FERMENTATION OF MICROALGAE BIOMASS THROUGH MILD ACID PRE-TREATMENT

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DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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APPROVAL FOR SUBMISSION

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ABSTRACT

Conversion of microalgae biomass to bioethanol is actively researched to establish a cost effective and sustainable production technology. The main challenge is break down the carbohydrates content in the biomass to obtain fermentable sugar for subsequent fermentation process. Study was focus on the effectiveness of phosphoric acid pre-treatment and capability of *Saccharomyces diastaticus* as the fermentation microbe. *Scenedesmus dimorphus* microalgae was used as the biomass due to its high carbohydrate content. The production process can be break down into two main processes, which is the acid pre-treatment of microalgae biomass follow by the fermentation of the pre-treated biomass. *S.diastaticus* was investigated in this study to explore it's capability in consuming complex sugar during fermentation process. One of the major challenges faced is to determine the effective condition for acid pre-treatment. The pre-treatement process have to produce high amount of fermentable sugar while at the same time avoiding the formation of inhibitors. According to the result, the optimum condition yield about 94% of bioethanol conversion is at 2.5% v/v phosphoric acid treated at 120 ^{o}C for 30 min. On the dry weight basis, the bioethanol yield is about 0.128 g bioethanol/ g biomass in 24 hr of fermentation time. This study proved that coordination of phosphoric acid pre-treatment process with *S.diastaticus* yeast provides a practicable method for the production of bioethanol from microalgae.

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CHAPTER 1

INTRODUCTION

1.1 Background of study

The use of bioethanol as an alternative energy source is becoming more crucial in the late years. Unlike conventional fuels that are produced from non-renewable source such as crude oil and natural gas, bioethanol is produced through the fermentation of biomass. Therefore, bioethanol is categorise as a renewable and environmental friendly energy resources. One of the advantageous properties of bioethanol is that it is a biodegradable fuel and exhibits clean combustion properties due to low impurities. On the contrary, conventional fuels contributes significantly to the environmental pollutions such as acid rain and green house effect (EPA, 2016). Besides, the use of bioethanol as fuel also reduces the carbon emission to the environment due to complete combustion. Bioethanol are mainly used to blend with gasoline to produce an environmental friendly fuel. By using bioethanol as an alternative, the reliance on conventional fuels can be reduced as well.

Ethanol can be produced through chemical synthesis. However, due to high cost and extreme condition required, the production of ethanol through chemical synthesis is not economical. The main process to produce bioethanol is through the fermentation of biomass. Currently, the industrialised process for bioethanol production use crops, mostly corn or sugarcane as raw material (Asada, 2012). Despite that, these raw materials are also being used in the food industry. Thus, it increases the demand of these raw materials which resulted in high cost. Besides agricultural crops, lignocellulosic materials such as wood and bagasse can also be used to produce bioethanol. These materials can be easily obtained at a relatively low cost as it is abundantly available on earth. However, the shortcoming of using

lignocellulosic materials as raw material is the difficulty to separate unwanted side products from the desired product, sugar. Besides, pre-treatment of lignocellulosic materials involved complex saccharification and hydrolysis process (Millati, 2005). Thus, the production of bioethanol from lignocellulosic materials have yet to be industrialised.

In the US year 2010, about 53% of the total renewable energy was produced using biomass as feedstock (EIA, 2011). This indicates that biomass is one of the renowned resources that were used to produce renewable energy. Generally, crops or agricultural waste are used as biomass to produce renewable energy. However, with the advanced technology and continuous research, microalgae are now one of the most promising biomass to produce renewable energy. Research shows that microalgae is a potential feed for the production of bioethanol due to high carbohydrate content. Microalgae are simple plants that are relatively small in size that grows using water resource as their habitat. Microalgae are unicellular organism that absorb water, nutrients, minerals and sunlight for growth. Microalgae convert sunlight, carbon dioxide, nutrients, and phosphorus via photosynthesis into a product that can be used as biomass (Y.Ghasemi, 2012). The converted energy is stored within the cell of microalgae. The stored energy consists of large amount of carbohydrates which can be extracted for bioethanol production. Besides, cultivation of microalgae can be easily done due to the rapid growth rate and stable growth at all seasons. Moreover, microalgae do not require much resource to grow compared to agricultural crops. The land required for cultivation of microalgae is also relatively smaller compared to crops (Slegers, 2015). Thus, it has the advantageous properties to replace other biomass for bioethanol production.

