BIOETHANOL FROM *Chlorella* sp. AS A BIOENERGY SOURCE

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ABSTRACT

High global demand of human energy consumption had caused the overexploitation of fossil fuels resources leading to critical need of environmental issues to develop a sustainable, ecofriendly and cost-effective renewable energy resources. Bioethanol produced from microalgae has been considered "third generation" biofuel to be known as one of the important renewable energy. Microalgae are one of a promising feedstock that rich in carbohydrates content which is a viable criteria for fermentation to ethanol. Pretreatment is an important step to release fermentable sugars before bioethanol fermentation. This study investigates the reducing sugar concentration of microalgal *Chlorella* species after pretreated with acetic and sulfuric acid in various concentrations (1%, 3%, 5%, 7%,9%), respectively and reducing sugar consumed after fermentation. 3,5-dinitrosalisilat acid (DNS) method, Fourier transform infrared spectroscopy (FTIR), Gas chromatography with flame ionization detection (GC-FID) were used to determine the reducing sugar concentration, functional groups of alcohol bonds after distillation and ethanol concentration. Two-way ANOVA analysis ($p<0.05$) was carried out to determine the difference between effect of types and concentrations of acid to ethanol content. The ANOVA result indicated there was a significant different of both factors in ethanol content. The highest ethanol yield 0.281 g ethanol/ g microalgal was found in bioethanol sample pretreated with 5% sulfuric acid while 0.23 g ethanol/ g microalgal was obtained by bioethanol sample pretreated with 5% acetic acid.

Keywords: bioenergy, microalgae, bioethanol, pretreatment, fermentation.

BIOETANOL DARIPADA MIKROALGA *Chlorella* sp. SEBAGAI SUMBER BIOTENAGA

ABSTRAK

Permintaan global yang tinggi untuk penggunaan tenaga manusia menyebabkan eksploitasi yang berlebihan dan berterusan terhadap bahan api fosil telah menimbulkan isu-isu alam sekitar. Hal ini membawa kepada keperluan yang kritikal untuk membangunkan sumber tenaga yang boleh diperbaharui, berkekalan, dan kos efektif. Bioetanol yang dihasilkan daripada mikroalgal telah dianggap sebagai sumber bahan api (biofuel) "generasi ketiga" yang boleh diperbaharui. Mikroalgal adalah salah satu bahan mentah yang menjanjikan di samping itu ia juga mengandungi karbohidrat yang kaya dan merupakan salah satu kriteria yang penting untuk penapaian proses etanol. "Pretreatment" adalah langkah amat penting bagi melepaskan gula yang boleh ditapis untuk proses penapaian kepada etanol. Kajian bertujuan menyiasatkan tentang kepekatan gula pereduksi bagi spesies *Chlorella* selepas dirawat dengan asid asetik dan asid sulfurik dalam pelbagai jenis kepekatan (1%, 3%, 5%, 7%, 9%). Kaedah- kaedah yang digunakan untuk menentukan kepekatan gula pereduksi, kumpulan fungsi alkohol selepas proses penyulingan dan kepekatan etanol adalah asid 3,5-dinitrosalisilat (DNS), transformasi infra merah spektroskopi fourier (FTIR), dan kromatografi gas dengan pengesanan pengionan api (GC-FID). Selain itu, analisis ANOVA dua-hala ($p < 0.05$) telah dijalankan untuk mengenal pasti kesan jenis dan kepekatan asid kepada kandungan etanol. Analisis ANOVA telah menunjukkan perbezaan yang signifikan antara kedua-dua faktor terhadap kandungan etanol. Hasil etanol yang tertinggi ialah 0.281 g etanol / g mikroalgal telah didapati dalam sampel bioetanol yang dirawat dengan 5% asid sulfurik manakala 0.230 g etanol / g mikroalgal diperolehi oleh sampel bioetanol yang dirawat dengan 5% asid asetik.

Kata kunci: biotenaga, mikroalgal, bioetanol, pretreatment, penapaian.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
%	:	Percentage
AAU	:	Alpha amylase unit
cm^{-1}	:	Reciprocal centimeters
Df	:	Degree of freedom
DNA	:	Deoxyribonucleic acid
GAU	:	Glucoamylase unit
Glu	:	Glucose
h	:	Hour
kHz	:	Kilohertz
kW	:	Kilowatt
L	:	Liter
MS	:	Mean square
Mg	:	Milligram
mL	:	Milliliter
M	:	Molarity
N	:	Nitrogen atom
N/A	:	Not applicable
Nm	:	Nanometer
psi	:	Pound per square inch
rpm	:	Revolutions per minute
spm	:	Strokes per minute
SS	:	Sum of squares
sp.	:	Unspecified species

U	:	Units
µL	:	Microliter
µm	:	Micrometer
v/v	:	Volume per volume
w/w	:	Weight for weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Combustion of fossil fuels to produce the energy cause the released of greenhouse gases (GHG) to the atmosphere which is one of the major contributions to the global warming phenomenon (Baicha et al., 2016; Chia et al., 2018; Pragya et al., 2013).

Recent report of International Energy Agency (IEA) stated that the highest potential alternative energy was derived from biofuels and waste which represented 10.0% of the world's energy supply, compared to 2.4% from the water and 1.1% from solar and wind energy (Baicha et al., 2016; IEA, 2017). Biomass is one of the alternative energy sources which can sustain the need of the present and future fuel demand (John et al., 2011).

Currently, the main source to produce the bioethanol are mostly derived from sucrose and starch crops (first-generation) as well as lignocellulosic materials (second-generation) as feedstock (Borines et al., 2013; Sirajunnisa & Surendhiran, 2016). Advantages of the use of algae, microalgae and cyanobacteria for the production of the third-generation biofuels has more than first- and second-generation biofuels due to their fast growth characteristics and capability to grow in several conditions as well as in wastewater (Carlos & Bertucc, 2016; Nigam & Singh, 2011). In addition, the productions from the grains, corns, oil-crop based biofuel are restricted due to inadequate agriculture land on earth and stress on arable land that utilize for agriculture crops in food production leading to a severe food shortage especially in developing countries where already more than 800 million people were suffered from hunger and malnutrition (Dragone et al., 2010; Sirajunnisa & Surendhiran, 2016).

The production of bioethanol from microalgae and cyanobacteria are considered as a practicable technological development as they show higher yield than certain crops such

as sugarcane and corns (Carlos & Bertucc, 2016). According to Zhang et al. (2014), the bioethanol production derived from the algae showed the optimum productivity which can be approximately two times higher than the ethanol productivity from sugarcane and five times higher than from corn. Algae contain of energy rich lipids and carbohydrates. Some of the microalgae cell wall are comprised of cellulose, mannans, xylans and sulfated glycans which can be broken down chemically and enzymatically into simple sugar form and converted into ethanol (Chaudhary et al., 2014; Sirajunnisa & Surendhiran, 2016).

1.2 Problem statement

Fossil fuels depletion due to the gradually growth of the worldwide population causing the depredation of current resource with the significant environmental impacts in energy shortage, worsen the climate change scenario and increase greenhouse gasses emission (Berardi, 2017). Many studies about the conversion of the biomass from agriculture crops to biofuels has been investigated. Research and developments in biofuels initiate from the production of fuel from first generation which use the sources of food (corn, wheat, barley and sugarcane) as feedstock evolve to algae metabolic engineering (Dutta et al., 2014). Production of the bioethanol in the world majority comes from terrestrial biomass which are essentially food (Borines et al., 2013; John et al., 2011). The used of the agriculture crops such as corns and grains in large quantities to produce bioethanol eventually lead to the contradiction about its possibility of food supply and food scarcity especially in the developing countries. Microalgae have been recently considered as the third-generation feedstock to produce bioethanol. The microalgae are fast growth and able to produce in large industrial scales. Moreover, microalgae are potentially to produce bioethanol because most of them have high carbohydrate contents which can be break down to fermentable sugars to produce bioethanol through fermentation process. Thus, this is another new approach of the sustainable energy. There is only some limited studies to

compare the bioethanol production from microalgae pretreated by strong acid and diluted acid. Therefore, this research aims to find out the bioethanol production of microalgae through pretreatment with different types of acids (by comparison of a strong and a weak acid) with various concentrations.

1.3 Research objectives

The main goal of this research is to compare and determine the production of bioethanol from microalgae after pretreatment with different types of acids (a strong and a weak acid) with various concentrations. Thus, the objectives of this research are as following:

1. to determine the reducing sugar contents of microalgal *Chlorella* pretreated with acetic and sulfuric acid in different concentrations during fermentation process.
2. to investigate the effect and relationship of types of acids with different concentrations towards the bioethanol fermentation.
3. to evaluate the bioethanol productivity from *Chlorella* by comparing the different concentrations of sulfuric acid and acetic acid via simultaneous saccharification fermentation (SSF).

1.4 Thesis outline

Chapter 1 are given a general idea of the introduction of biofuels produced from microalgae and problem statements in this study.

Chapter 2 are presented with a literature surveys about the pretreatments and fermentation process to obtain the bioethanol production.

Chapter 3 stated the methodology in this study and testing methods that applied to evaluate the bioethanol produced from microalgal *Chlorella*.

Chapter 4 focused on the results obtained in this experiment and discussion of the findings.

Chapter 5 contained a summary of the work done in this research and recommendation proposed for the future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Sustainable energy

The global energy consumption has been increased steeply in the past decades especially in developing countries (Chia et al., 2018; Ho et al., 2013a). The world energy resources has been widely use due to the fast growth of the world population. The energy sources based on fossil fuels in the form of oil, gas, coal or uranium are estimated to be exhausted in 55-75 years (Baicha et al., 2016). Fossil fuels which are the primary and non-renewable energy source for the world are predicted to be run out of source in the middle of century (Alaswad et al., 2015; Chia et al., 2018). Moreover, increasing global population which is projected to exceed 9 billion by 2050 leading to the overexploitation of the resources and fasten the scarcity of arable land to its limit (Dutta et al., 2014). Hence, this is critical to develop the alternative energy resources and adopt policies to minimize the utilization of fossil reserves, maintain the environmental sustainability and cost-effective, and reduce the release of greenhouse gasses (John et al., 2011; Singh et al., 2011). Among those mitigation measures, the most reliable way is development of the renewable technologies to produce the energy that is sustainable, efficient, cost-effective and reduce the emission of greenhouse gases to almost zero in 2100 (Baicha et al., 2016; John et al., 2011).

Biofuel are considered as a renewable fuels derived from biological sources that can be used to produce electricity, heat and fuel source (Singh et al., 2011). Recent statistical report of International Energy Agency (IEA) revealed that the total primary energy supply by fuel that the energy produce from biofuels and waste increasing steadily from 2.3% in 1973 to 5.7% in 2016 with the total of 3740 and 5257 (Mtoe), respectively (IEA, 2017). Therefore, it is expected biofuels will be emerged to one of the most strategically sustainable energy source (Nigam & Singh, 2011). Biofuels can be categorized into first-,

second-, third- and fourth-generations based on the type of the feedstocks (Shuba & Kifle, 2018). First-generation feedstock mostly comes from the agricultural crops which has raised a controversial arguments on food security and food supply (Koizumi, 2015; Veeramuthu et al., 2017). Therefore, the growing interest to develop a sustainable biofuel has been changed to second- and third-generation biofuels which have no food-fuel conflicts and better environmental benefits in terms of reducing greenhouse gases emissions (Gambelli et al., 2017). Second-generation biofuels are mainly produced from lignocellulosic materials such as industrial organic wastes, forest and agricultural residues (Lee et al., 2015; Vassilev & Vassileva, 2016). In addition, third-generation biofuels derived from microalgae feedstock such as bioethanol, biodiesel, biohydrogen and biomethane have been investigated and getting more interests worldwide (Chen et al., 2015; Kumar et al., 2017).

2.2 Algae biomass

Algae, macroalgae (commonly known as seaweeds) and microalgae which are the third-generation bioethanol source have secure a wide attention as an alternative source of biomass to produce ethanol (Chaudhary et al., 2014; Lam & Lee, 2015). The production of the bioenergy from the algae harvest from the marine or freshwater need not compete with food production in agriculture field (Li et al., 2014). Algae are a fast growing microorganism on Earth and they do not compete the arable land with food crops. They are tolerate to environmental stresses including growing in brackish and saline water or non-arable land and they can be cultivated from the wastewater released from industries (Sirajunnisa & Surendhiran, 2016).

Microalgae are one of the oldest microorganisms existing in the Earth and represent an abundance of photosynthesis species dwelling in the diverse environment (John et al., 2011; Lam & Lee, 2015). Microalgae are able to capture the carbon dioxide (CO_2)

efficiently from varies sources such as atmosphere, gas released from industrial activity, and soluble carbonate salts (Dragone et al., 2010). Microalgae are unicellular and microscopic plants (Katiyar et al., 2017) which considered to be an abounding resource for carotenoids, lipids, and polysaccharides (Chaudhary, 2014). Microalgae are convincing alternative resources for bioethanol production in comparison with conventional plant crops and their carbohydrate contents are mainly in the form of starch and cellulose which are easier to break down into fermentable sugars via microbial fermentation (Chen et al., 2013; El-Dalatony et al., 2016). The cell wall composition of microalgae are also different with lignocellulosic crops due to absent of lignin and very hemicelluloses contents which requires less harsh pretreatments and reduces the overall bioethanol production cost (Daroch et al., 2013; Pancha et al., 2016). Table 2.1 shows the carbohydrates content in different species of microalgae.

Table 2.1: Carbohydrates content in different species of microalgae (all results are presented in *%* dry weight).

Biomass	**Carbohydrate**	**References**
Anabaena cylindrica	25–30	(Dragone et al., 2010)
Aphanizomenon flos-aquae	23	(Milano et al., 2016)
Chlamydomonas reinhardtii	17	(Dragone et al., 2010)
Chlorella sp.	19.5	(Phukan et al., 2011)
Chlorella sorokiniana	35.67	(Chen et al., 2014)
Chlorella vulgaris	20.99	(Wang. et al., 2013)
Chloroccum sp.	32.50	(Harun et al., 2010)
Dunaliella salina	32.00	(Dragone et al., 2010)
Dunaliella tertiolecta	21.69	(Shuping et al., 2010)
Euglena gracilis	14–18	(Dragone et al., 2010)
Isochrysis zhangjiangensis	23.2–47.7	(Feng et al., 2011)
Isochrysis galbana	7.7–13.6	(Fidalgo et al., 1998)
Isochrysis sp.	5.2–16.4	(Martín-Juárez et al., 2017)
Nannochloropsis oceanica	22.70	(Cheng. et al., 2014)

Table 2.1, continued.

