature (with fixed RH) and increasing RH (with fixed temperature) on the OP of EVOH with 44mol% ethylene looked neutral.

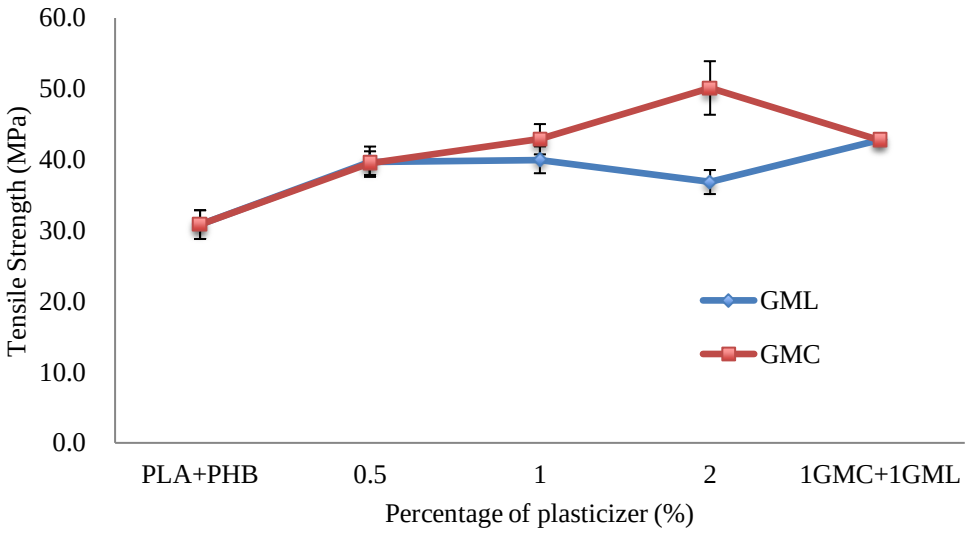

Fig V-1. Tensile strength of films in step 1

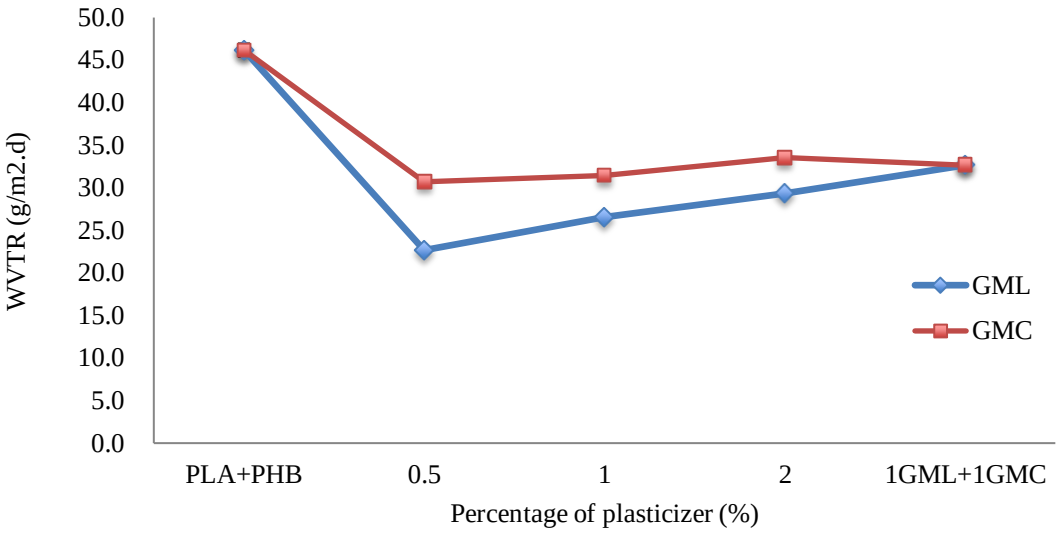

Fig V-2. WVTR of films in step1

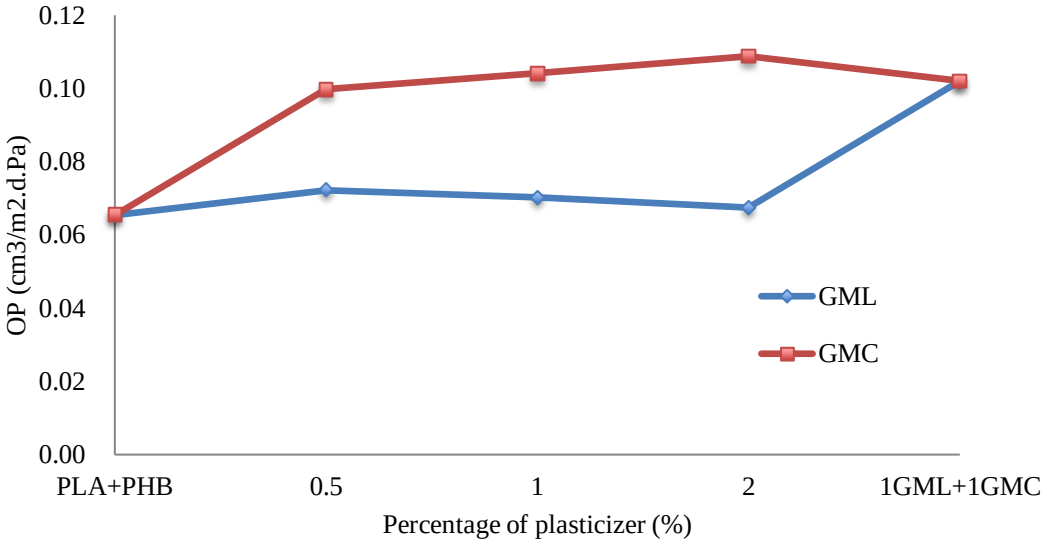

Fig V-3. OP of films in step1

3.2. Detailed physical and mechanical properties in step 2

In step two, 5% CIN was added into each of these two films. Then PLA-PHB based active films were compared with EVOH copolymer containing 5% CIN. The detailed formulations are shown in Table V-1.

Table V-1. Formulation of films

	Base	Plasticizer	Additive
A	PLA-PHB (3:1)	0.5% GMC	5% CIN
B	PLA-PHB (3:1)	0.5% GML	5% CIN
C	95% EVOH	——	5% CIN

The thickness of film A, B and C was 47.6 ± 5.8, 51.4 ± 4.0 and 42.8 ± 2.2 μm respectively. Detailed mechanical properties of film A, B and C were determined and are shown in Table V-2. In study of Cardoso et al (Cardoso et al., 2017), the addition of oregano essential oil (OEO) to poly (butylene adipate co-terephthalate) (PBAT) resulted in the maximum tensile strength (about 17.3 MPa) at an OEO concentration of 4.01 g, while the opposite was observed for elongation at break. Xavier et al (Xavier, Babusha, George, & Ramana, 2015) developed antimicrobial PHB films by adding vanillin (4-hydroxy-3-methoxybenzaldehyde) into the polymers and the tensile strength of the film was 1.89 ± 0.32 MPa. As shown in Table V-2, tensile strength of film A and B was 42.9 ± 1.6 and 44.3 ± 1.3 MPa respectively. It was greatly improved compared to PLA-PHB film and much higher than EVOH based films in Table V-2, which is an important

improvement for biodegradable film. It is likely that small molecules of plasticizer and CIN penetrated into the amorphous phase space of the macromolecular PLA-PHB. In contrast, it increased intermolecular force and reduced of slippage at the same time, which lowered elongation at break. PLA-PHB based film had higher WVTR than that of EVOH based