Research shows that microalgae species such as *Chlorella*, *Dunaliella*, *Chlamydomonas* and *Scenedesmus* contain higher amount of starch and glycogen which are suitable for biofuel production (John, 2011). The carbohydrate content within *S.dimorphus* are mainly starch and cellulose. These carbohydrates are essential for the production of bioethanol. However, it is insoluble in nature due to the presence of cell wall, thus the degradation of the cell wall is needed prior to fermentation. One of the method to degrade the cell wall is through pre-treatment of biomass into fermentable sugar. There are several types of pre-treatment methods. One of it is using acid hydrolysis to break cell wall and convert starch to glucose.

Sulphuric acid is the most common acid used for hydrolysis of cell wall as sulphuric acid can produce a high yield of glucose at a faster rate. Besides acid hydrolysis, enzymes such as α-amylase or amyloglucosidase can be used for the pre-treatment of microalgae (Kolusheva, 2007). Enzymatic activity can take place at a lower temperature compare to acid hydrolysis. Besides, enzymatic hydrolysis can produce a higher yield of glucose as well. After the pre-treatment of biomass, it is then fed to yeast for fermentation process.

Yeast is used for the fermentation of glucose to bioethanol. Due to the absence of lignin compound in microalgae, the process involved for the conversion of starch to ethanol is much simpler and straight forward (Ho, 2012). The starch content in microalgae is high due to high photosynthesis rate. Yeast such as *S.cerevisiae* or *S.diastaticus* is used to convert the carbohydrate to ethanol. However, the starch content is bounded by cell wall. Thus, the microalgae need to be pre-treated before feeding it to the yeast. *S.diastaticus* is a wild type yeast that are able to excrete amyloglucosidase enzyme to convert soluble starch to bioethanol. The enzyme excreted is capable of breaking down starch to simple sugar and *S.diastaticus* ferment it to form bioethanol.

1.2 Problem statement

Microalgae is a potential feedstock for the production of bioethanol. The starch content in microalgae is covered by a layer of cell wall. In order to extract the starch content, degrading or hydrolysis of this cell wall has to be carried out. The effective hydrolysis of cell wall will simplify the extraction and degradation process. Direct fermentation of microalgae without pre-treatment is not applicable as the cell wall inhibits the fermentation process (Chikako, 2012). The common methods that were used to hydrolyse the cell wall are by using acid or alkaline chemical. Acid is more commonly used as it provides a higher rate of reaction, simple procedures and relatively cheaper compared to other methods. Sulphuric acid is the common acid used for the hydrolysis of microalgae. The use of sulphuric acid at high temperature, 100-125 ^{o}C will result in higher yield of glucose and the reaction time is faster, approximately 20 min (Nguyen, 2009). However, during acid hydrolysis, the

formation of side products is difficult to be removed from the mixture and this side products are capable of poisoning the yeast.

On the other hand, phosphoric acid is a type of weak acid which reduces the risk of degradation of carbohydrates to form inhibitors. The use of mild phosphoric acid to pre-treat the microalgae biomass allow the degradation of cell wall and partial hydrolysis to form a mixture of complex and simple sugar. The use of mild acid avoids the degradation of carbohydrates and reduces the acidity of the environment. Another benefit of using phosphoric acid is that during neutralisation with sodium hydroxide, it releases sodium phosphate which can act as a nutrient for the yeast to grow. Besides, phosphoric acid is also a less toxic chemical compound compared to sulphuric acid.

Enzyme such as α-amylase and amyloglucosidase can also be used for the hydrolysis of cell wall of the microalgae. Enzymatic hydrolysis can provide a higher yield whereas the production of side-product is avoided. In addition, enzyme are more environmental friendly compared to the used of acid. However, the drawback for the use of enzyme is due to high initial cost of enzyme. It is also difficult to extract the enzyme from the final product as well. In addition, the treatment time needed for the pre-treatment of microalgae is also longer compared to acid hydrolysis (Harun, 2010).