Biomass	Carbohydrate	References
Pavlova lutheri	28.25	(Ryu et al., 2012)
Porphyridium cruentum	40	(Biller & Ross, 2011)
Prymnesium parvum	25–33	(Dragone et al., 2010)
Scenedesmus dimorphus	21 - 52	(Dragone et al., 2010)
Scenedesmus obliquus	10–17	(Dragone et al., 2010)
Spirulina platensis	31.20	(Jena et al., 2011)
Spirogyra sp.	33 - 64	(Milano et al., 2016)
Spirulina sp.	20	(Biller & Ross, 2011)
Tetraselmis maculate	15	(Martín-Juárez et al., 2017)
Tetraselmis suecica	15–50	(Bondioli et al., 2012)
Tetraselmis sp.	24	(Schwenzfeier et al., 2011)

Chlorella is an unicellular microalga which grows in fresh water and exist on Earth since the pre-Cambrian period which is 2.5 billion years ago and its genetic integrity has remained consistent (Safi et al., 2014). *Chlorella is* capable to synthesis and convert a large quantities of carbohydrates into biomass for bioethanol production due to its high proton efficiency (Kumar et al., 2016). The name *Chlorella* originated from the Greek word *chloros* (Χλωρός), which refers to green, and the Latin suffix *ella* meaning to its microscopic size (Safi et al., 2014). Besides, *Chlorella* has high carbohydrate contents (20- 30 w/w dry) which is a potential feedstock to convert into bioethanol (Brennan & Owende, 2010). This microalgal is capable to be cultivated through autotrophic, heterotrophic techniques or mixotrophic (which combining both autotrophic and heterotrophic) environment (Liang et al., 2009).

2.3 Bioethanol

Biofuels can be categorized into two groups which are primary fuels such as firewood, wood pellets, wood chips, animal waste, crop residues and landfill gas; secondary group

which consists of bioethanol, butanol, biodiesel, and biohydrogen (Maity et al., 2014; Tan et al., 2015).

The production of bioenergy as an emerging sustainable energy has gained the interest especially in those economic developed countries (Katiyar et al., 2017). Compared to the fossil fuels, bioethanol is more benefits to environment because it is one of the renewable energy source that is considered as one of high possibility to replace the gasoline to act as a transport fuels (Li et al., 2014). The sources of the production of bioethanol are mainly generated from the staple food crops such as corns, wheat, or sugar cane which are well established as one of the food source for many people (Li et al., 2014). The use of these food crops as feedstock are not ideally due to its high price of raw materials which compromised around 40-75% of total ethanol production cost (Jang et al., 2012). The production of first generation fuel has raised a controversial debates on food supply and food security due to the large conversion of agricultural crops to biofuels (Koizumi, 2015; Tomei & Helliwell, 2016) and the dilemma of unstable food prices in the market because they are food source and feed crops globally (Vassilev & Vassileva, 2016). Nevertheless, the growing interest in biofuels has been switched to second and third generation which are no food-fuel conflict and have better environmental performance in terms of reducing greenhouse gasses emission (Gambelli et al., 2017). Second-generation feedstocks mainly produced from lignocellulosic materials from forest and agriculture residues and industrial organic wastes such as straw, grass, woods, sawdust and others have the limitation due to the difficulty and high costs to convert lignocellulosic biomass into biofuel in pretreatment process (Ho et al., 2013a; Lee et al., 2015; Vassilev & Vassileva, 2016). Third generation biofuels produced from microalgae as feedstocks have garnered interest worldwide (Bibi et al., 2017; Chen et al., 2015; Kumar et al., 2017).

2.4 Different types of pretreatment of microalgae biomass

Pretreatment is an important step to break down carbohydrates or starch polymers into monomer such as glucose to produce bioethanol (Tanadul et al., 2014). Then, the fermentable sugars will be metabolized by microorganisms that carry out bioconversion into bioethanol via fermentation (Miranda et al., 2012). Pretreatment is one of the most crucial steps to minimize the crystallinity degree of the cellulose matrix and increases the surface area to improve the digestibility of the substrate or bio-availability for bacterial enzymes to hydrolyze the biomass more resourcefully (Ellis et al., 2012; Harun et al., 2011). Many methods, including chemical methods, enzymatic hydrolysis and mechanical methods, have been conducted in order to disrupt cell wall of microalgae in order to release more carbohydrates and processing them into monosaccharides (Keris-Sen & Gurol, 2017; Shokrkar et al., 2017; Zhao et al., 2013). Details of these pretreatment methods were described in section 2.4.1, 2.4.2, 2.4.3 and section 2.4.4.

Chemical pretreatment is one of the most accessible method because chemical substances are easier to obtain and more cost effective in comparison to engineered enzymes. When chemical substances are stored properly, they are easier to handle as they have higher durability. Furthermore, most of the chemical substances which keep in a proper storage are less hassle and high durability compare to enzymes (Aditiya et al., 2016). In addition, it is relatively easier to carry out the mild condition chemical hydrolysis of microalgae-based cellulose which is not associated with lignin compared to with lignocelluloses (Chen. et al., 2013).

2.4.1 Acid hydrolysis

The release of monosaccharaides molecules from long chains of polysaccharides can be enhanced by using chemicals such as acid to break their bonds (Jambo et al., 2016). In most studies, the common acids used to convert feedstock to fermentable sugars were

sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) (Kim. et al., 2011; Matthew J. Scholz, 2013; Zhou et al., 2011). Shokrkar et al. (2017) had conducted a study to investigate the efficiency of microalgae in acid hydrolysis with different concentrations of HCl (0.5, 1, 2M) and H_2SO_4 (0.5, 1, 2M), 0.5M H_2SO_4 and 2.5% (w/v) $MgSO_4$, 0.5M H_2SO_4 and 2.5% (w/v) $CaCI_2$ respectively, autoclaved at 121℃ with different reaction times (10, 20, 30, 40 min). The findings of the results showed a higher acid concentration had improved the sugar extraction yield (Shokrkar et al., 2017).

Moreover, Miranda et al. (2012) reported acid pretreatment was more efficient than the physical treatment because physical disruption method did not really create a huge destructive impact on the cell wall of microalgae to release fermentable sugars. The study also highlighted the acid treatments (H_2SO_4 and HCl 2N) at temperature 120 ℃ had higher sugar extraction efficiency which were 8.2% and 8.1%, respectively. Dilute acid pretreatment was commonly used due to strong acid will cause an excessive degradation of the complex subtract and leading to the loss of fermentable sugars (Paudel et al., 2017). Therefore, it was not a surprised that unwanted compounds such as weak acids, furaldehyde and phenolic compounds were found in acid hydrolysate which could interfere the growth and glucose conversion reaction of *S. cerevisiae* during fermentation process (Wang. et al., 2014) . The formation of inhibitors after acid pretreatment can have a negative effects on the fermentation process (Sritrakul et al., 2017). Thus, acid hydrolysate need to be neutralized before fermentation process and it could directly increase the production cost. Many experimental studies have been carried out to identify the efficient pretreatment methods without the use of acid hydrolysis (Kim et al., 2015; Sanchez Rizza et al., 2017).

2.4.2 Alkaline hydrolysis

Different concentrations of sodium hydroxide (NaOH) solutions (0.5%, 0.75%, 1%, 2%, and 3%) with specific temperature and specific period of time were investigated in order to free and breakdown the *Chlorococcum infusionum* cell wall for fermentation process (Harun et al., 2011). The highest bioethanol yield was 26.1% (g ethanol /g algae) with 0.75% (w/v) NaOH at 120 *°C* for 30 min while the lowest bioethanol yield was 10.66% with 1% (w/v) of NaOH at 100 *°C* for 60 min. This study indicated there was some potential for bioethanol production from microalgae through alkaline pre-treatment. Besides this, four different alkaline agents (potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) and aqueous ammonia (NH_4OH)) were used to pretreat *T. suecica* and *Chlorella* sp. biomass into a 0.3M of alkaline agent at 90 *°C* for 75 min (Kassim & Bhattacharya, 2016). The highest reducing sugar concentration for *T. suecica and Chlorella* sp. were obtained by using 0.3 M (1.68% w/v) of KOH and 0.3 M (1.19% w/v) NaOH, respectively. Nevertheless, insignificant effects on reducing sugar production during the alkaline pretreatment process were found by using Na_2CO_3 and NH_4OH. The results indicated only a small amount of the reducing sugar produced from both pretreatments have similar reducing sugar produced in control sample (in the presence of alkali solution in room temperature). The observation in this study indicates the pretreatment condition such as selection of alkaline reagent, alkaline concentration and temperature play a major role in the alkaline pre-treatment process.

2.4.3 Supercritical carbon dioxide (CO_2)

Supercritical fluid method displays the interaction between fluid properties (density, diffusivity, surface tension, viscosity) and operational conditions (temperature, pressure, concentration of biomass) facilitates the separation of the desirable products (D'Hondt et al., 2017). Research on supercritical CO_2 to extract carbohydrate from microalgae for

bioethanol production has been reported (Harun et al., 2010). After the pretreatment which carried out at 60 ^{o}C with 400 mL min^{-1} of carbon dioxide, the cell wall of the *Chlorococum* sp. was disrupted due to the high pressure and process temperature. The disruption led to release of carbohydrates entrapped within cell wall. This study indicated microalgae pretreated with supercritical CO_2 gave 60% higher ethanol concentration for all samples than the biomass without pretreatment. Most of the current reports that were related to utilization of supercritical CO_2 were mainly for lipid extraction as compared to carbohydrates. Thus, there is a potential to conduct more studies of supercritical CO_2 extraction of carbohydrates from microalgae for bioethanol production.

2.4.4 Ammonia fiber explosion (AFEX)

AFEX is a pretreatment that allows ammonia to penetrate into cell wall with the presence of water, to create a cleavage of diferulate linkages which cross link polysaccharides, lignin ferulate and lignin diferulate linkages (Mathew et al., 2016). Biomass was treated with liquid anhydrous ammonia at a specific temperature (normally range between 60 ^{o}C to 100 ^{o}C) during AFEX pretreatment under a high pressure (about 250-300 psi) (Teymouri et al., 2005) for a limited period of time to vaporize ammonia and allow its recovery and recycling (Tomás-Pejó et al., 2011). At the end of pretreatment, large pores in the middle lamella and outer cell wall formed due to the expeditious pressure released caused the decompression of ammonia at cell wall periphery which led to increased enzyme activity on AFEX treated biomass (Mathew et al., 2016). AFEX pretreatment showed a significant improvement in saccharification rates especially in various herbaceous crops and grasses (Ye Sun, 2002). Furthermore, one of the benefits of AFEX pretreatment was no formation of by-products inhibitors such as furans (Chiaramonti et al., 2012). In fact, most of the experimental studies focused AFEX pretreatment on lignocellulosic biomass for lipid extraction instead of bioethanol

production from microalgae. In general, ammonia is a commodity chemical that is widely applied, and the cost is cheaper than sulfuric acid, at about one-fourth of the price on a molar basis. This implies AFEX pretreatment is more economically viable than dilute acid pretreatment (Kim et al., 2003). However, one concern of AFEX process is the recovery of ammonia after pretreatment which may lead to increase in both capital and operational costs (Bals et al., 2011).

2.5 Enzymatic hydrolysis

Recently, the production of bioethanol from enzymatic hydrolysis has greater potential as compared to acid hydrolysis (Jambo et al., 2016). Enzymatic hydrolysis of cell wall utilizes less energy than chemical hydrolysis although the amount of selective enzyme used for effective saccharification is generally high (Simas-Rodrigues et al., 2015). In an evaluation of enzymatic hydrolysis between seawater *Porphyridium cruemtum* (SPC) and freshwater *Porphyridium cruemtum* (FPC) with cellulase and pectinase, it was identified that the optimum pectinase and cellulase loading volume for saccharification were at 4.8 mg pectinase /g and 7.2 mg cellulase /g of SPC and FPC, respectively. The study also found out an ethanol conversion yield of 65.4% and 70.3% in SPC and FPC respectively, after 9 h fermentation (Kim et al., 2017). In addition, Choi et al. (2010) performed a study with enzymatic pretreatment of algal biomass *Chlamydomonas reinhardtii* UTEX 90 consisted of two parts, liquefaction by α-amylase from *B. licheniform*is at pH 6.0 for 70 *℃* to 90 *℃* followed by saccharification amyloglucosidase from *Aspergillus Niger* with optimal condition, 0.2% enzyme at pH 4.5 under 55 *℃* and 30 min to produce 235 mg ethanol/ g algae. Based on the experimental studies, parameters such as enzyme concentration, pH value, temperature and time appeared to have a great influence to the production of bioethanol in enzymatic hydrolysis.

Moreover, Harun and Danquah (2011a) used *Chlorococum humicola* which was hydrolyzed by cellulose from *Trichoderma reesei* in four different concentration ratios at different temperatures within range of 28 *ºC*– 60 *ºC*, and pH within range of 2.5 - 7.5 for 72 h obtained a saccharification yield of 68.2*%* (w/w) under the conditions of 40 *ºC*, pH 4.8 with a microalgal biomass concentration of 10 g/L. In general, comparing to the acid pretreatment, enzymatic hydrolysis had more advantages than acid pretreatment. It was because the hydrolysis conducted at mild conditions was able to produce a higher glucose yields and did not form toxic by-products such as formic acid, levulinic acid, hydroxymethyl furfural (HMF), furfural and other phenolic compounds that may degrade the production of bioethanol during fermentation process (Chen. et al., 2013; Zabed et al., 2017). However, the selection of the pretreatment technology for a specific raw material depends on several factors which directly and indirectly affect the enzymatic hydrolysis step. The factors are sugar-release patterns, combination of the substrate, type of pretreatment and concentration and efficiency of the enzymes used for the hydrolysis to have a great impact on biomass digestibility (Alvira et al., 2010).

2.6 Combined pretreatment

There are many pretreatment methods apply on microalgae which include chemical, physical and enzymatic pretreatments. Physical methods require higher energy consumption and complex operations (Marie et al., 2015). Chemicals methods are widely use but they will produce many inhibitors after pretreatment which will interrupt the fermentation process and even though enzymatic hydrolysis has its inherent advantages however costs and highly specificity enzyme apply to certain algal may hinder its commercial application (Yuan et al., 2016). Compared with the aforementioned, there were also some reports in literatures applied the combined pretreatment to study the

efficiency of the bioethanol production. A summary regarding the combined pretreatment and the results were given in Table 2.2.

Combined sonication and enzymatic hydrolysis on microalgal *Chlamydomonas Mexicana* to determine the efficiency of bioethanol production via SHF and SSF was conducted by El-Dalatony et al. (2016). From the experiment, 88.2% theoretical bioethanol yield was obtained after 48 h SSF process. Then, the SSF process was selected to optimize the bioethanol yield through repeated-batches by using immobilized yeast cells achieved a total energy recovery of 85.81% from the microalgal biomass in the form of bioethanol. Residual biomass of *Chlorella* sp. KR-1 after lipid extraction was used for saccharification through simple enzymatic with Pectinex at pH 5.5 at 45 *°C* then chemical pretreatment 0.3N HCl at 121 *°C* for 15 min through SHF process able to produce 0.16 g ethanol /g residual biomass.

Furthermore, another combined pretreatment study was conducted to examine the enzymatic hydrolysis of *Chlorococum humicola* with the use of cellulase from *Trichoderma reesei*, ATCC 26921 after cell disruption by ultra-sonication (Harun & Danquah, 2011a). Ultra-sonication was used to destroy the cell wall of the microalgae followed by the hydrolysis at different range of temperatures (28- 60 *°C*), pH within the range of 2.5- 7.5 for 72 h and substrate concentration with constant enzyme dosage (20 mg cellulase). The highest glucose yield in this experiment was 64.2% (w/w) at a temperature of 40 *°C*, pH 4.8 and a substrate concentration of 10g /L of microalgal biomass. Besides this, Karatay et al. (2016) used different combined pretreatments such as sonication, temperature with pressure, acid hydrolysis with and without autoclaving in order to find out the most appropriate pretreatment process to optimize the cell disruption on halophilic microalgae *Dunaliella* sp. for bioethanol production. The most effective

combined method was 1% H_2SO_4 acid with autoclaved which produced 0.91 ± 0.05 g/L bioethanol after a 72 h fermentation with a 30g /L microalgae loading.