films. Cardoso et al (Cardoso et al., 2017) indicated that higher OEO content caused greater WVTR, which was quite similar to the observation in this study. The addition of CIN caused higher WVTR. It is because of the hydrophilicity of aldehyde group in CIN. Also, the WVTR of film A is lower than that of film B. Adding CIN increased WVTR of EVOH copolymer (1.3 to 2.6 g/[m^2.d]) but decreased OP (0.104 to 0.084 cm^3/m^2.d.Pa) under the mechanical property testing conditions (23 ℃ and 50% RH) . Film A and B had similar, or even lower OP rates compared to film C and EVOH copolymer. Low OP rate is ideal for preventing growth of aerobic bacteria and prolonging food shelf life. Film A and B had much lower haze and higher transmittance than PLA-PHB film with the addition of CIN, which could make the packaging more attractive. While excessive transmittance of light like film C and EVOH copolymer may cause the accelerating decomposition of photosensitive food.

Table V-2. Physical and mechanical properties of films.

	Tensile strength (MPa)	Elongation at break (%)	WVTR (g/[m^2.d])	OP (cm^3/m^2.d.Pa)	Haze (%)	Transmittance (%)
A	42.9±1.6[a]	6.9±1.1[a]	69.5±0.9[d]	0.0805	4.2±0.2[c]	82.4±0.3[c]
B	44.3±1.3[a]	5.7±0.3[a]	77.7±0.6[e]	0.0846	2.6±0.2[b]	80.8±0.3[b]
C*	29.5±2.5[b]	44.0±2.4[c]	2.6±0.0[b]	0.0842	1.2±0.8[a]	87.3±0.1[e]
EVOH*	26.8±1.8[b]	86.9±2.0[d]	1.3±0.0[a]	0.104	4.4±0.9[c]	86.3±0.6[d]
PLA-PHB	30.8±2.0[b]	13.6±1.22[b]	22.3±0.1[c]	0.0654	75.3±0.5[d]	19.1±0.4[a]

Values in same column for each attribute followed by letters (a–e) indicate significant differences ($P<0.05$). (* data partially from our previous work (Y. Ma et al., 2017))

The SEM images are shown in Fig. V-4 at a scale of 1:3000. From the tensile fracture surfaces, vertical micro cracks could be found in film C compared to EVOH copolymer. This might be caused by acetalization reaction. While film A and B not only had micro cracks in vertical, but also in horizontal, which made it have higher tensile strength than the other two films.