For the fermentation process, *S.cerevisiae* is one of the conventional yeast used due to high fermentation rate. However, the drawback of *S.cerevisiae* is that it does not consume complex sugar to produce bioethanol. Complex sugar such as starch and xylose are not consumable by *S.cerevisiae,* thus the saccharification or decomposition of these sugar is required. On the other hand, *S.diastaticus* is capable of fermenting both complex and simple sugar to bioethanol (Amutha, 2001). Thus, by coordinating acid pre-treatment with the use of *S.diastaticus*, high temperature acid pre-treatment can be avoided where the side products that inhibit the fermentation process will not be produced. Low concentration acid can be used to hydrolysed the microalgae biomass. The cell wall covering the starch will be hydrolysed and some of the starch is converted to simple sugar. The mixture of starch and simple sugar are fed to *S.diastaticus*. Unlike *S.cerevisiae*, *S.diastaticus* can convert these sugar to bioethanol.

The extraction of starch from microalgae and the conversion of starch to fermentable sugar is a technology challenge that can be solved with alternative pathway (Adenle, 2013). As mentioned previously, the cell wall protects the microalgae from being fermented. Thus, the degradation or hydrolysis of cell wall has to be carried out before conversion of starch to glucose can occur.

Research is undergoing in order to obtain the highest yield of bioethanol from microalgae through an economical method. In this study, mild acid will be used to hydrolyse the cell wall and partially convert starch to soluble sugar. After conversion of starch, the hydrolysate will be fed to yeast, *S.diastaticus* for fermentation to produce bioethanol.

1.3 Aims and objective

The focus of the study is based on the following objectives :

1) To characterize the composition of microalgae *S.dimorphus* biomass.
2) To investigate the effects of phosphoric acid pre-treatment on microalgae biomass under various mild condition.
3) To explore the fermentation performance of *S.diastaticus* with hydrolysate from microalgae biomass.

1.4 Scope of study

The scope of study for this research are as followed :

1) Analyse the composition and suitability of *S.dimorphus* for bioethanol production. The analysis focus on the type of carbohydrate content presence.
2) Study the effect of acid pre-treatment condition on microalgae using phosphoric acid. The parameters involve are the temperature of acid pre-treatment, phosphoric acid concentration, and the treatment time.

3) Evaluate the effect of hydrolysate in fermentation process using starch fermenting yeast, *S.diastaticus*. The fermentation process is mainly batch reaction and the yield of bioethanol and remaining sugar content is determined using analytical equipments.

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae - 3rd generation of biofuel production (bioethanol)

Microalgae are considered as the 3rd generation of biomass for the production of biofuel due to its chemical composition. Microalgae has high content in carbohydrates, proteins or lipids. The high content of carbohydrate are due to high photosynthesis rate where large amount of starch are stored in the cell. Besides, the growth rate of microalgae are high. Large amount of chloroplast are presence in the cell which provide a large surface area to absorb sunlight, thus increasing the photosynthesis rate. Photosynthesis converts simple inorganic materials to complex organic materials (Pacheco, 2015). The organic compound produced can be used as biomass. This biomass is suitable for biofuel production as it is rich in carbohydrates or lipids. Microalgae with high carbohydrates content can be used as a feedstock to produce bioethanol whereby microalgae with high lipids content can used to produce biodiesel. Besides, microalgae is capable of generating biomass all year long without requiring large area of land as compared to crops (Gendy, 2013). Thus, microalgae is categorise as the third generation biomass due to its advantageous properties.

2.1.1 Biochemical profile of microalgae

Microalgae are unicellular microorganisms with a wide variety of shape and forms. Besides, microalgae are commonly grown in aquatic medium (Richmond, 2013). In aquatic medium, the presence of water, carbon dioxide and nutrients are abundantly available. Thus, allowing the rapid growth of microalgae. Due to simple cellular

structure and high photosynthesis rate, the growth of microalgae is much faster compared to the growth of crops or terrestrial plants. Thus, cultivation of microalgae is much simpler and have higher growth rate when it meets with the optimum growth condition of microalgae. The absence of lignocellulosic materials in microalgae provides a much simpler and straight forward pre-treatment process. Besides, the biomass used to produce biofuel is environmental friendly and non-toxic (Scragg, 2009).

Microalgae are made up of prokaryotic or eukaryotic cell which grow via photosynthesis. There are mitochondrion, chloroplast and nuclei within the cell which are the main components that promote the growth of microalgae. At the outer layer of the cell, there is a cell wall enclosing the organelles. The cell wall serve to hold the cell structure in shape where it also keeps the nutrients within the cell itself. This cell wall serve as a protective layer that separates the cell from outer compound. Due to the presence of cell wall, the starch content within the microalgae could not be extracted directly, which is one of the main obstacle for bioethanol production (Asada, 2012).