Keris-Sen and Gurol (2017) used mixed microalgal cultures predominantly *Chlorococcum* sp. were pretreated with different ozone doses at 0.25- 2.0g O_3 g/L dry weight biomass (DWB) then performed at different enzyme concentrations obtained the highest glucose yield of 80.6% (w/w) of total carbohydrates under the condition of 2.5 g/L biomass concentration after 4 h hydrolysis time for 0.5g O_3 g/L DWB ozone dose and 1.2mL/g DWB enzyme concentration. This study showed ozone pretreatment with enzymes saccharification attained a much higher glucose yield compared to the use of enzymes only. In addition, hydrothermal fractionation combined with enzymatic method was studied by Kim et al. (2011) resulted in a production of 11.8g ethanol/ L.

Eighteen identified microalgae strains collected from coastal water Pearl River Delta were selected for further biomass and ethanol production. The outcomes in this findings found out microalgal *S. abundans* PKUAC 12 was the superior feedstock compared to other microalgae strains for bioethanol production after combined pretreatment with dilute acid and cellulase from *Trichoderma resei* (Guo et al., 2013). Furthermore, another combined pretreatment of liquid hot water (LHW) and enzymatic hydrolysis showed a reliable approach to destroy the cell structures to produce bioethanol from lyophilized microalgae, *Scenedesmus* sp. WZKMT (Okuda et al., 2008; Yuan et al., 2016). The mixtures were maintained at different temperatures (100, 120, 140, 160, 180 and 200 *°C*) for different timings of 20, 40, 60, and 80 min (Yuan et al., 2016). After LHW pretreatment, enzymatic hydrolysis was continued to pretreat the mixtures. This resulted a concentration and recovery of glucose at 14.223g/ L, 89.32%, respectively with an optimal condition at 147 *°C*, 41 min, and a solid-to-liquid ratio at 1:13 (w/v).

Table 2.2: Different types of combined pretreatment to microalgae and the result obtained from various studies.

Microalgae	Pretreatment	Condition	Fermentation process	Results	References
Chlamydomonas Mexicana	Sonication and enzymatic	Sonication: frequency 40kHz, 2.2kW, 15min and enzymatic: ≥1 unit per mg of solid, pH 5.0, 1 h, 50 *°C*	i. *S. cerevisiae*, SSF, pH 5, 30 *°C* , 72 h, 120 rpm ii. *S. cerevisiae* with Ca-alginate , SSF, pH 5, 30 *°C* , 72 h, 120 rpm repeated batch fermentation for 7 cycles	i. 10.5 g/L ethanol production ii. 9.64 g/L ethanol production	(El-Dalatony et al., 2016)
Chlorella sp. KR-1	Enzymatic and chemical (HCl)	Enzymatic: 0.8mL enzyme/g residual biomass, pH 5.5, 45 *°C*; chemical: dilute HCl, 121 *°C*, 15 min	*S. cerevisiae*, SHF, pH 6, 30 *°C*, 20h, 180 rpm	0.16 g ethanol/g residual biomass	(Lee et al., 2015)
Chlorococum humicola	Ultrasonication and enzymatic	Ultrasonication: frequency at 40kHz, 130W, enzymatic: 0.02 g enzyme/ g substrate 4.5, 40 *°C*	-	Highest glucose yield was 64.2% (w/w).	(Harun & Danquah, 2011a)
Dunaliella sp.	Autoclave and chemical (H_2SO_4)	Autoclave: 121 *°C*, 15 min and chemical: 1% H_2SO_4, 121 *°C*, 15 min	*S. cerevisiae*, pH 6, 72h	0.91 ± 0.05 g/L ethanol production	(Karatay et al., 2016)

Table 2.2, continued

Microalgae	Pretreatment	Condition	Fermentation process	Results	References
Mixed microalgal culture (dominated by *Chlrococcum* sp.)	Ozone and enzymatic	4 h for 0.5 g O_3 gL^{-1} dry weight biomass (DWB) ozone dose and enzymatic:1.2 mL^{-1} g^{-1} DWB, pH4.8, 50 *℃*, 4 h, 160rpm	-	Highest glucose yield was 80.6% (w/w) of total carbohydrate	(Keris-Sen & Gurol, 2017)
Schizocytrium sp.	Hydrothermal and enzymatic	Hydrothermal: 115.5 *℃*, 46.7 min reaction time, 25% (w/w) solid loading and enzymatic (14,000 α-amylase units (AAU)/g and 350 glucoamylase units (GAU)/g)	*E. coli*, SSF, 37 *℃*, 72 h, 150rpm	11.8 g /L	(Kim et al., 2011)
Scenedesmus abundans PKUAC 12	Chemical (H_2SO_4) and enzymatic	Chemical: 3% H_2SO_4, 110 *℃*, 30 min and enzymatic: 10 mg αmylase and 10 mg glucoamylase, pH 5.5, 30 min	*S. cerevisiae*, pH 5.5, 30 *℃*, 48 h, 200 rpm	0.103 g of ethanol/ g biomass	(Guo et al., 2013)
Scenedesmus sp. WZKMT	Liquid hot water (LHW) and enzymatic	LHW: 1:13 (w/v), 147 *℃*, 40 min and enzymatic: 0.125% (w/v), pH 4.5, 37 *℃*, 48 h, 150 rpm	-	Concentration and recovery of glucose 14.223 g/L 89.32%	(Yuan et al., 2016)

2.7 Bioethanol production through fermentation process

Fermentation is a metabolic conversion activity of monosaccharides to bioethanol and other by-products with the present of fermentative microorganism in supporting conditions namely temperature and pH range (El-Dalatony et al., 2016). An introduction of specific fermentation agent (yeast or bacteria) is commonly used in the fermentation process. Theoretically, 1 kg of glucose and xylose can produce 0.49 kg of carbon dioxide with ethanol yield of 0.51 kg (Aditiya et al., 2016). Microorganisms *E.coli, Z. mobilis and S. cerevisiae* have promising bioethanol production with high efficiency and widely utilized in industrial activity, especially *Saccharomyces* (Carlos & Bertucc, 2016). Some of the research studies carried out experiments with different fermentative microorganisms during fermentation process as shown in Table 2.3.

2.7.1 Fermentative microorganisms

2.7.1.1 Yeast

Yeast can be defined as ascomycetous or basidiomycetous fungi whose asexual growth by budding or fission reproduction which do not form their sexual states within or upon a fruiting body (Kurtzman et al., 2011; Mohd Azhar et al., 2017). Yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were known as crabtree-positive yeast because they could accumulate ethanol in the presence of oxygen (Piskur et al., 2006). There are some other yeasts strains such as *Candida shehatae, Kluyveromyces marxianus, Kluyveromyces fragilis, Pichia stipitis,* and *Pachysolen tannophilus* have been used in fermentation process (Dutta et al., 2014; Mohd Azhar et al., 2017; Mussatto et al., 2012; Saini et al., 2017).

Saccharomyces cerevisiae has been widely used due to high bioethanol production from hexoses, high tolerance to bioethanol and other inhibitory compounds (Balat et al., 2008). In a comparison study of ethanol production from seawater *Porphyridium*

cruentum (SPC) and freshwater *Porphyridium cruentum* (FPC) with dry yeast *Saccharomyces cerevisiae*, KCTC 7906 to perform fermentation at 30 ℃ for 7 h via separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). This study indicated that SSF process was more efficient than SHF for bioethanol production from both SPC and FPC with the ethanol yield of 2.77 and 2.98 mg/ mL, respectively (Kim et al., 2017). Besides this, mixed culture *Chlorella vulgaris* YSL001 and *Uronema belkae* YSL010 were pretreated with ultra-sonication, heat and enzymatic hydrolysis then added fermentative bacterial and *Dekkera bruxellensis* ATCC34447 yeast in different fermenters to perform fermentation showed a higher ethanol yield by using yeast as fermentative microorganism than mixed bacterial culture (Hwang et al., 2016).

Karatay et al. (2016) reported microbial growth and fermentation rates were greatly influenced by pH value of growth medium and it indirectly affected the bioethanol productivity. Furthermore, four different yeasts (*Saccharomyces cerevisiae*, *Brettanomyces custersainus, Pichia stipites, and Klebsiella oxytoca*) were studied to compare the bioethanol production from microalgae *Microcystis aeruginosa* in a shaking incubator under 25 ℃ at 150 rpm for 30 h. Results showed the highest ethanol content was obtained by *S. cerevisiae in* the fermented samples (Khan et al., 2017). Even though *S. cerevisiae* was able to convert many fermentable sugars into ethanol efficiently but it was not able to ferment pentoses. In contrary, *Brettanomyces custersainus* was capable to convert pentoses and *Pichia stipites* was able to convert xylose to ethanol faster than any other microorganisms (Khan et al., 2017; Kumar. et al., 2009).

2.7.1.2 Bacteria

The popular ethanologenic bacteria are *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis*. However, *Z. mobilis* is well known for its ability to produce

bioethanol rapidly from glucose-based feedstock and able to achieve 5% higher yields and up to five-fold higher volumetric productivity when comparing with traditional yeast fermentations in comparative performance trials (Balat et al., 2008). *E. coli* has several advantages in biofuels production due to its high growth rates, ability to grow in low-cost mineral media in anaerobic condition, which significantly reduces the production cost, and ability to use different carbons source such as carbohydrates, polyoils and fatty acids (Gonçalves & Simões, 2017; Wang et al., 2017). Three different algae species: *Undaria pinnatifid*, *Chlorella vulgaris,* and *Chlamydomonas reinhardtii* were used to study the saccharification conditions at several temperatures, acid concentrations, pH value, and durations with some ethanolic *E. coli* W3110 strains. Maximum ethanol yield of 0.4g ethanol/g biomass was obtained by pretreated *C. vulgaris* with *E. coli* SJL2526 (Lee et al., 2011). Although *E.coli* was capable to ferment a wider range of sugars than *S. cerevisiae* and *Z.mobilis* strains but the ethanol yield was lower due to formation of other fermentation products in higher rates and final concentration (Xiros et al., 2013).

Z. mobilis is recognized as a safe status ethanologenic bacteria that has many industrial biocatalyst characteristics and the investigation of DNA restriction-modification systems in *Z. mobilis* also helps to increase the transformation proficiency for more control strain development (Yang et al., 2016). Pre-cultured *Z. mobilis* cells were inoculated in the solution of *Scenedesmus obliquu*s CNW-N after acid hydrolysis via separate hydrolysis and fermentation (SHF) showed an ethanol concentration of 8.55 gL^{-1} which represented theoretical yield of nearly 99.8% (Ho et al., 2013). A comparison study between *Z. mobilis* and *S. cerevisiae* from algae *Spirogyra* exhibited that *Z. mobilis* was more effective than *S. cerevisiae* to produce bioethanol which were 9.70% and 4.09%, respectively after a 96 h fermentation process (Sulfahri et al., 2011).

Table 2.3: Comparison of ethanol yield from various microalgae by using different fermentative microorganisms.

Microalgae	Pretreatment	Fermentative microorganism	Fermentation condition	Maximum ethanol production	References
Chlorococum sp.	Supercritical fluid	*S. bayanus*	30 ℃ , 60 h, 200rpm	3.83 g/L	(Harun et al., 2010)
Chlorococcum infusionum	Chemical (NaOH)	*S. cerevisiae*	Process: SHF, 72 h, 200rpm	0.26 g ethanol/g algae	(Harun et al., 2011)
Chlamydomonas reinhardtii UTEX 90	Enzymatic	*S. cerevisiae* S288C	Process: SSF 30 ℃, 40 h, 160rpm	235 mg ethanol /g algae	(Choi et al., 2010)
Chlorella	Chemical (HCl and $MgCI_2$)	*S. cerevisiae* Y01	30 ℃ , 48 h, 200rpm	22.60 g/L	(Zhou et al., 2011)
Chlorella variabilis	Viral and enzymatic	*E. coli* KO11	pH 6.5 35 ℃, 3 days, 150rpm	0.326 g/g carbohydrate consumed	(Cheng et al., 2013)

Table 2.3, continued.

Chlorella vulgaris	Chemical (H_2SO_4)	*E. coli* SJL2526	Process: SHF, pH 7.0, 37 *°C*, 170rpm	0.4g ethanol/g algae	(Lee et al., 2011)
Chlorella vulgaris FSP-E	Chemical (H_2SO_4)	*Z. mobilis*	Process: SHF pH 5 – 6, 30 *°C* within 12 h	11.66 g/L	(Ho et al., 2013a)
Porphyridium cruemtum	Enzymatic	*S. cerevisiae* KCTC 7906	Process: SSF pH 4.8, 37 *°C* , 9 h	Theoretical yield 65.4% (seawater) and 70.3% (freshwater)	(Kim et al., 2017)
Scenedesmus obliquus CNW-N	Chemical (H_2SO_4)	*Z. mobilis* ATCC29191	Process: SHF pH 6, 30 *°C* within 4 h	8.55 g/L	(Ho et al., 2013)

2.8 Bioethanol production through fermentation process

2.8.1 Separate hydrolysis and fermentation (SHF)

SHF is a fermentation process based on separation of hydrolysis to degrade the feedstock into monosaccharides continuously by fermentation process with fermentative microorganism that converts fermentable sugars into ethanol (Jambo et al., 2016). The advantages of this process are low cost of chemicals, short residence time and simple equipment system (Li et al., 2014). SHF can produce ethanol at optimum temperature independently but the accumulation of glucose and cellobiose during hydrolysis have inhibitory effects that result in the formation of end-product inhibition as well as high probability of unpreventable contamination (Jambo et al., 2016; Xiros et al., 2013). Moreover, SHF is less favorable than SSF due to the additional neutralization and purification steps are needed to be carry out before fermentation process to prevent the formation of undesirable by-products (Lam & Lee, 2015).

2.8.2. Simultaneous saccharification and fermentation (SSF)

In contrast with SHF, SSF is a simultaneous fermentation which conducts both hydrolysis and fermentation in single step in a single reactor. The feedstock, enzyme and yeast were added together in an orderly manner to release fermentable sugars rapidly and converted into bioethanol (Jambo et al., 2016). According to Balat (2011), the carbohydrates polymers are converted to fermentable sugars with the presence of cellulases and xylanases. SSF process is more effective than SHF process however it requires a compatible conditions with similar pH, temperature, and optimum substrate concentration (Ballesteros et al., 2004). From a comparative study between SHF and SSF conducted by El-Dalatony et al. (2016), SSF showed a greater efficiency of fermentation process than SHF for bioethanol production from microalgae *Chlamydomonas Mexicana* with production of 10.5 g /L ethanol and 8.48 g /L of ethanol, respectively. Moreover,

Kim et al. (2017) reported the red microalgae *Porphyridium* cruentum showed SSF was more efficient than SHF for bioethanol production from both seawater *P.*cruentum and freshwater *P.*cruentum with ethanol conversion of 65.4% and 70.3%, respectively. Many studies stated SSF was more preferable than SHF due to its ability to reduce the cost, processing time, required only a small amount of enzymes, less contamination, low inhibitory effects and high ethanol production rate (Aditiya et al., 2016; Dahnum et al., 2015; Jambo et al., 2016; Sirajunnisa & Surendhiran, 2016).