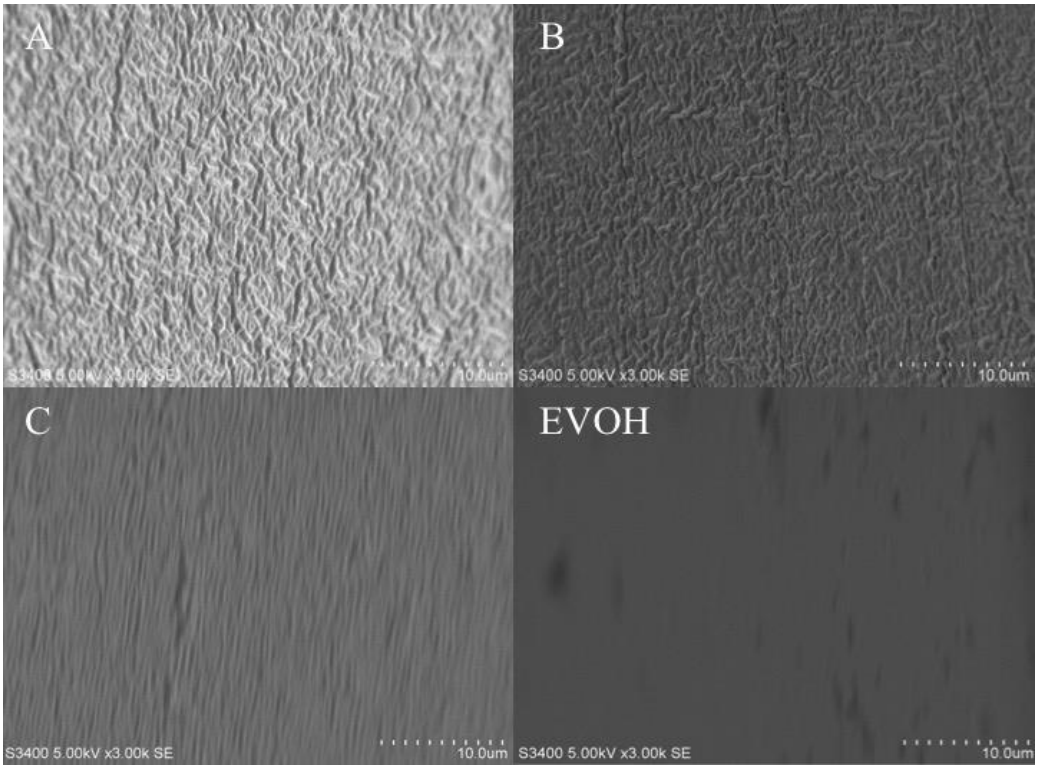

Fig V-4. SEM images of the films (photos C and EVOH are from our previous work (Y. Ma et al., 2017))

Thermal stability was analyzed by TGA test. As shown in Fig. V-5, film A and B had similar thermogravimetric curve. Different plasticizers did not affect thermal stability of the films. The first downward trends of these three films were coincident and were about 5% in weight. Those were the loss of CIN. Film A and B had sharply decrease of weight in a range of 266℃ to 376 ℃ while film C decomposed between 373℃ and 477℃. Although PLA-PHB based films showed lower decomposition temperature compared to non-biodegradable EVOH copolymer, they are stable enough for common use.