Different microalgae have different biochemical composition that is applicable to different industries for various purpose. Most algae primary comprise of lipids, carbohydrates and proteins with varying composition. The high content of lipids, carbohydrates and proteins in microalgae allows them to be used as a biofuel production feedstock. The main biofuel produced using microalgae as feedstock are biodiesel and bioethanol. Various study were done to determine the method to maximise the extraction of carbohydrates from microalgae and the improvement on the conversion efficiency of biomass to bioethanol on a economical basis. Starch is used as a model compound for the analysis of carbohydrates.

However, within the cell of microalgae, not all of the carbohydrates are in the form of starch. There are carbohydrates that serve as a component for the structuring of cell wall and as storage for energy supply. Different types of carbohydrates are accumulated in different types of microalgae. Thus, the amount of carbohydrates content is quantified by hydrolysis of various carbohydrates to monosaccharide for HPLC analysis. For bioethanol production, the amount of carbohydrates is one of the main factor that determines the yield of bioethanol produced. Based on the study

done by Biller (2014), *Scenedesmus* species have higher carbohydrates contents compared to other species at a range of 19-46% w/w.

2.1.2 Microalgae *S. dimorphus* for bioethanol production

Chlorella, Dunaliella, Chlamydomonas and *Scenedesmus* are the microalgae species which have relatively higher weight percentage of carbohydrate based on dry basis (Becker, 1994) compared to other species. Unlike other species which might have higher concentration of lipids or higher concentration of protein, the four species mentioned above have higher concentration of carbohydrates which is one of the main precursors for bioethanol production. As mentioned before, photosynthesis of microalgae produces carbohydrate mainly in the form of starch which is stored within the cell. The content of starch in these microalgae is high which is capable in providing a higher yield of bioethanol production. However, the cell wall have to be hydrolysed before the extraction of starch can be taken place.

Table 2.1: Amount of Carbohydrates from Various Species of Microalgae on a Dry Matter Basis (%) (Adapted from Becker 1994; Harun et al 2010)

Algae	Carbohydrates (% / wt biomass)
Scenedesmus dimorphus	21-52
Chlamydomonas reinhardtii	17
Chlorella vulgaris	12-17
Chlorella pyrenoidosa	26
Dunaliella bioculata	4
Dunaliella salina	32
Tetraselmis maculate	15
Spirulina maxima	13-16

Based on Table 2.1, *Chlorella, Dunaliella, Chlamydomonas* and *Scenedesmus* species have relatively higher carbohydrates content than other microalgae. *Scenedesmus dimorphus* is chosen as the biomass to be used in this study as the carbohydrates content in the cell is higher than others. *Scenedesmus* is capable of having a rapid growth rate under low nutrient conditions (Yu, 2012). However, nitrogen limitation will result in decrease of cellular content of thylakoid membrane, activation of acyl hydrolase and stimulation of the phospholipid hydrolysis (Xin,

2010). In another words, in reduced nitrogen medium, the content of lipids and triglyceride in microalgae cell increases. Thus, cultivation of *Scenedesmus* in reduced nitrogen medium is not suitable for bioethanol production.

Scenedesmus is capable of uptake nitrogen and phosphorus as nutrients for its growth. With this capability, *Scenedesmus* is introduced as a biological method for wastewater treatment plant to remove these components (Pacheco, 2015). Nitrogen and phosphorus have to be removed from wastewater as it is not suitable for consuming and these components are toxic. Thus, by coordinating with wastewater treatment plant, the cultivation cost for *Scenedesmus* can be lowered as the microalgae feed on the nutrients in the wastewater can be later utilise for bioethanol production.

S.dimorphus can be cultivated to produce protein, carbohydrates or lipid-rich biomass in a single cultivation platform. Similarly with other types of microalgae, *S.dimorphus* requires water, light, carbon dioxide and inorganic nutrients to grow. The productivity of culture for *S.dimorphus* is affected by pH, carbon dioxide content, salinity and temperature of medium. The desired component can be cultivated within *S.dimorphus* by varying the cultivation conditions and parameters. *S.dimorphus* can produce a biochemical composition as high as 35% protein, 60% carbohydrates or 37% lipid in outdoor setting (Wang, 2013). With the manipulation of low nitrogen content and high light exposure through low inoculation density, *S. dimorphus* is capable of providing a highest productivities of protein and carbohydrate at a rate of 0.2 and 0.7 g L^{-1} d^{-1} respectively (Wang, 2013). The high content of carbohydrates in *S.dimorphus* where no lignocellulosic materials are presence allows *S.dimorphus* to be used as a feedstock for bioethanol production. Besides, *S.dimorphus* is simple in structure, easily harvested and consist of large amount of starch. The carbohydrate extracted is valuable for production of bioethanol.