2.9 Economic sustainability among the industries

Microalgae have driven the economic impacts in biotech, food, pharmaceutical, agriculture and cosmetic industries (Cardozo et al., 2007). Bioethanol production from microalgae require less energy consumption compared with biodiesel production and the ethanol yields are more comparable to those sugary or lignocellulosic substrates (Baicha et al., 2016). Microalgae are getting more relevant among the industries especially biofuels. A systematic supply chain is essential to maximize the profitability of algae biofuel (Bibi et al., 2017). Bioethanol obtained from microalgae may offer a suitable alternative way to replace petroleum-based fuels in the future (Sivaramakrishnan & Incharoensakdi, 2018). Biofuels such as diesel, gasoline and bioethanol produce from microalgae can be used for transportation or directly transported for electricity generation (Milano et al., 2016). Bioethanol has more efficiency advantages over gasoline such as higher octane number (108), wider flammability limits, greater flame speeds and higher heat of vaporization that allow for a higher compression ratio and shorter burning time (Balat & Balat, 2009). In U.S. and Australia, Algae Biofuels operated by PetroSun was set up in June 2006 to investigate about the production of biodiesel, ethanol, methanol methane and hydrogen from microalgae (Sirajunnisa & Surendhiran, 2016). The company also provided feedstock, half or up to 150 million gallons per year to another company

BioAlternatives. Furthermore, a total of 8000 gallons of liquid biofuel per acre per year were generated by Algenol in U.S. which is a company started bioethanol production from microalgae in 2006 by (Bibi et al., 2017; Sirajunnisa & Surendhiran, 2016). In California U.S., Sapphire Energy, Inc was founded in 2007 with an investment greater than $100 million to produce 100,000 gallons/ year of fuel-grade ethanol (Bibi et al., 2017). The development of the algae biofuel market was predicted to increase by year 2030 and will dominate 75% of the market share (Gambelli et al., 2017; Vo et al., 2018).

2.10 Environmental influences of bioethanol from microalgae

Scarcity and inconsistent price of fossil hydrocarbons are emerging issues. Production of biofuels from renewable supplies especially from algae is an ingenious notion that can be thought in relation to economic sustainability and ecological preservation. Biofuel produced from algae has a high performance to serve as a bioresource due to the ability of algae to use a large amount of CO_2 resulting into production of biomass. The reduction of CO_2 level provides energy facilities with zero or almost zero emissions on both air contaminants and greenhouse gasses (Bibi et al., 2017). In addition, a study evaluated the emissions of bioethanol in different spark ignition (SI) engines found out the carbon monoxide (CO) emissions decreased when using blended ethanol fuel in SI engine (Thangavelu et al., 2016). Bioethanol-blended fuel for automobiles can reduce the reliance on petroleum and cut down the greenhouse gas emission from vehicles (Balat et al., 2008). Bioethanol has been considered as eco-friendly biofuel because it can reduce the interfacial tension of petrol with reverence to water that allows the ethanol-gasoline non aqueous phase liquid (NAPL) to pass through minor pore spaces and penetrate easily from vadose zone to water table underground (Bibi et al., 2017).

2.11 Challenges and future outlook of algae bioethanol

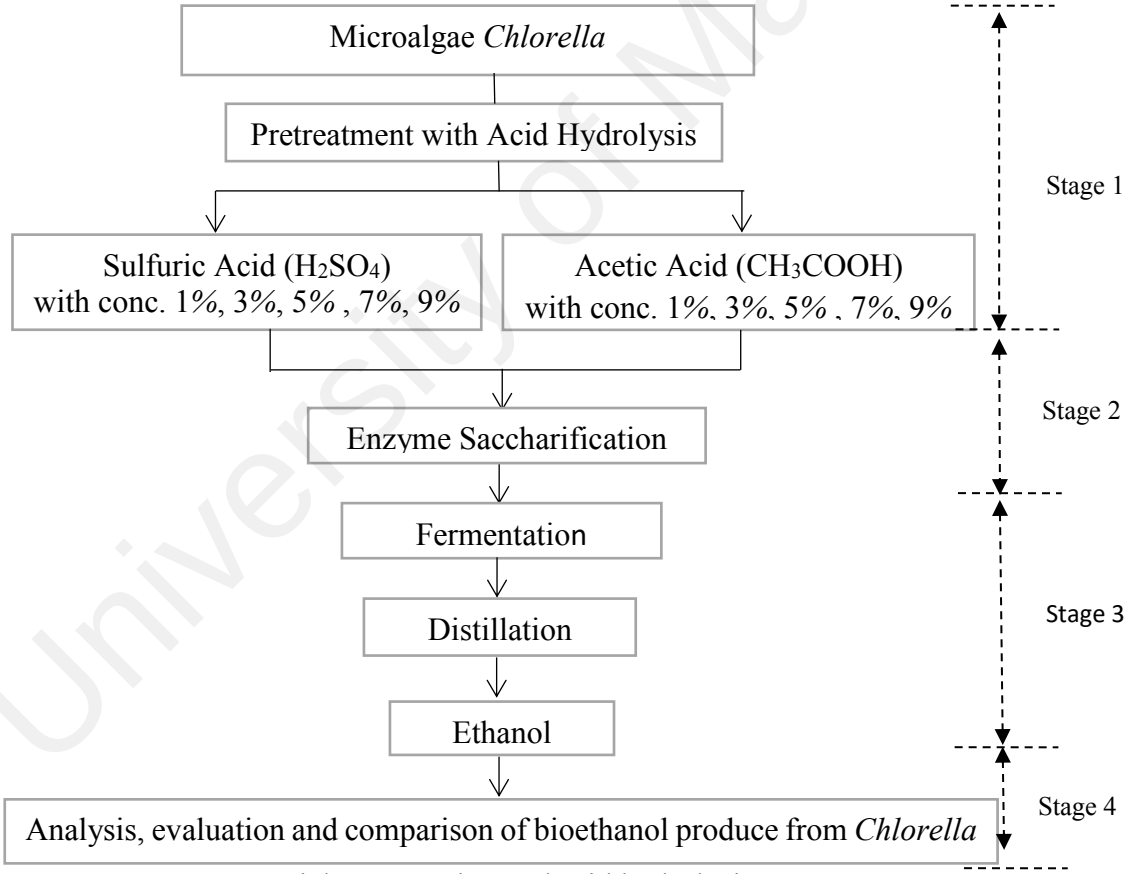
Microalgae have driven the economic impacts in biotech, food, pharmaceutical, agriculture and cosmetic industries (Cardozo et al., 2007). They are getting more popular among the industries including biofuels which may offer a good sustainable way to replace petroleum-based fuels in future (Sivaramakrishnan & Incharoensakdi, 2018). Bioethanol has garnered popularity in both developing and developed countries since the utilization of fossil petrol caused harm to the global environment and oil reserves (Aditiya et al., 2016). However, one major barrier is the inconsistency of technologies in bioethanol production. Due to high production cost and difficulty in achieving revenue, investors are less inclined to commercialize bioethanol in a larger scale (Jambo et al., 2016). However, researchers are still focusing on the improvement of algae bioethanol technologies and looking for a better transgenic algae strain to obtain reproducible results (Singh et al., 2011). Furthermore, bioethanol production from microalgae are also facing some hurdles such as high capital cost of facilities, lack of implementation of relevant policies and insufficient government support in the commercialization process (Bibi et al., 2017; Jambo et al., 2016).

A concept called "fourth generation algal biofuels" or "photosynthetic biofuels" which make use of synthetic biology of algae and cyanobacteria involve in a direct application of photosynthesis for the generation of fuels and chemicals through a metabolic engineering process where a single photosynthetic microorganism can be acted as catalysts and processors to synthesis and secretes ready to use products (Melis, 2012). This significant approach provides high biofuel productivity and more economic sustainability by cutting the biomass separation and processing costs associated with traditional approach (Daroch et al., 2013).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Process outline

In stage 1, microalgal biomass is pretreated with two types of acid with different concentrations. After that, enzyme pretreatment with α-amylase for liquefaction and amyloglucosidase for saccharification are performed in stage 2. Then, cultivated yeast *S. cerevisiae* are added and fermentation process is conducted under a specific condition for 84 h in shaking incubator to produce bioethanol in stage 3. Evaluation and analysis of bioethanol produce from *Chlorella* are tested in stage 4.



Note: Stage 1- Materials preparation and acid hydrolysis.
Stage 2- Enzymatic pretreatment with α-amylase for liquefaction and amyloglucosidase for saccharification.
Stage 3- Bioethanol production through SSF process.
Stage 4- Testing ethanol contents via gas chromatography equipped with flame ionization detector system (GC-FID); analysis and comparison of the obtained experimental results.

Figure 3.1: Research frame of bioethanol production from microalgal, *Chlorella*.

3.2 Material and enzymes

The material used in this study was microalgae *Chlorella* powder purchased from the market of South Korea. 10g of microalgae *Chlorella* was weighed with SHIMADZU ATX224 analytical balances (as shown in Figure 3.2).

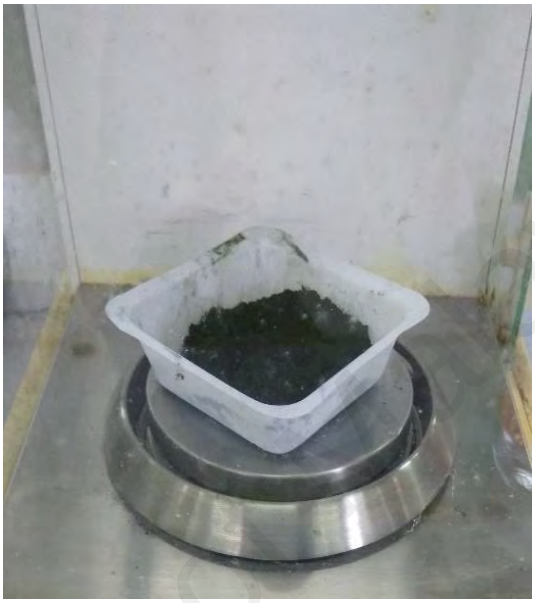


Figure 3.2: Microalgal *Chlorella* sample.

Enzyme α-amylase from *Bacillus licheniformis* Type XII-A with an enzymatic activity of more than or equal to 500 U/g protein and amyloglucosidase from *Aspergillus niger* with an enzymatic activity of greater than or equal to 300 U/mL were used as a catalyst. Both enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA), α-amylase acted as liquefaction and amyloglucosidase performed saccharification.

3.3 Methodology

There were several processes involved in this production of bioethanol from *Chlorella* species. Firstly, the microalgae was pretreated with acetic and sulfuric acid in various concentration (1%, 3%, 5%, 7% and 9%). Next, the hydrolysates were pretreated with enzymes α-amylase and amyloglucosidase to perform liquefaction and saccharification before fermentation. Cultivated yeast *S. cerevisiae* was added before fermentation.

Fermentation process was conducted under a specific condition for 84 h in shaking incubator to produce bioethanol.

3.3.1 Dilute acid preparation

Acetic acid (from Merck (glacial) 100% concentration) and sulfuric acid (from Friendemann Schmidt Chemical 95- 97% concentration) were used in this experiment. Both of the acids were diluted in conical flasks to concentrations of 1%, 3%, 5%, 7% and 9%, respectively. Distilled water was added first then only added acid to prevent splashing concentrated acid out from the flask due to exothermic reaction. Micropipette and measuring cylinder were used to obtain the accurate volume of concentration. Total of 10 samples with different concentrations of acetic acid and sulfuric acid mixtures were prepared in 200 ml glass reagent bottles with cap, respectively as shown in Figure 3.3 and 3.4. Dilute acid with different concentrations were prepared (Table 3.1) by using formula as below:

$$M_1V_1 = M_2V_2 \quad (3.1)$$

Whereas,

M_1= concentration in molarity of the concentrated solution

V_1= volume of the concentrated solution

M_2= concentration in molarity of the dilute solution

V_2= volume of the dilute solution

Table 3.1: Different concentrations of acid were mixed with distilled water.

Type of Acids / Concentration (%)	Acetic Acid (CH_3COOH)	Sulfuric acid (H_sSO_4)
	Ratio (Acid: Distilled water) in mL	Ratio (Acid: Distilled water) in mL
1	1.55 : 148.45	1.5 : 148.5
3	4.7 : 145.3	4.5 :145.5
5	7.8 : 142.2	7.5 : 142.5
7	10.9 : 139.1	10.5 : 139.5
9	14.1 : 135.9	13.5 : 136.5

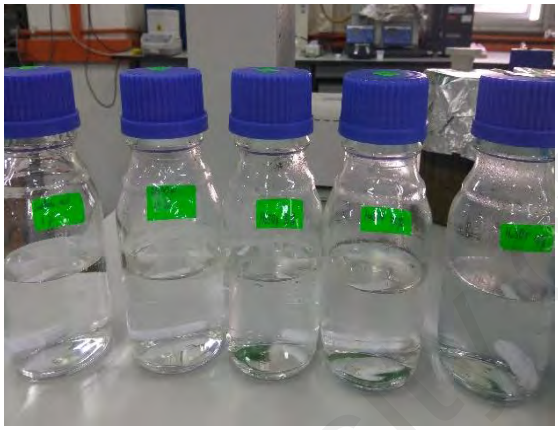


Figure 3.3: Acetic acid with different concentrations.

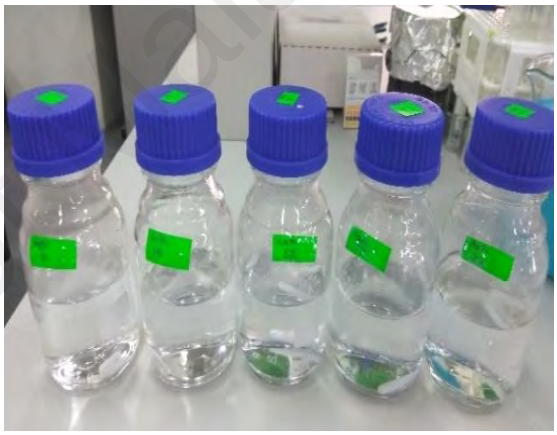


Figure 3.4: Sulfuric acid with different concentrations.

3.3.2 Acid hydrolysis pretreatment

Chemical pretreatment with dilute acid H_2SO_4 are widely used to pretreat the microalgae for bioethanol production due to its fast and inexpensive (Lam & Lee, 2015; Li et al., 2014). 10 g of microalgal *Chlorella* was measured by using a weighing machine. Then, the microalgal powder was added to the mixtures with different concentration of acids. Next, magnetic stirrer bar was added to each bottle. The mixtures which contained acid and microalgal powder as shown in Figure 3.5 were stirred on the DAIHAN Scientific multi hotplate stirrer SMHS-3 model for 5 min at 400 rpm to ensure the microalgal powder was dissolved completely.

After five minutes, the glass bottles were put inside the Thermoline drying oven SOV70B model at 120 °*C* for 30 min as shown in Figure 3.6. The acid concentrations and heating temperature were modified according to Harun and Danquah (2011b). A zero percent of acid concentration was set as control. After 30 min, the oven was cooled down to 60 °*C* and all bottles were taken out from oven. The bottles were cooled down to room temperature before adjusted pH value of the hydrolytes. The acid hydrolytes were adjusted to pH 6.0 by using sodium hydroxide (NaOH).

Figure 3.5: Mixture were stirring on the hot plates.

Figure 3.6: Samples were kept in the oven under specific condition for acid hydrolysis.