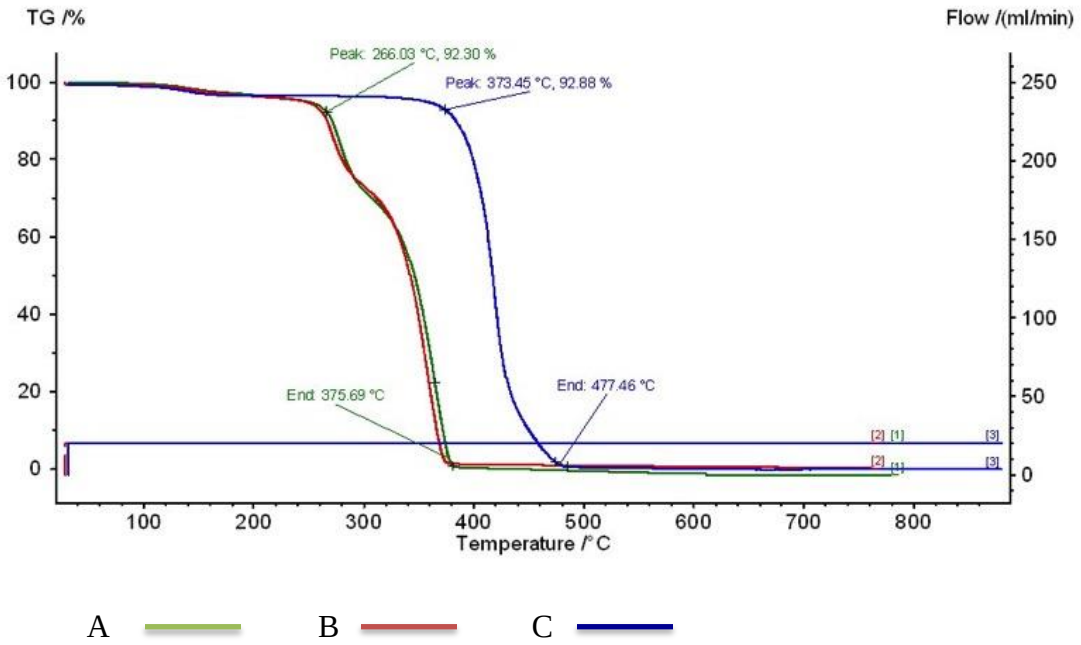

Fig V-5. TGA Analysis of the films (Curve C were from our previous work (Y. Ma et al., 2017))

3.3. Release of CIN from films into food simulations

As shown in Fig. V-6, film A and B had highest migration rate and releasing concentrations of CIN in 65% ethanol solution, but released little amount of CIN in the other three simulations. The maximum migrated CIN concentration from film A, B and C in simulations was 55.6 μg/mL, 59.0 μg/mL and 55.1 μg/mL respectively. While the CIN concentrations of film A and B were less than 2.5 μg/mL in other three simulations. This indicates that PLA-PHB based films are more suitable for packaging high lipid food. In the simulation of 65% ethanol solution, the CIN releasing rates from PLA-PHB based films (films A and B) were much slower than that of EVOH based film (film C). The maximum releasing concentrations were reached on day 10. While the CIN concentration of film C almost reached the peak within 4 h. It indicated that PLA-

PHB based film might have a property of slowly-releasing. In Fig. V-6 (c), the concentration of CIN decreased after 200 h in water, it is because that CIN is easy to volatilize or phase separate in water, while it is soluble in organic solvents such as alcohol (Y. Ma et al., 2017).

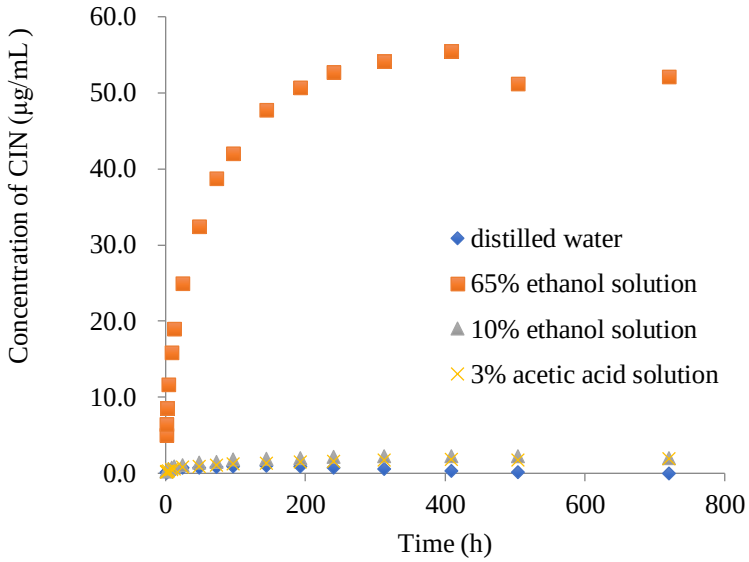

Fig V-6 (a). Concentration of CIN from film A into simulations

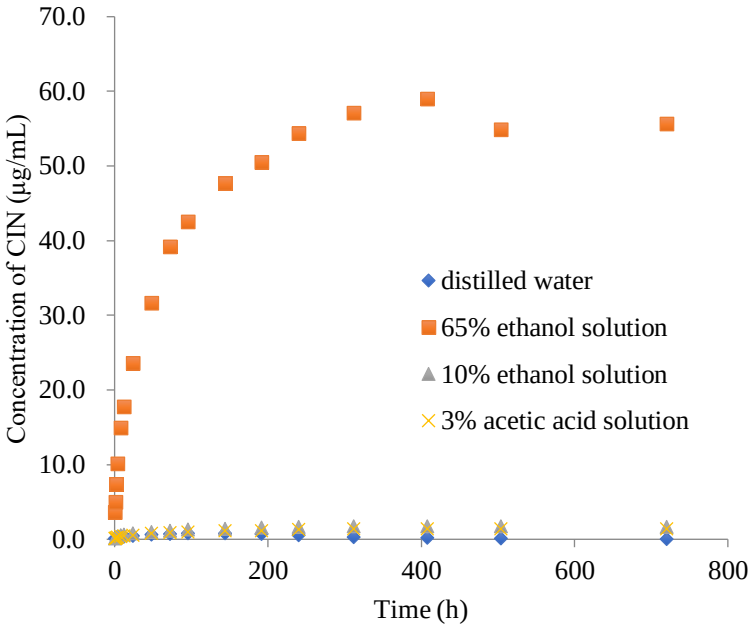

Fig V-6 (b). Concentration of CIN from film B into simulations

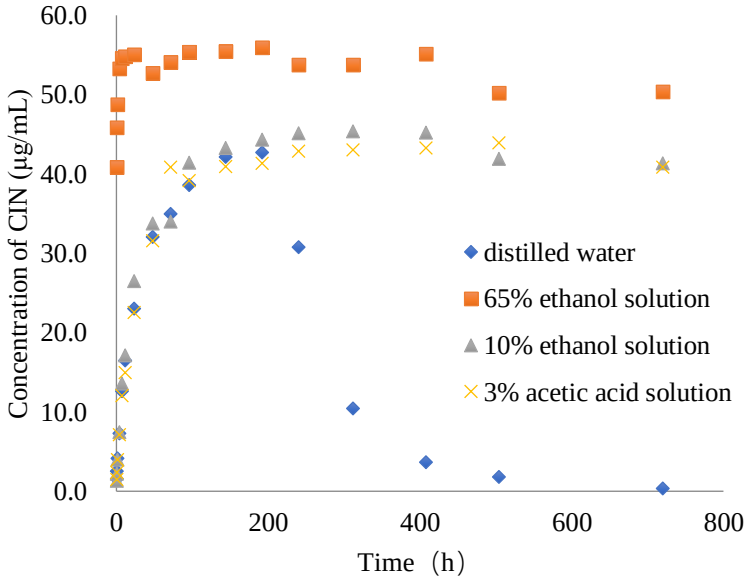

Fig V-6 (c). Concentration of CIN from film C into simulations (This figure is from our precious work (Y. Ma et al., 2017))

As shown in Table V-3, in 65% ethanol solution, film A and B had lower D value compared to film C. This once again verified that film A and B might have the ability to slowly release CIN. In the other three simulations, the amount of migrated CIN from film A and B was so little that made D value insignificant.

Table V-3. Diffusion coeffcients of CIN released from films

	A	B	C
65% ethanol solution	1.51E-13± 1.28E-14	1.16E-13± 1.76E-14	4.00E-12± 1.77E-13

3.4. Antioxidant activity (DPPH method)

As shown in Fig. V-7, by using DPPH method, the radical scavenging rate of film A, B, C was 15.7%, 13.8% and 4.7% respectively. PLA-PHB based films showed stronger antioxidant activities than that of EVOH based film. This is probably because of the lower casting temperature of PLA-PHB compared to EVOH copolymer, which minimizes the loss of CIN.

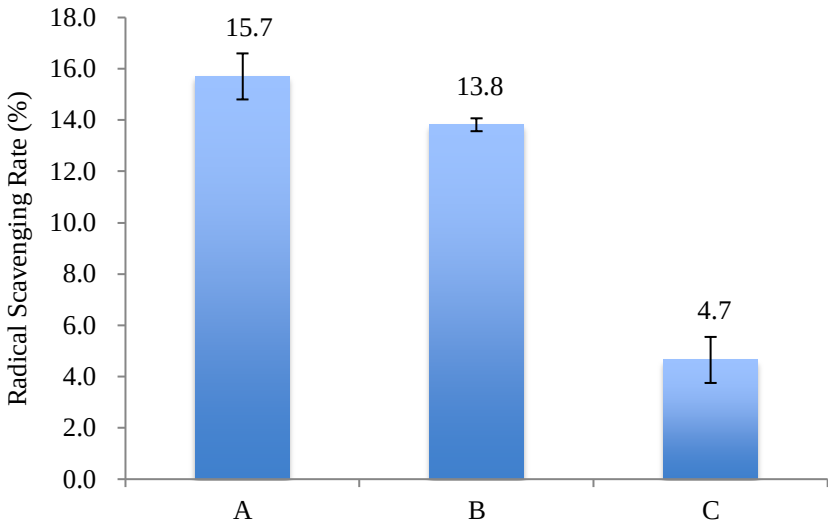

Fig V-7. Radical scavenging rate of the films (column C is from our previous work (Y. Ma et al., 2017))

3.5. Antibacterial activity

The counting results of film C were all zero, while the results showed that antibacterial effects of film A and B were not significant. This might be that TSB culture medium is quite similar to water. This result agreed with the phenomenon showed in releasing test, CIN was difficult to release from PLA-PHB based films to distilled water.