3.3.3 Enzyme pretreatment

Enzyme pretreatment step was carried out after acid hydrolysis. Enzyme pretreatments is importance to convert the complex organic compounds into simpler fermentable sugars that is essential to use in the fermentation process (Sulfahri et al., 2011). Once the solution was adjusted to pH 6 at room temperature 110 U/g of α-amylase was added to the solution and heat at 90 °*C*, stroke speed 130 spm for 90 mins in a water bath shaker for liquefaction. After the reaction, the bottles were taken out and 66 U/mL of amyloglucosidase was added into solution and heat at 70 °*C*, stroke speed 130 spm for 240 mins in the Memmert WNE 45 water bath shaker for saccharification reaction (Figure 3.7). The parameters were modified according to Sebayang et al. (2017). α-amylase was used to split the

molecular starch which is a polymer from 1.4 bond-α-glycosides into glucose, maltose, and dextrin (Sulfahri et al., 2011). Amyloglucosidase was used because it targeted to break 1,4-linked alpha-D-glucose residue into beta-D-glucose which are a fermentable sugar (Pancha et al., 2016). The pretreated solutions were adjusted to a pH range within 4.5 to 5.0 because this is the optimum pH range for yeast to work well during fermentation to convert the fermentable sugars into ethanol (Mohd Azhar et al., 2017). In this experiment, the samples were labelled as A1, A3, A5, A7, A9 (which mean pretreated microalgal samples with 1%, 3%, 5%, 7% and 9% acetic acid) and S1, S3, S5, S7, S9 (which mean pretreated microalgal samples with 1%, 3%, 5%, 7% and 9% sulfuric acid). A control without any pretreatment was used to monitor and compare the ethanol productivity with other pretreated samples.

Figure 3.7: Samples were kept inside water bath shaker at specific condition.

3.3.4 Yeast cultures media preparation

The yeast of *Saccharomyces cerevisiae* (*S. cerevisiae*) Type II, which was purchased from Sigma-Aldrich, was used for fermentation process in this experiment. 5 g of dry yeast *S. cerevisiae* was activated by adding 50 mL of warm distilled water in a beaker. Then, the yeast solution was put inside an incubator under 32 °*C* for 6 h.

Then, the yeast peptone dextrose was prepared by 2 g of yeast extract, 4g bacterial peptone, 4 g of glucose and 12 g agar in 200 mL of distilled water. The broth was sterilized in an autoclave at 121 °*C* for 15 min. After that, the nutrient broth was poured on the petri dish. When the liquid nutrient broth on the petri dish was condensed, the yeast cultivation process was conducted in a UV laminar flow chamber (Labcaire class II cabinet BH120) to prevent contamination (Figure 3.8). A micropipette was used to pipette 250 µL of activated yeast solution on the petri dish. Then, sterilized hockey stick was used to spread the activated yeast on petri dish. After that, the petri dish was completely sealed with parafilm and wrapped with aluminum foil. The petri dishes were put in an incubator at 32 °*C* for 5 days.

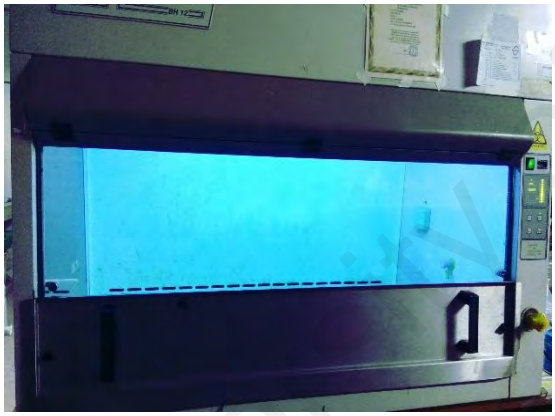


Figure 3.8: UV laminar flow chamber was switch on before yeast cultivation process.

Figure 3.9: The cultivated yeast grew on the petri dish after five days.

After 5 days, the cultivated yeasts were growing on petri dish in the individual spots (Figure 3.9). In order to get the pure yeast culture, zig zag plating was performed. A yeast spot was taken from each of the petri dish and transferred to another empty petri dish with agar by using an inoculation loop (Figure 3.10 and Figure 3.11). Then, the petri dish was completely sealed with parafilm and wrapped with aluminum foil. All of the petri dishes were put in an incubator at 32 °*C* for three days.

Figure 3.10: The single yeast colony grew on the petri dish after three days.

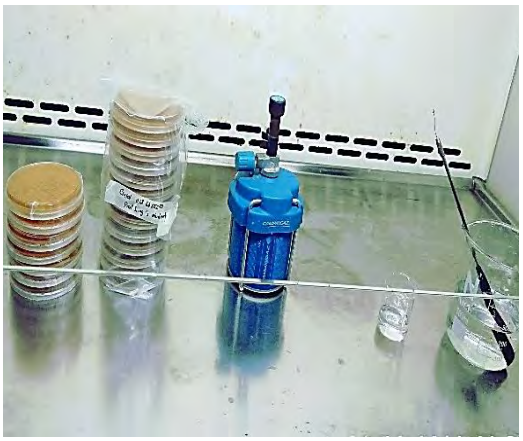


Figure 3.11: Yeast cultivation with inoculation loop.

After 3 days, single colony grew on the petri dish was transferred to another agar plate with streak plating method by using an inoculation loop (Figure 3.12). Then, the petri dish was completely sealed with parafilm and wrapped with aluminum foil. All of the petri dishes were put in an incubator at 32 °*C* for 3 days. On the third day, the cultivated yeasts were used for fermentation process (Figure 3.13).

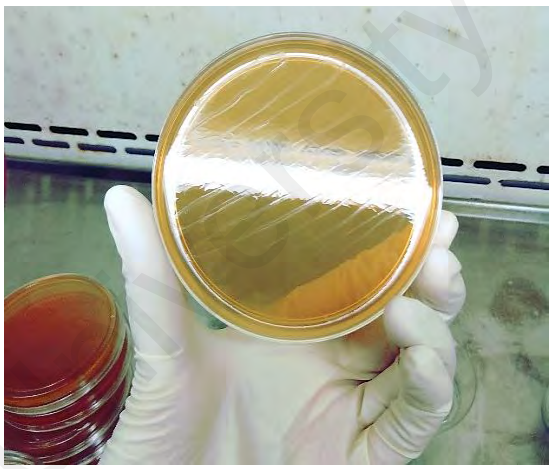


Figure 3.12: Streak plating method.

Figure 3.13: Cultivated yeast ready to use for fermentation process.

3.3.5 Fermentation

a) Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation is a single process that combines the saccharification and fermentation processes. It requires less sterile condition and small

amount of enzymes to operate in a shorter fermentation time and less cost (Simas-Rodrigues et al., 2015).

In this experiment, 1.5% (w/v) of cultivated yeast cells, *Saccharomyces cerevisiae* was added in each reagent bottles containing 1 g of yeast extract, 0.4 g of potassium dihydrogen phosphate (KH_2PO_4) and 0.2 g of ammonium chloride (NH_4Cl) for every 100 mL of hydrolysate. The fermentation process was carried out in a PROTECH SI-50D shaking incubator under temperature condition of 32 °*C* with velocity agitation 150 rpm for 84 hours (Figure 3.14 and Figure 3.15). Samples were taken for every 12 h to observe the changes of reducing sugar during fermentation process. The fermentation parameters were modified based on Dahnum et al. (2015) and Lam and Lee (2015).

Figure 3.14: Bottles tightly sealed with parafilm and wrapped with aluminum foil.

Figure 3.15: Bottles with cap were put inside the incubation shaker under specific condition.

3.3.6 Distillation

After 84 h of fermentation, the ethanol was extracted through distillation process. The IKA RV10 control rotary evaporator was used in this process under a manual condition of temperature, pressure and rotary speed of 65 °*C*, 186 bar and 120 rpm, respectively as shown in Figure 3.16. Vaporized fermentation solution was vacuumed through a pump and dropped down into the round bottle flask after condensed, ethanol solution was collected.

Figure 3.16: Rotary evaporator used during distillation process.

3.4 Testing Method

3.4.1 Reducing sugar content

Reducing sugar concentration (g/L) was analyzed using 3,5-dinitrosalisilat acid (DNS) method (Miller, 1959) by employing a glucose standard curve (Figure 3.19). The sample was estimated by adding 1 mL bioethanol samples (every 12 h till 84 h) with 100x dilution factor to mixed with 1 mL of DNS reagent. The test tubes with diluted samples and DNS solution were then immersed into water bath shaker for 5 min to produce a dark orange red color (Figure 3.17).

An ultraviolet-visible (UV-Vis) spectrophotometer (SPEKOL® 1500, Analytik Jena, Berlin, Germany) was used to analyze the amount of reducing sugar (Figure 3.18). DNS reacts with free carbonyl group of reducing sugar at an alkaline condition with a maximum absorption at 540 nm forming 3-amino-5-nitrosalicyclic acid to show a quantitative spectrometer measurement of reducing sugar (Negrulescu et al., 2012).

Figure 3.17: Test tubes with samples and DNS solution in water bath shaker.

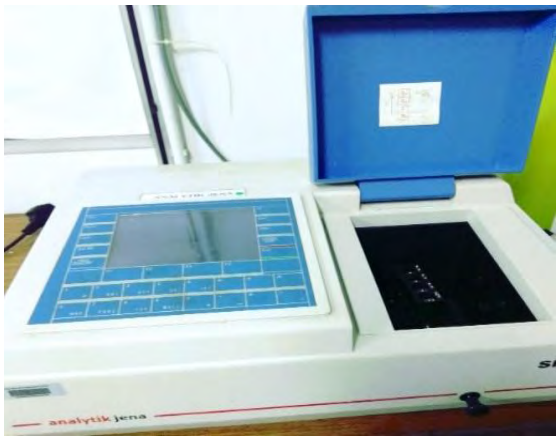


Figure 3.18: Ultraviolet-visible (UV-Vis) spectrophotometer.

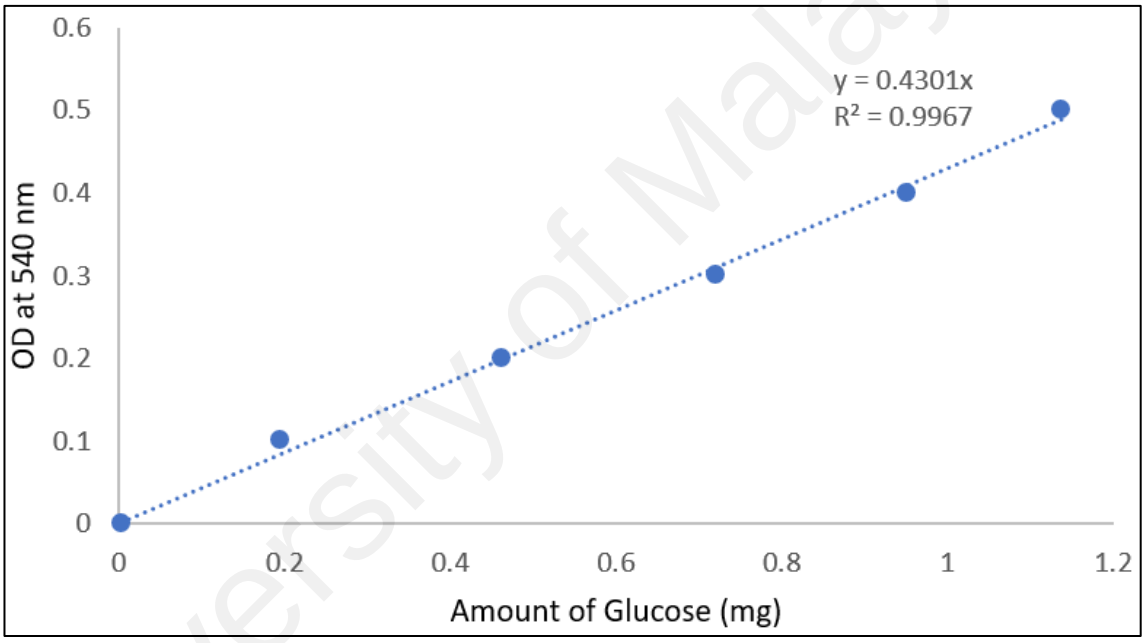


Figure 3.19: An internal glucose standard curve by using DNS method.

3.4.2 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy is an useful quantitative analytical technique to examine the functional group of alcohols bonds after distillation. It is a not just provides a rapid and reproducible measurement it also capable to quantify the presence functional groups, molecules or whole biochemical fractions (Mayers et al., 2013). Absorption happens when the individual chemical bond in a molecule vibrates at specific frequencies and matches the infrared radiation (Movasaghi et al., 2008). Thus,

PerkinElmer Spectrum 400 FTIR/ FT-FIR Spectrometer with a region of 4000- 400 cm^{-1} was used to identify the chemical structure of bioethanol from microalgal *Chlorella* (Figure 3.20).

Figure 3.20: Fourier transform infrared (FTIR) spectroscopy.

3.4.3 Gas chromatography (GC) with flame ionization detection (FID)

A gas chromatography equipped with a flame ionization detector (GC–FID) was used (Figure 3.21). Ethanol concentration at the end of the fermentation was measured with a gas chromatograph (7697A headspace, Agilent) with a flame ionization detector system. The system was programmed with a thermal conductivity detector and 30 m x 320 µm x 1.8 µm, column (Agilent) with hydrogen as carrier gas, the oven temperature, loop temperatures and detector temperature were set as 80 °*C*, 90 °*C* and 100 °*C*, respectively. The concentration of ethanol in the samples were calculated using a calibration curve of different standard ethanol concentration run by GC-FID. The standard to determine the ethanol was modified from European Standard EN14110.

Figure 3.21: Gas chromatography with FID.

3.5 Data and Statistical Analysis

The reducing sugar amount measured by an ultraviolet-visible (UV-Vis) spectrophotometer were evaluated through the glucose standard curve to obtain the reducing sugar concentration (g/ L) by applying the equation from the curve, y= 0.4301x. The data presented are the averages of three replicates (n= 3). The experimental data of the ethanol concentration which determined by GC-FID were analyzed statistically using two-way ANOVA (SPSS Statistical software) with the significance level $p< 0.05$, to identify the relationship of two factors which are concentrations and type of acids to the ethanol contents.

The concentration of ethanol of the samples were measured by gas chromatography equipped with a flame ionization detector (GC–FID). The yield percentage (%) of the bioethanol produced from both of the weak and strong acid were calculated as the following equation:

$$\text{Yield percentage} = \frac{Actual\ yield}{Theoretical\ yield} \quad (3.2)$$

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Reducing sugar testing by DNS method

The reducing sugar concentrations of pretreated microalgal *Chlorella* with acetic (CH_3COOH) and sulfuric acid (H_2SO_4) in different concentrations (1%, 3%, 5%, 7% and 9%, respectively) during fermentation process as shown in Table 4.1. The samples were taken every 12 h from each bottle. The reducing sugar concentration obtained from the control sample without pretreatment was only 20.25 g/L. However, the highest reducing sugar concentration after pretreatments was resulted in sulfuric acid with 9% concentration (S9) which generated 34.02 g/L; following by acetic acid with 9% concentration (A9) which obtained a reducing sugar concentration of 31.91 g/L.

By comparing the results between untreated control sample and pretreated samples with acids hydrolysis shows pretreatment is the important step to disrupt the cell and extract more intracellular carbohydrates from the microalgal (Velazquez-Lucio et al., 2018). The lowest reducing sugar concentration after pretreatments was found in acetic acid with 1% concentration (A1), 24.82 g/ L. The result indicates that the reducing sugar yield are affected by types and concentrations of acid used to during pretreatment (Borines et al., 2013; Park et al., 2016).