Based on the active properties of film A and B, salmon was selected for preservative effect tests, as salmon is an aquatic product with a high fat content.

3.6. Application of films to salmon fillets

3.6.1. Changes in TBC of salmon dices during storage

At the beginning period of storage, film C showed lowest TBC among the three films in Fig. V-8. It is because that film C reached maximum releasing concentration of CIN at early stage of storage. After day 9, the trend lines of film A and C continued to rise while film B was gradually stabilized. This could be explained by releasing test described above. Film C had highest migration speed of CIN while film B showed maximum migration rate of CIN after day 10 in high lipid simulation. The TBC of salmon dices in film A and C were above 6.0 CFU/g before day 15, which was the maximum acceptable level for fish (Song et al., 2011). Meanwhile, the TBC of salmon in film B was still below the permissible limit on day 17. In previous work, we had shown that film C can prolong the shelf life of packaged food by 3-4 days (Y. Ma et al., 2017). According to the results showed in Fig. V-8, film B could even delay the change for at least 2 more days compared to film C. Film A and B had similar properties while film B showed better preservative effects. This might because film B had higher WVTR than that of film A.

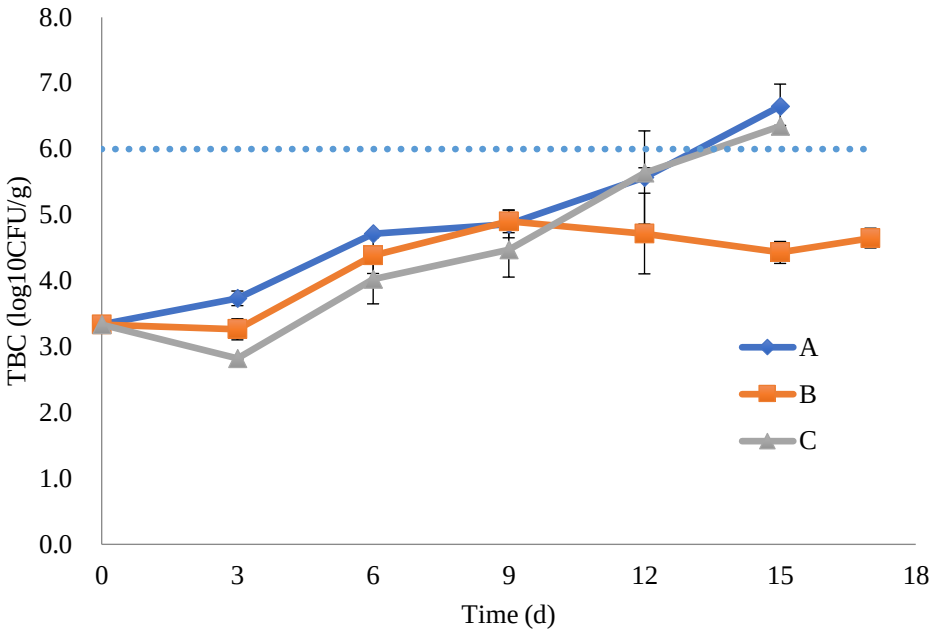

Fig V-8. Total bacteria counts of salmon

3.6.2. Changes in TVB-N of salmon dices during storage

The TVB-N values of 15-20 mg N/100 g indicated a seafood was still in a good quality condition (Yang et al., 2014). During storage, the TVB-N values of salmon dices increased gradually in all three films in Fig. V-9. On day 15, the TVB-N of salmon dices film A, C was 24.3 mgN/100g and 21.2 mgN/100g respectively. While in film B, it didn't reach 20 mgN/100g until day 17. The results of TVB-N were matched with that of TBC.

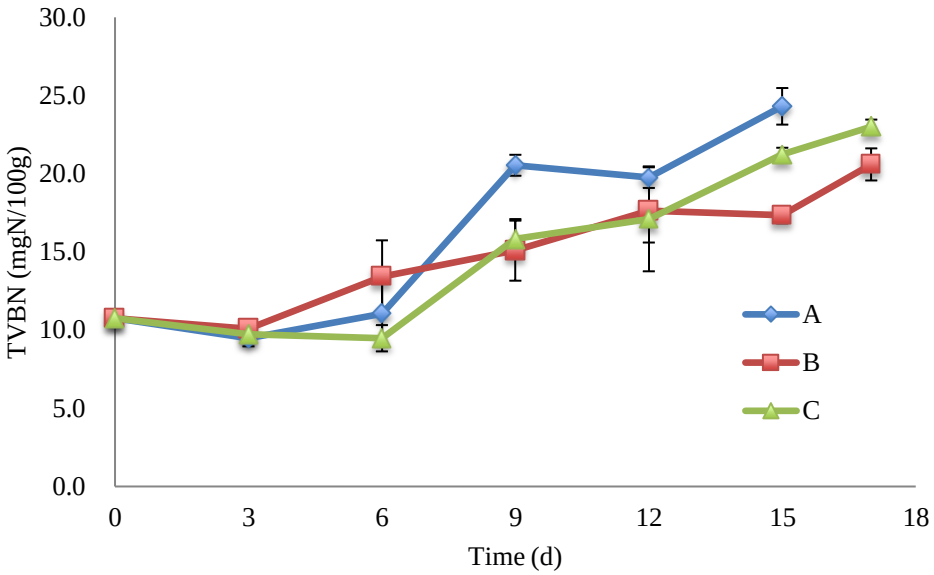

Fig V-9. TVB-N results of salmon

3.6.3. Changes in TBA of salmon dices during storage

A level of 0.2 mg MDA/100g has been denoted as the limit of acceptability (Rode & Hovda, 2016). In Fig. V-10, the TBA of salmon dices reached the limitation on day 6 to 9 for film A, on day 12 for film C, and on day 17 for film B. The results agreed with those of TBC and TVB-N tests.

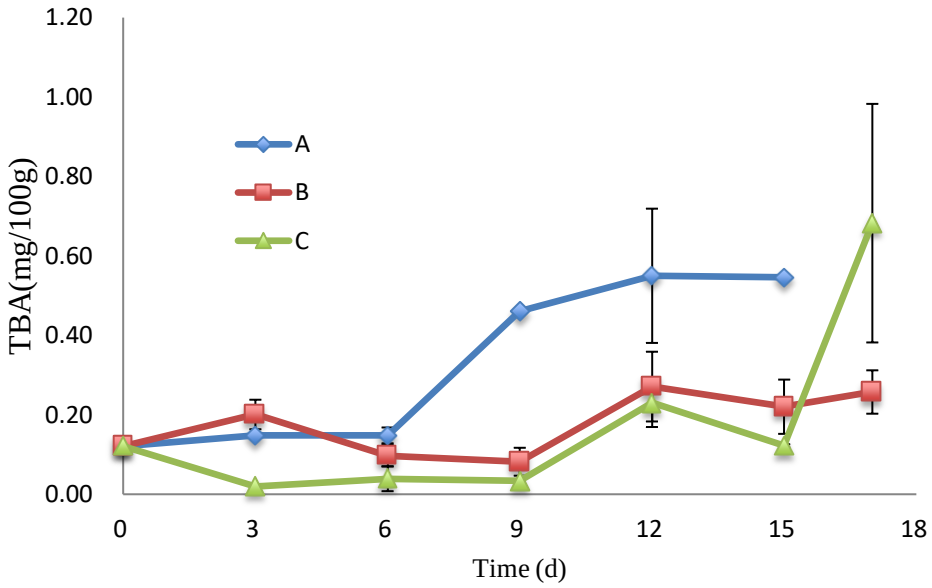

Fig V-10. TBA Results of salmon

4. Conclusion

In step 1, the formulations with 0.5% plasticizers (GMC or GML respectively) showed best basic mechanical properties. In step 2, tensile strength of film A, B and C was 42.9 ± 1.6, 44.3 ± 1.3 and 29.5 ± 2.5 MPa respectively. PLA-PHB based film showed much better tensile strength and excellent OP rate compared to EVOH based film. These considerable changes are significant in biodegradable films. EVOH based films had lower WVTR compared with PLA-PHB based films. PLA-PHB based films had equal or even better antioxidant and antibacterial ability under certain conditions. Film A and B showed higher radical scavenging rate compared to EVOH based film. In high lipid food simulation, the maximum CIN releasing concentrations of film A and B were similar to that of film C, and the releasing rates were much slower, which might lead

to a property of slow release. In preservative tests, film C showed best effects during early stage of storage. On day 15, the results of TBC, TVBN and TBA indicated that salmon dices in film A and C spoiled. However, the changes were delayed by at least 2 days in salmon dices packed with film B. Salmon dices in film B changed stably throughout the storage time, which verified slow release. This study showed that it could be feasible to use biodegradable active packing as an alternative to replace non-biodegradable packaging for chilled salmon.

Chapter VI Conclusions and Future Work

1. Summary of Results

In this study, an effective biodiesel production procedure directly from wet microalgae *(Chlorella vulgaris)* was established. With the assistant of RF heating, the cell wall of algae was disrupted, and esterification/transesterification reactions were promoted. Compared with other studies, the whole process in this work avoided high temperature or pressure, saved large quantities of solvents and eliminated the process of drying algae. It is safer and more economically feasible for industrial application. The highest FAME yield was 79.5 ± 3.0%.