Table 4.1: Reducing sugar content of control sample, pretreated microalgal *Chlorella* with acetic (CH_3COOH) sulfuric acid (H_2SO_4) in different concentration during fermentation. The results represent the average value of three replicates (n = 3).

Acids with various conc. (%)	After pre-treatment	Fermentation time (h)							Difference (g/L)
		12	24	36	48	60	72	84	
		Reducing sugar (g/ L)							
A1	24.82	22.62	23.27	20.04	19.14	16.97	9.76	7.57	17.25
A3	26.22	24.42	23.74	22.24	19.01	16.01	9.12	7.18	19.04
A5	30.05	28.62	23.44	22.37	19.91	17.76	9.08	5.63	24.42
A7	29.03	28.58	24.26	22.09	20.80	15.69	11.96	9.86	19.17
A9	31.91	30.15	25.74	24.85	21.78	21.42	15.78	15.66	16.25
S1	27.68	25.89	21.98	19.61	19.46	17.25	9.29	7.87	19.81
S3	30.75	30.05	21.89	20.00	18.96	17.46	8.95	8.27	22.48
S5	32.04	31.81	25.87	22.49	20.67	17.72	8.90	6.95	25.09
S7	31.55	30.15	29.96	24.90	20.21	19.61	13.16	9.73	21.82
S9	34.02	33.63	28.86	27.27	21.87	21.55	19.61	17.37	16.65
Control*	20.25*	17.72*	15.01*	13.24*	11.87*	9.51*	7.87*	5.63*	14.62*

* control sample without pretreatment.

Figure 4.1 and Figure 4.2 shows the variation of reducing sugar concentration in pretreated microalgal *Chlorella* with acetic acid (CH_3COOH) and sulfuric acid (H_2SO_4) in different concentrations during 84 h fermentation process.

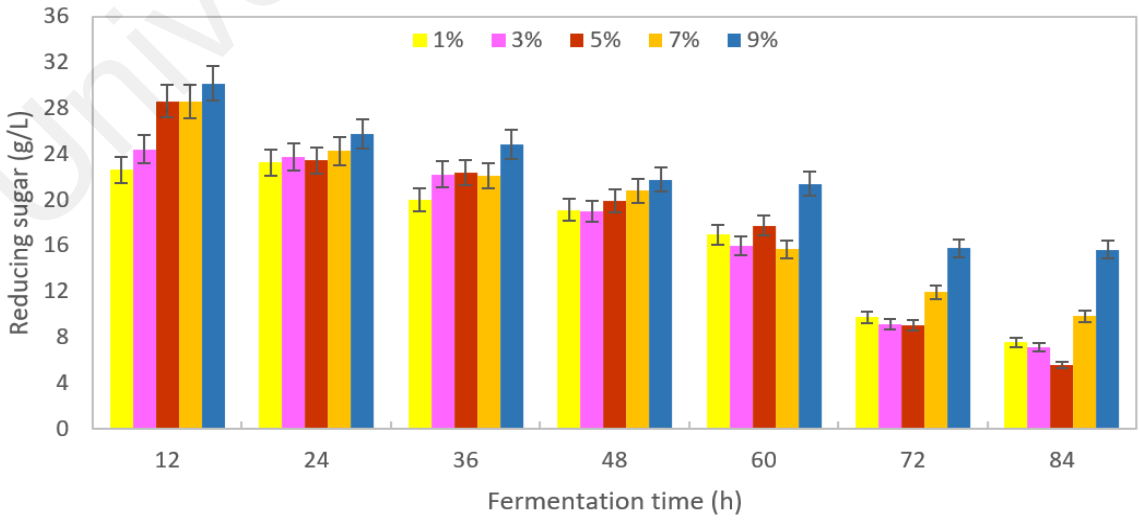


Figure 4.1: Reducing sugar concentration of pretreated microalgal *Chlorella* with acetic acid (CH_3COOH) in different concentrations versus fermentation time. The results represent the average value of three replicates (n = 3).

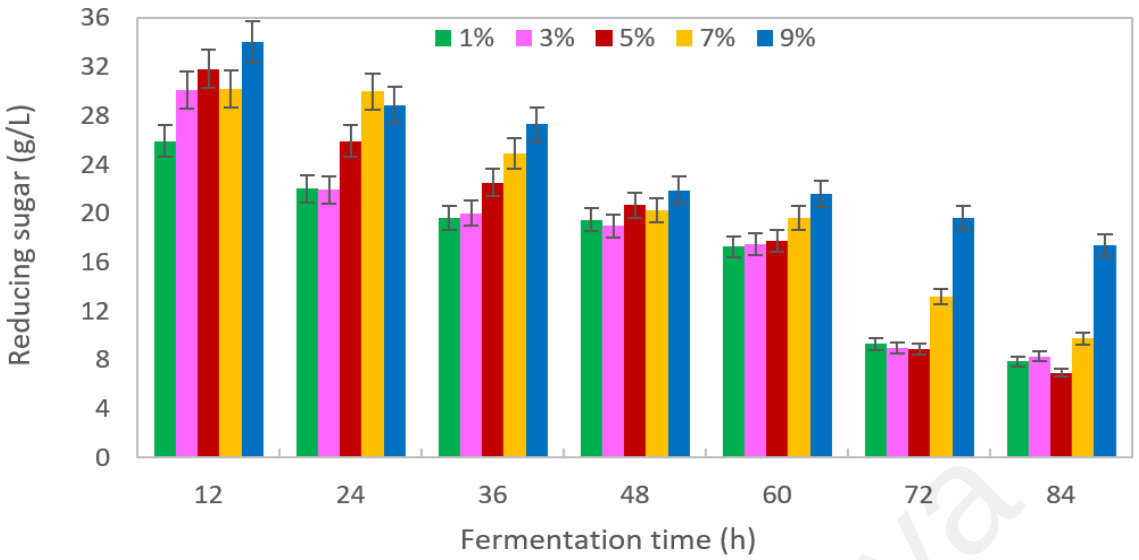


Figure 4.2: Reducing sugar concentration of pretreated microalgal *Chlorella* with sulfuric acid (H_2SO_4) in different concentrations versus fermentation time. The results represent the average value of three replicates (n = 3).

Differences of the reducing sugar concentrations were calculated and compared after 84 h fermentation process (Table 4.1). The reducing sugar content was highly consumed by sulfuric acid with 5% concentration (S5) and acetic acid with 5% concentration (A5) from 32.04 g/ L to 6.95 g/ L and 30.05 g/ L to 5.63 g/ L, respectively. Figure 4.3 and 4.4 show the major differences of reducing sugar concentration of S5 and A5 after 84 h fermentation which were 25.09 g/ L and 24.42 g/ L, respectively. The sample pretreated with 5% of dilute acetic acid achieved almost same amount of the reducing sugar concentration than the 5% dilute sulfuric acid. As a result, the pretreated samples of both dilute sulfuric acid and acetic acid were comparable. However, dilute mineral acid still existing some of the limitations including high corrosivity comparing to the use of organic acid (Mosier et al., 2005). This is a clear indication that acetic pretreatment is more comparable and advantageous over dilute acid pretreatment due to better recovery of fermentable sugars, reduce recovery process cost and could be a promising industrial feasibility for the production of value bioenergy yields (Saha et al., 2016). On the contrary, S9 and A9 both generated the highest reducing sugar concentration after pretreatment but they only obtained the least consumed sugar recorded as 16.65 g/ L and 16.25 g/ L,

respectively. This low sugar yields results may have been due to high concentration of acid during pretreatment which convert the monosaccharides to some inhibitors such as furfural, causing the decreases of the fermentable sugars to be consumed (Khan et al., 2017; Saha et al., 2016). An increase of reducing sugar concentration was observed in both of the acid hydrolysis's samples when concentration increased from 1% to 5%. This result concur with the report conducted by Miranda et al (2012), who reported a decreased of reducing sugar content when microalgae *Scenedesmus obliquus* was treated with acid solutions with a concentrations above 2N (5.4% v/v). In addition, it has been stated by Chng et al. (2017), a low concentration of acid with high temperature is more desirable than a high concentration acid because it will not cause the degradation of the fermentable sugars to other unfavorable compounds thus reduce the yield of hydrolysis. According to Nguyen et al. (2009), a low concentration of acid pretreatment is more considerable than a high concentration acid due to lesser amount of neutralizing agent needed in the following step and less tendency to corrode the experimental equipment.

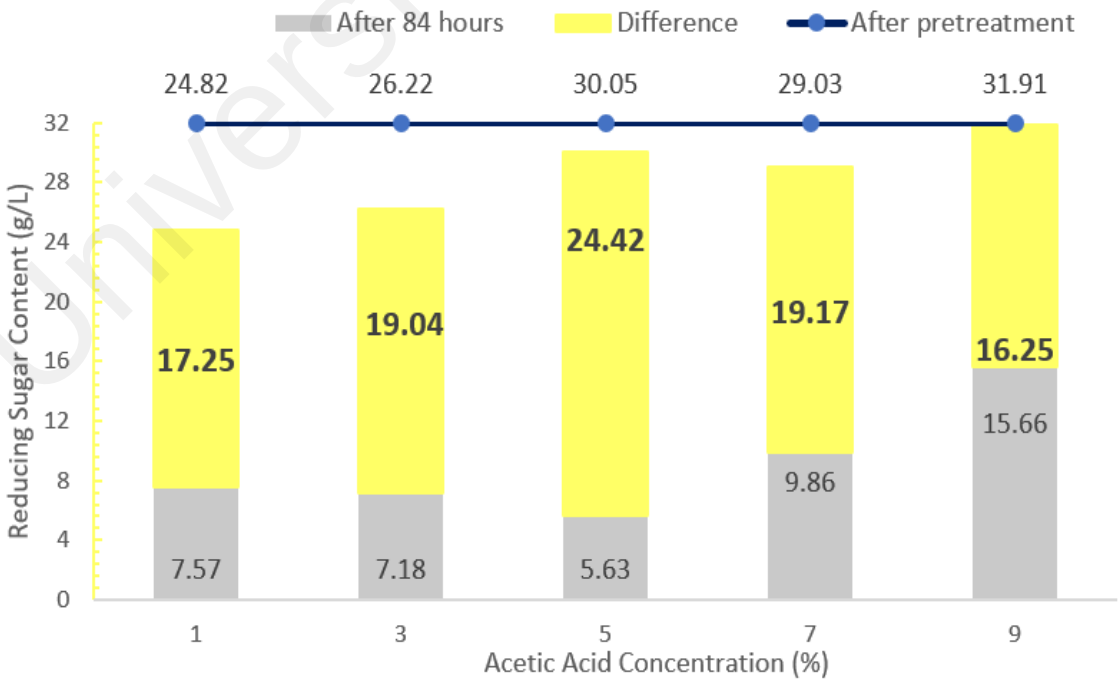


Figure 4.3: Difference between reducing sugar concentration of *Chlorella* pretreated by acetic acid (CH_3COOOH) with various concentrations after 84 hours fermentation process.

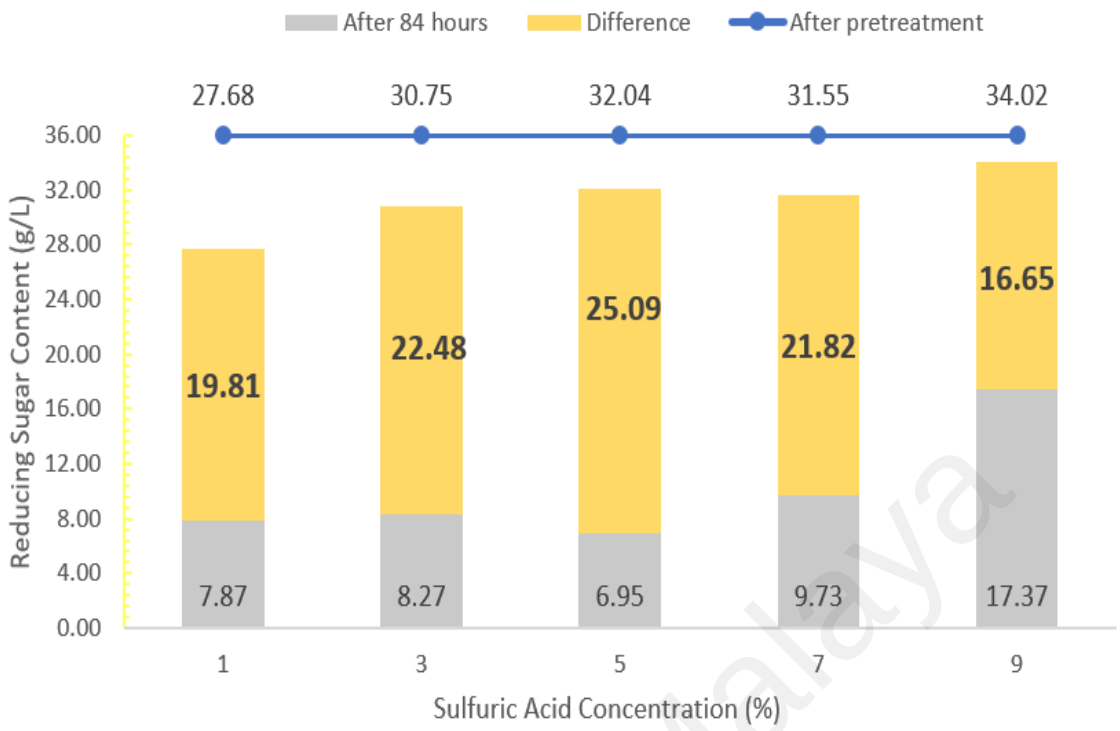


Figure 4.4: Difference between reducing sugar concentration of *Chlorella* pretreated by sulfuric acid (H_2SO_4) with various concentrations after 84 h fermentation process.

4.2 FTIR analysis

Fourier Transform Infrared Spectroscopy (FTIR) spectrum from 10 samples of bioethanol produced from microalgal *Chlorella* species pretreated with acetic and sulfuric acid with different concentrations (1%, 3%, 5%, 7% and 9%) were obtained (Figure 4.5a to 4.5e and Figure 4.6a to 4.6e). A region of 4000- 400 cm^{-1} was used to evaluate the bioethanol from microalgal *Chlorella.* FTIR analysis allows relatively rapid analysis of liquid samples and ability to quantify the functional group present in the bioethanol produced.

i) Acetic Acid

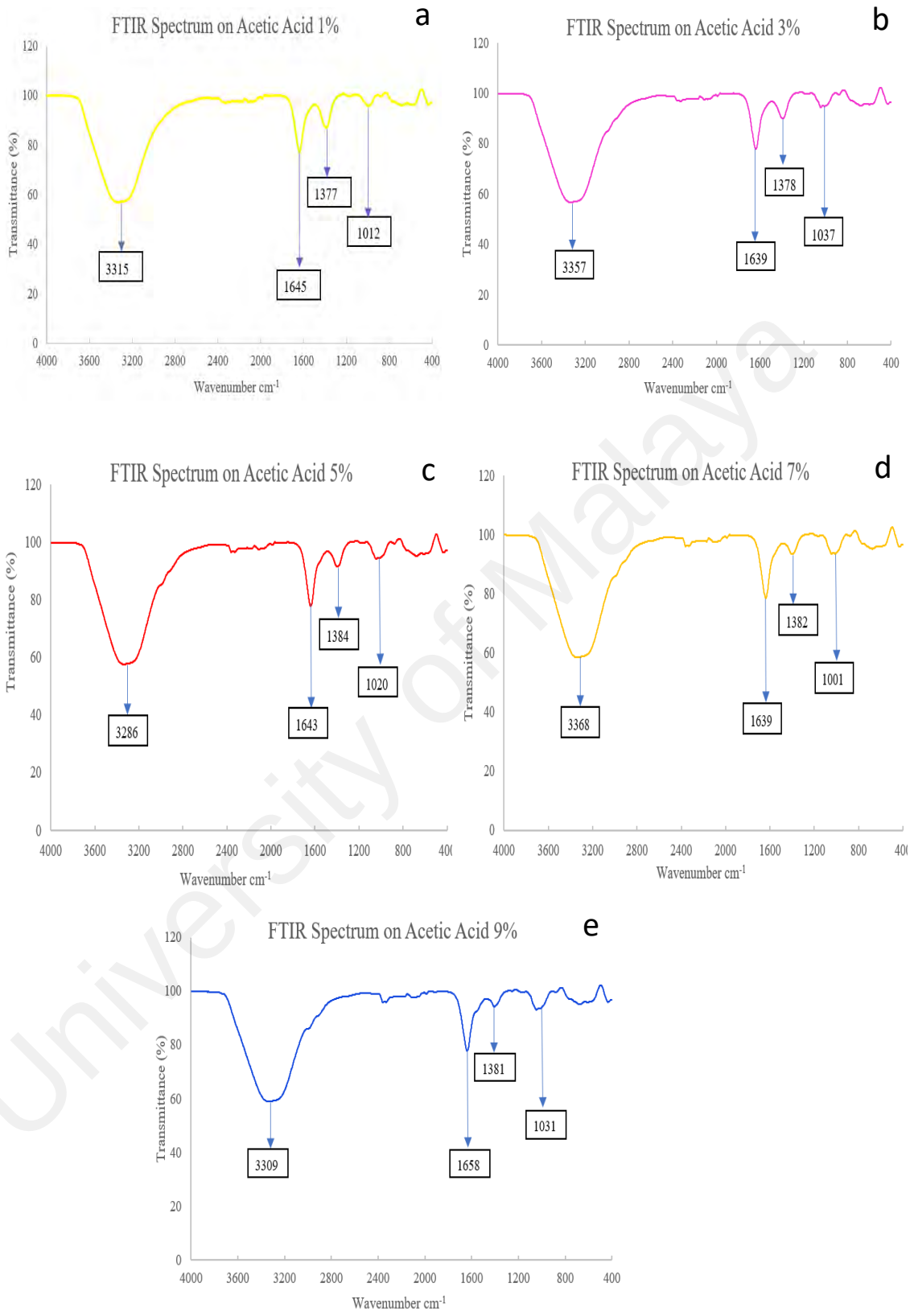


Figure 4.5a to 4.5e: FTIR spectra of bioethanol produced from pretreated *Chlorella* with different concentration (1%, 3%, 5%, 7%, 9%) of acetic acid (CH_3COOH).

ii) Sulfuric Acid

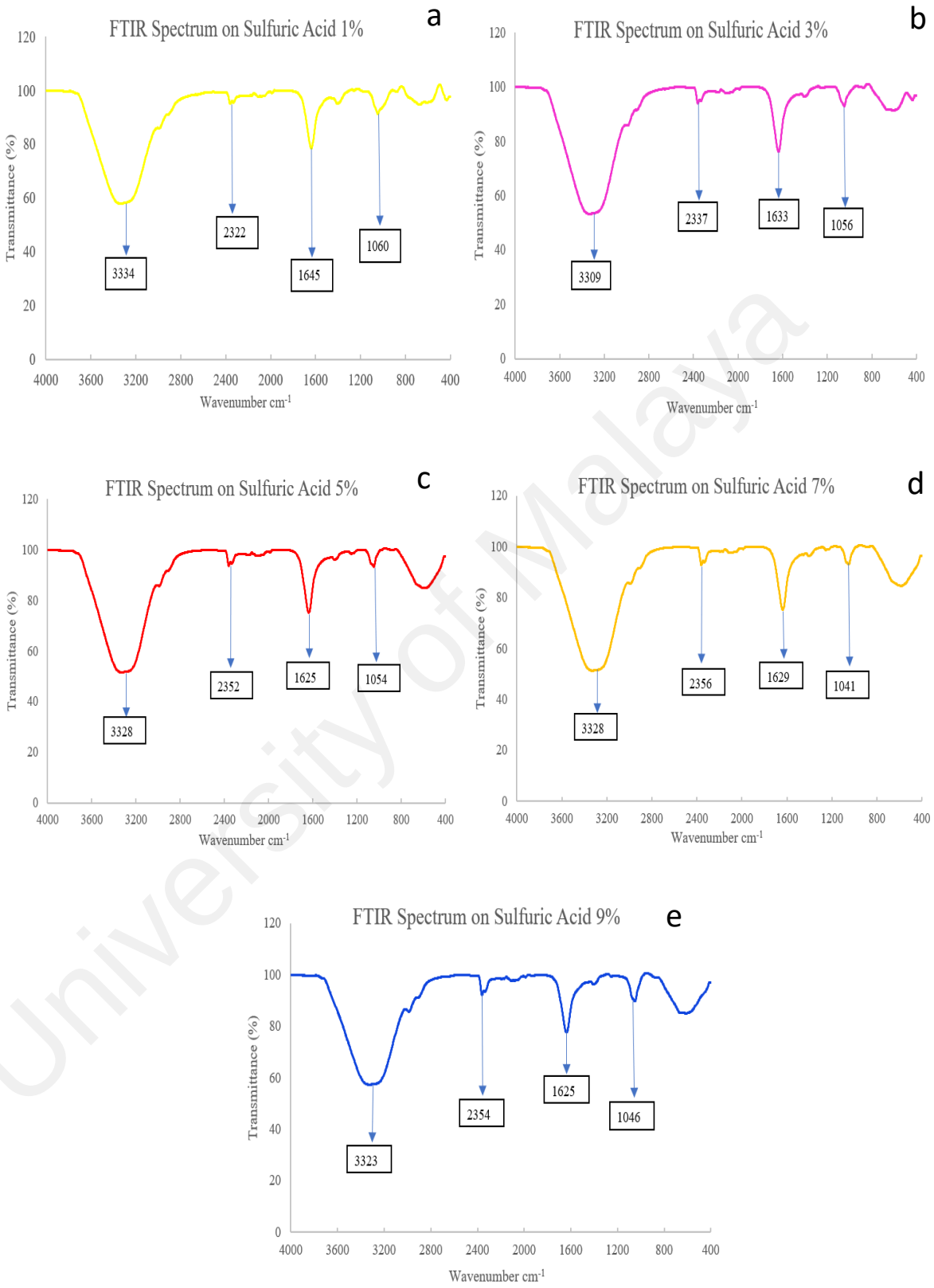


Figure 4.6a to 4.6e: FTIR spectra of bioethanol produced from pretreated *Chlorella* with different concentration (1%, 3%, 5%, 7%, 9%) of sulfuric acid (H_2SO_4).

Figure 4.7 and Figure 4.8 show the comparison of FTIR spectrum analysis from 10 bioethanol samples produced from microalgal *Chlorella* species pretreated with acetic and sulfuric acid in different concentration (1%, 3%, 5%, 7% and 9%).

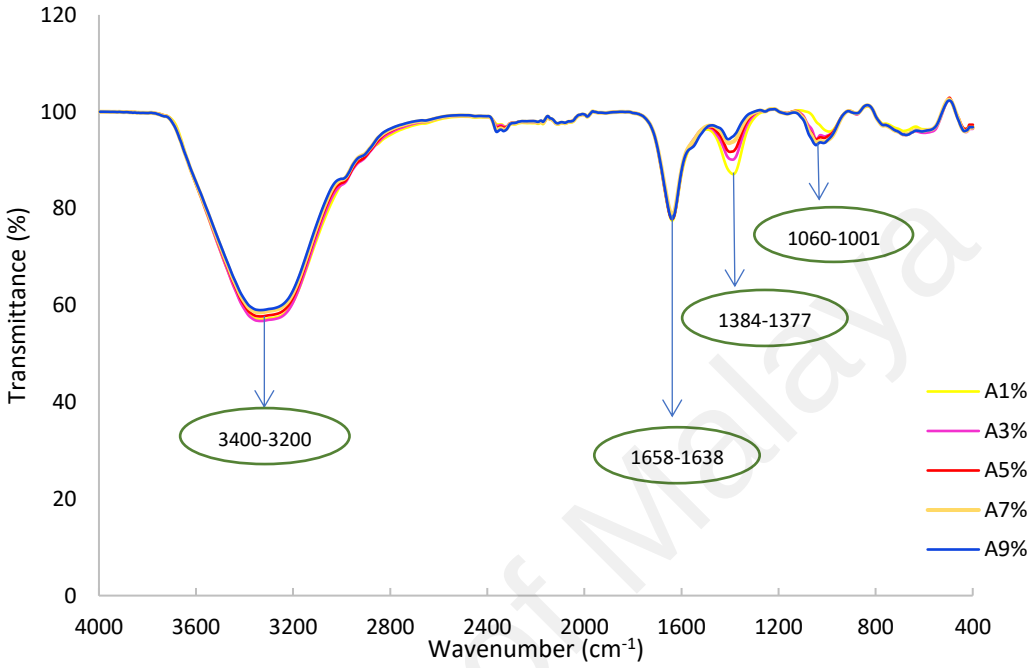


Figure 4.7: FTIR spectroscopy analysis illustrates the comparison of bioethanol produced from *Chlorella* with various concentrations of acetic acid.

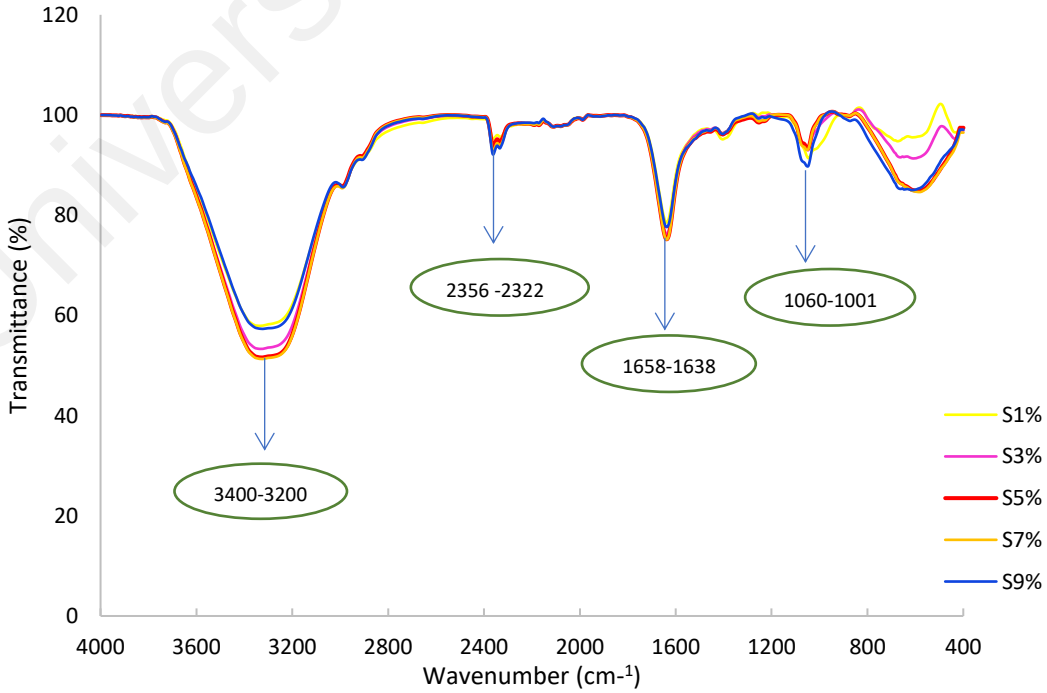


Figure 4.8: FTIR spectroscopy analysis illustrates the comparison of bioethanol produced from *Chlorella* with various concentrations of sulfuric acid.

In Table 4.2, the FTIR results showed some common peaks in both bioethanol produced from microalgal *Chlorella* pretreated with acetic and sulfuric acid within ranges of 3400 - 3200 cm^{-1}, 2356 - 2322 cm^{-1}, 1658 - 1638 cm^{-1}, 1384 – 1377 cm^{-1}, and 1060-1001 cm^{-1}. The peak in 3400 – 3200 cm^{-1} represents the hydroxyl (OH) group in the samples (Kassim & Bhattacharya, 2016). The existence of absorption wave 1658-1638 cm^{-1} represents an existence of alkene group with variable C= C bonds between atoms with medium intensity (Sebayang et al., 2017). According to Veale et al. (2007), peak between 1060- 1001 cm^{-1} can be indicated as reflection of ethanol and glucose in the wave regions between 1200- 800 cm^{-1} due to the absorptions bands of C-O and C-C stretch vibration. Moreover, major components of microalgal biomass such as carbohydrate showed the peak near 1100- 900 cm^{-1} (Pancha et al., 2016).

Table 4.2: Absorption bands obtained from FTIR Spectra of bioethanol produced from *Chlorella* pretreated with acetic and sulfuric acids.

Summary of IR Absorption Table for Acetic Acid							
Main peaks (cm^{-1})					**Functional Group**	**Bond**	**Range (cm^{-1})**
1%	**3%**	**5%**	**7%**	**9%**			
3315	3357	3286	3368	3309	alcohols, phenols	O–H stretch, H–bonded; broad	3400–3200
1645	1639	1643	1639	1658	Alkenes	–C=C– stretch, medium	1648-1638; 1658- 1648
1377	1378	1384	1382	1381	Alkane	-C-H-bending, medium	1450- 1375
1012	1037	1020	1001	1031	alcohols, carboxylic acids, ethers	C-C, C-O, stretch	1200- 800

Table 4.2, continued.

Summary of IR Absorption Table for Sulfuric Acid							
Main peaks (cm^{-1})					Functional Group	Bond	Range (cm^{-1})
1%	3%	5%	7%	9%			
3334	3309	3328	3328	3323	alcohols, phenols	O–H stretch, H–bonded; broad	3500–3200
2322	2337	2352	2356	2354	carbon dioxide	O=C=O stretch, strong,	2349
1645	1622	1625	1629	1625	conjugated alkene	-C=C-, stretch, medium	1650-1600
1060	1056	1054	1041	1046	alcohols, carboxylic acids, ethers	C-C, C-O, stretch	1200- 800

4.3 GC-FID ethanol concentration analysis

Agilent 7697A Gas Chromatograph (Agilent) equipped with a headspace sampler and a flame ionization detector (FID) was used to test the concentration of ethanol presence in the samples after 84 h fermentation. Figure 4.9 represents the gas chromatogram from one of the samples (S3) with the peak area (pA) and retention time.