Then, the biodiesel production procedure was further improved by using the RSM. The production procedure consisted of two stages of conversion: acid-catalyzed first stage and base-catalyzed second stage. A three-variable, five-level CCD was employed to optimize the parameters of HCl to MeOH ratio (v/v), MeOH volume and RF heating time in the first stage of conversion. The results showed that both of the two conversion stages were indispensable. The response surface plots illustrated that the MeOH volume had the largest impact on FAME yield. The maximum predicted FAME yield of 93.1% was obtained at HCl to MeOH ratio 4.27 (v/v), MeOH 28.5 mL and RF heating time 19.2 min. The validation experiments confirmed that the RSM model was adequate.

In addition to the utilization of the lipids in *Chlorella vulgaris*, the carbohydrate residual could also be used for further production of bio-products. The total carbohydrates in *C. vulgaris*

consist of 31.5 ± 0.4% glucose and 10.2 ± 0.2% galactose (based on dry biomass). After biodiesel production, the glucose was the dominant reducing sugar in RSMA with content of 23.0 ± 0.7% (based on dry biomass). The enzyme Combination D was selected as the best formulation with glucose yield of 59.66 ± 3.00 % (RFMA) after 72 hours. After applying the same enzyme combination to RSMA and RWMA, the glucose yield was 53.74 ± 1.17 % and 32.23 ± 0.95 %, respectively. The combination of RF pretreatment and CTec2 could maximized cell wall disruption, thus made cellulose and hemicelluloses in the cell walls and starch within cells easier to interact with enzymes, and consequently increased sugar yield. The solvents applied during biodiesel production did not show significant inhibitory effect on saccharification of RSMA. The enzyme adsorption for RFMA and RSMA were more efficient than that for RWMA. RF pretreatment could significantly increase the affinity of enzyme to be attached onto microalgae samples. The SEM images visually presented the cell morphology of microalgae samples after RF pretreatment and saccharification, which verified the results of saccharification and enzyme adsorption. Taken together, the results showed that it is feasible to use microalgae residual after biodiesel production as carbohydrate feedstock to be hydrolyzed for fermentable sugar production, which can be further used as the substrate for valuable biofuel and biochemical production.

PHB is one of the biochemical product which could be obtained through fermentation by bacterial using the fermentable sugar. Normally, PHB could be melted with PLA to produce biodegradable packaging material. In this study, PLA−PHB based films containing bioactive

elements were developed and characterized. Seven formulations containing different contents of plasticizers (mono-caprylin glycerate (GMC) or glycerol monolaurate (GML)) were initially developed. After basic mechanical property tests, the PLA-PHB based films with 0.5% GMC or GML were selected as two formulations for further study. In the subsequent experiments, 5% cinnamaldehyde (CIN) was added into each of the two formulations selected and EVOH copolymer (widely used non-biodegradable packaging as control here) respectively. More mechanical and active properties were investigated. The results showed that PLA-PHB based films possessed some better mechanical properties than those of EVOH based film, and better active properties when applied to high lipid food simulant. In a preservative test, changes in total bacterial counts (TBC), thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N) were carried out on salmon dices packed with films and stored at 4±1 ℃. It was only 4.65 CFU/g on day 17 for the salmon packed with PLA-PHB based film with GML as plasticizer meanwhile the TBC of salmon dices sealed in other two films reached 6.65 and 6.35 CFU/g respectively on day 15.

2. Conclusion

This study provided an effective approach for comprehensive utilization of wet microalgae. The lipids were used for biodiesel production and the carbohydrates were saccharified to fermentable sugar, which could be further used as the substrate for valuable biofuel and

biochemical production. Although this pathway was developed on *Chlorella vulgaris*, it can be applied to any other microalgae which are rich in lipids and carbohydrates.

As one of the biochemical product, PHB was melted with PLA to produce biodegradable packaging material. This results showed that it could be feasible to use biodegradable active packing as an alternative to replace non-biodegradable packaging in the near future.

3. Future Work

In this study, the utilization of algal carbohydrate only achieved the step of fermentable sugar. Since our group had successfully produced PHB from fermentable sugar using engineered *E. coli* through fermentation, this part was not repeated. However, the previous method was achieved through separate hydrolysis and fermentation (SHF). In the future, we'd like to compare SHF method with simultaneous saccharification and fermentation (SSF) method. Also, we would like to try the possibility to produce PHB using other strains of bacterial. What's more, as the substrate, the fermentable sugar can be used for valuable biofuel and biochemical production, which provides unlimited possibilities for future research. And we can also apply RF heating to disrupt cells of other feedstock, such as bacterial, for other utilization.

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