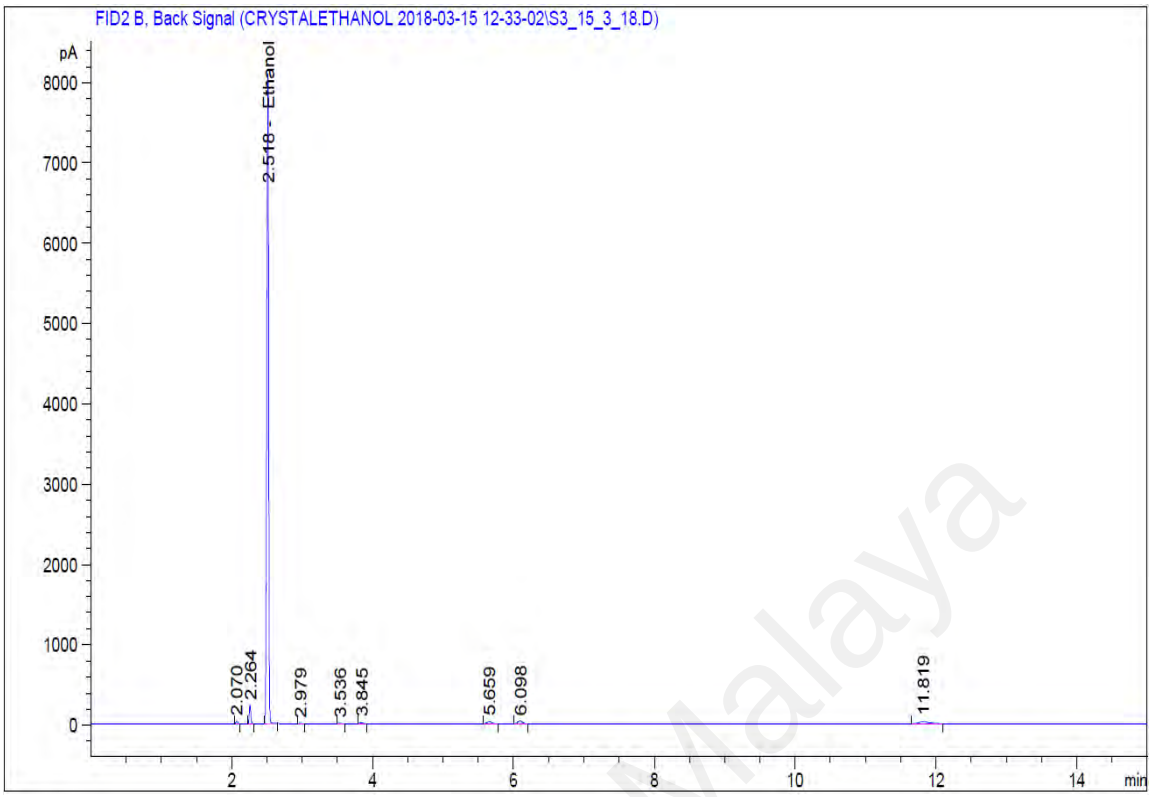


Figure 4.9: GC-FID chromatogram for ethanol (EtOH) to analyze bioethanol produced from *Chlorella* according to modified European Standard EN14110.

In Table 4.3, the highest concentration of ethanol was achieved by S5 (2.714%) followed by A5 (2.219%). To determine the effect of pretreatment bioconversion, an untreated control sample was subjected to fermentation process. An ethanol concentration of 0.675 % was found in the untreated control sample. This result was much lower than those pretreated samples with acid hydrolysis. However, it shows the important to carry out pretreatment before fermentation in order to increase the ethanol productivity (Babujanarthanam & Kavitha, 2014). Moreover, the lowest ethanol concentration in sulfuric acid was 0.767% found in S9 sample while in acetic acid obtained an ethanol concentration of 0.802% in A9 sample. Greetham et al. (2016) mentioned that the presence of the low concentration of weak acid can improve the yeast tolerance to the inhibitors such as hydroxymethylfurfural (HMF) and furfural, improved the glucose utilization to produce a higher bioethanol yield. Thus, the ethanol concentration of A9 sample was slightly higher than the ethanol concentration of S9 sample. In contrary, A1

and S1 had achieved a higher ethanol concentration of 1.107% and 1.372% than A9 and S9. It is possible because of low acid concentration can help to enhance the downstream fermentation by releasing essential nutrients (Bensah & Mensah, 2013). This result is corresponds to the experiment of the acid pretreatment of softwood conducted by Larsson et al. (1999), the decreased of the ethanol yield with an increasing concentration during pretreatment.

Table 4.3: Ethanol concentration analysis by GC-FID.

Type of Acids / **Concentration(%)**	**Ethanol concentration (%)**		
	CH_3COOH	**H_2SO_4**	**Control**
0	N/A	N/A	0.675
1	1.107	1.372	N/A
3	1.314	1.669	
5	2.219	2.714	
7	1.335	1.535	
9	0.767	0.802	

Figure 4.10 shows the comparison of ethanol concentration (%) of pretreated samples with acetic and sulfuric acids in different concentrations and a control sample without pretreatment after 84 h fermentation. The highest ethanol concentration was found in samples pretreated with 5% acid concentration. A low ethanol concentration was detected in the untreated control compared to those pretreated samples.

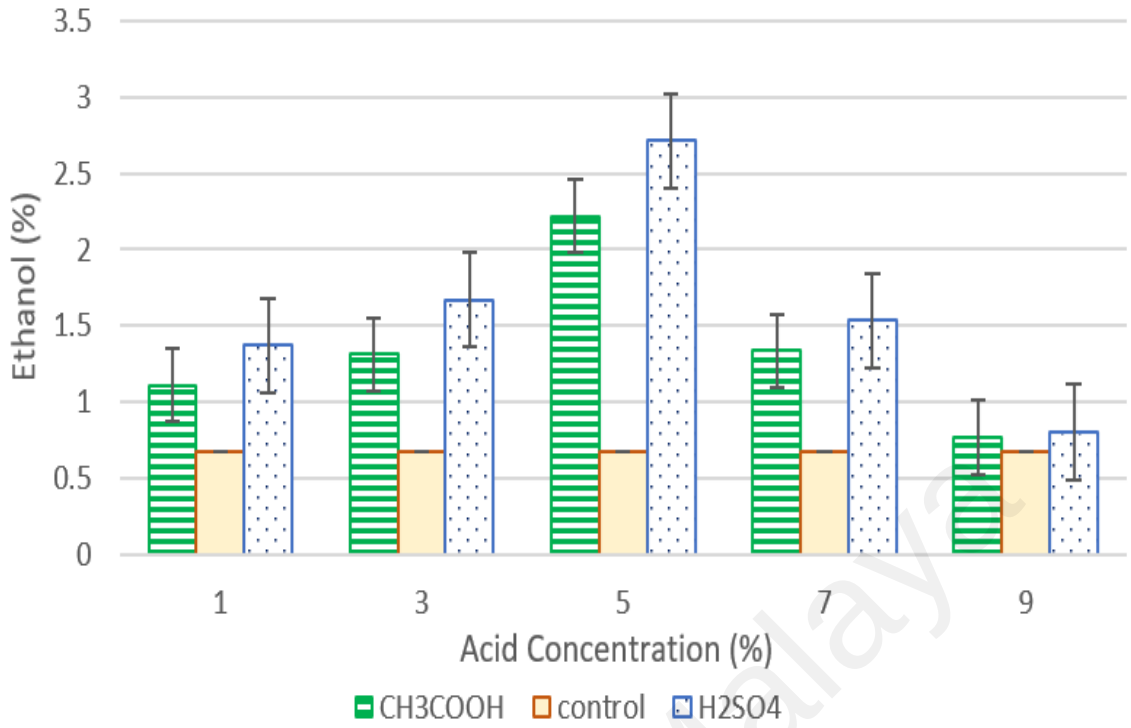


Figure 4.10: Ethanol concentration (%) of pretreated samples with acetic and sulfuric acids in different concentration after distillation.

4.4 ANOVA two-way analysis of ethanol concentration

The result in Table 4.4 indicates there is a statistically significant ($p < 0.05$) effect of the two factors which are concentrations (p value= 0.025) and type of acids (p value= 0.001) to the ethanol contents of the 10 bioethanol samples produced from microalgal *Chlorella*. Higher concentration of the acid pretreatment leading to the degradation of the fermentable sugars and conversion to other products (Miranda et al., 2012).

Table 4.4: Two-way ANOVA analysis of two main factors (concentration of acids and types of acids) related to the ethanol concentration tested via GC-FID.

Source	Dependent Variable	SS	Df	MS	F -Ratio	P value
Concentration of acid	Ethanol	0.182	1	0.182	7.709	0.025*
Type of acids	Ethanol	3.033	4	0.758	6.388	0.001*

note: *significant value P< 0.05

4.5 Determination of ethanol yield

Table 4.5 shows the calculation of ethanol yield (g ethanol/ g dry microalgae). Among the samples, the highest ethanol yield 0.281 g/g was found at the S5 followed by 0. 230 g/g obtained by A5. The ethanol yield of the untreated control sample was only 0.068 g/g. In fact, Babujanarthanam and Kavitha (2014) have been reported the corresponding highest ethanol yield produced from red algal *Gelidiella acerosa* after dilute acid and enzymatic pretreatments was 0.214 g ethanol/ g red algae while the untreated red algae was only obtained 0.046 g/g of ethanol yield.

Thus, the result of ethanol yield for the pretreated and untreated microalgae in this experiment showed slightly higher compared to the ethanol produced by red algal *Gelidiella acerosa*. Likewise, there are some typical bioethanol production yields through fermentation from microalgal feedstock using *S. cerevisiae* have been reported do not exceed 0.3g ethanol/ g of dry weight algae (Choi et al., 2010; Guo et al., 2013; Harun et al., 2011; Nguyen et al., 2009).

In contrast to S5 and A5 ethanol yield, the reason for poor ethanol yield obtained in acetic acid with 9% concentration (0.084 g ethanol/ g biomass) and sulfuric acid with 9% concentration (0.080 g ethanol/ g biomass) may because of the existing of high concentration of fermentation inhibitors such as formic acid, levulinic acid and 5-hydroxymethylfurfural (HMF) during hydrolysis pretreatment in the hydrolysate. This reaction eventually leading to a negative effects on the growth of fermentative organisms and indirectly produce a lower production of desired end products (Pancha et al., 2016; Park et al., 2016).

Table 4.5: Ethanol yield (g/g) of acetic and sulfuric acid in various concentrations after distillation. Control without any pretreatment was used to monitor and compare with the pretreated samples.

Type of Acids / Concentration(%)	Ethanol yield (g/g)		
	CH_3COOH	H_2SO_4	Control
0	N/A	N/A	0.068
1	0.110	0.137	N/A
3	0.133	0.169	
5	0.230	0.281	
7	0.134	0.154	
9	0.080	0.084	

4.6 Theoretical yield of ethanol using stoichiometric calculation

Stoichiometric calculation is the relative amount of the reactants and the products represented in a balanced chemical reaction. Stoichiometric calculation can help to estimate the theoretical yield of bioethanol produced from microalgal. From the balanced chemical equation, 1 more of glucose is able to produce 2 moles of ethanol, which means that 1:2 mole ratio between glucose and ethanol (Gombert & van Maris, 2015). Thus, the maximum theoretical yield of ethanol from glucose is 0.511g/g of glucose (Borines et al., 2013; Markou et al., 2013).

$$C_6H_{12}O_6(aq) \rightarrow 2C_2H_5OH\ (aq) + 2CO_2(g) \quad (4.1)$$

Table 4.6 shows the stoichiometric calculation of the samples. All of the theoretical yield of ethanol of the samples were higher than their actual yield of ethanol. The stoichiometric calculation shows the highest volume per volume ethanol concentration (v/v, %) in S5 with 2.475% and A5 with 2.371%, respectively. The results of stoichiometric calculation are corresponded to the actual yield of ethanol which also showed the highest ethanol yield achieved by pretreated samples of S5 and A5,

respectively. In general, actual yield is lower than theoretical yield because the microorganisms may use some of the glucose for growth during fermentation process (Balat & Balat, 2009). Moreover, the production of bioethanol using fermentative microorganism depend on temperature, pH level, osmotic tolerance, and resistance for inhibitors, growth rate and genetic stability during fermentation process (Balat & Balat, 2009). Thus, the theoretical yield of ethanol is generally less than the actual yield of ethanol.

Table 4.6: Stoichiometric yield of ethanol (*%*) of acetic and sulfuric acid in various concentrations. (Control sample without any pretreatment was used to monitor and compare the pretreated samples.)

Type of Acids / **Concentration(%)**	**Stoichiometric Calculation of Ethanol Yield (%)***		
	CH_3COOH	**H_2SO_4**	**Control**
0	N/A	N/A	1.420
1	1.675	1.924	N/A
3	1.849	2.183	
5	2.371	2.475	
7	1.862	2.119	
9	1.578	1.617	

*Calculations of theoretical values are based on the assumption that all of the reducing sugar are fermentable.

Table 4.7 shows the yield percentage of the bioethanol from both of the acetic (weak acid) and sulfuric (strong acid). Based on the results, the highest yield percentage was generated by pretreated sample S5, 11.35% followed by A5 which resulted in 9.7%. The low yield percentage obtained may have been due to the various mixture of sugars (reducing sugar concentration) such as hexoses and pentoses released in the hydrolysate cannot be fully utilized by *S. cerevisiae* to convert them into bioethanol (Agwa et al., 2017). This is because *S. cerevisiae* is well known in capable to convert many fermentable sugars into bioethanol but it is unable to ferment pentoses and xylose (Kumar. et al., 2009;

Phwan et al., 2018) . Hence, it may be is one of the reasons to explain calculation of low yield percentage in all the samples.

Table 4.7: Yield percentage (%) of the bioethanol.

Type of Acids / **Concentration (%)**	**Yield Percentage (%)**		
	CH_3COOH	**H_2SO_4**	**Control**
0	N/A	N/A	4.79
1	6.57	7.12	N/A
3	7.19	7.74	
5	9.70	11.35	
7	7.20	7.27	
9	5.07	5.20	

CHAPTER 5: CONCLUSION

5.1 Conclusion

This experiment shows that *Chlorella* encompass a potential and sustainable renewable biofuel for bioethanol production. However, types of acids and the concentrations of acid using for pretreatment can be significantly affected the reducing sugar concentration and the ability to convert the fermentable sugars into bioethanol during the fermentation process. By yeast fermentation, 0.281g of ethanol/g of microalgal biomass could be obtained from 5% concentration of sulfuric acid hydrolysis and enzymatic pretreatment while with an 5% of acetic acid and enzymatic pretreatment can produced a 0.23g of ethanol/ g of microalgal. This study provides an essential information for possibility to replace the strong acid to weak acid in moderate concentration during pretreatment can give a similar effect to produce bioethanol in a more sustainable and less negative environmental impact approach. Besides types of acid is considered to be one of the most important parameters that influence the bioethanol production from microalgae, the concentrations of acid and the fermentative microorganism also play an important part in this experiment. The findings from this study are essential for cost effectiveness and low energy consumption of bioethanol production from microalgae.

5.2 Recommendations

It is recommended that further research in the future may focus on the study of organic acids which is less negative environment impacts in pretreatment technology and optimize them to increase bioethanol productivity from microalgae. In order to obtain an economically feasible conversion process, development of membrane techniques that help to remove the inhibitors inside the hydrolytes after pretreatment and improve the fermentability of the bioethanol production are necessary. Furthermore, genetic

engineering which is one of the powerful biotechnological tools is required to develop more genetically modified the strains of carbohydrates-rich microalgae and fermentative microorganism to increase the bioethanol yield under the stress condition.

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