2.1.3 Challenge of using microalgae as feedstock for bioethanol production

According to EIA, in year 2010, about 83% of energy are generated from fossil fuel. Although the production of energy using fossil fuel as feedstock is well developed and cost effective, the pollutants emitted from fossil fuel are enormous in amount. The pollutants such as carbon monoxide, nitrogen oxides, sulphur oxides and particulate matters causes severe pollution to the environment. Acid rain, global warming, greenhouse effect and air pollution are some of the consequences of using fossil fuel for energy generation (UCS, 2016).

Due to the heavy pollution caused by combustion of fossil fuel, many countries are focusing on the alternative or renewable energy which is environmental friendly as well as sustainable. Among the renewable energy resource, biomass is one of the main contributors for renewable energy generation. Besides biomass, the natural energy such as wind energy, solar energy, geothermal energy and hydroelectric are utilise for the generation of energy (EIA, 2011).

For bioethanol production, the main feedstock are crops such as corn and sugarcane. The cost of conversion of starch from crops to ethanol is considerably economical. However, due to competition with food supply and land, the cost of using crops as feedstock is high. Another feedstock are lignocellulosic materials. Lignocellulosic materials are abundantly available thus, the cost of obtaining this material is low. However, due to the complex structure and chemical composition of lignocellulosic materials, the pre-treatment and extraction process is complicated (Hu, 2009). Due to the complexity process to pre-treat lignocellulosic materials, the production of ethanol using this material is not industrialised due to high cost.

Microalgae are the new generation or also known as third generation biomass for bioethanol production. The drawback of using crops and lignocellulosic material for bioethanol production is overcome by microalgae. The main advantage of using microalgae as feedstock is that microalgae have high growth rate, capable of accumulating high amount of biomass based on weight percent and it does not significantly compete with the use of land for growth (Schlagermann, 2012). Besides, microalgae has metabolic flexibility where normal plants don't. This flexibility allows the cultivation process to adjust the parameters and species to produce the highest desired chemical composition within the microalgae (Tredici, 2010). For

example, this flexibility can promotes higher carbohydrate yield which will increase the amount of ethanol produce during fermentation process. In addition, the cultivation for microalgae can be operated all year long whereas crops normally doesn't due to climate change. Microalgae is capable of providing a higher productivity per unit area compared to the other feedstock. Besides, for the production of the same amount of biofuel, the utilisation of resource for crops and microalgae are different. For example, the water needed for crops to grow is around 3,000 litres whereas microalgae only need 10 to 20 litres for 1 litres of biofuel production (Fraiture, 2008). This indicates that microalgae have the superiority in various aspect while producing the same yield of bioethanol.

2.2 Pre-treatment of microalgae for fermentable sugar production

Pre-treatment of microalgae is an important process to convert the insoluble carbohydrate into soluble fermentable sugar. Direct addition of microalgae to the yeast is not applicable as the carbohydrates in the microalgae cell is not accessible and digestible by yeast. This is due to the presence of cell wall which prevent sugar to be readily dissolve in the medium (Andersen, 2013). There are various types of carbohydrates stored within the cell (Hu, 1998). Thus, the disruption of the cell wall by pre-treatment is needed in order to release the trapped starch and other type of carbohydrates. The released carbohydrates can be used as a feedstock for fermentation to bioethanol. When the cell wall is not degraded, the fermentation activity could not take place which result in zero production of bioethanol.

The traditional way for the treatment of microalgae are using acid at high concentration and high temperatures. A pH of 1 to 2 and temperature of 150 to 230 ^{o}C at high pressure is the range that are mostly used. This extreme condition caused high amount of by-products formed which capable of inhibiting the fermentation process.

In order to produce monomeric sugar constituents which are fermentable by yeast, two main type of hydrolysis methods are used. Acid hydrolysis is one of the method used to pre-treat the microalgae biomass prior to fermentation. Acid

hydrolysis are high in efficiency whereas low cost as acid is not expensive. Another type of hydrolysis method is enzymatic hydrolysis. Alkaline pre-treatment of microalgae is yet to be used as conventional hydrolysis agent. However, alkaline is capable of producing salvation and saponification reaction which removes the cross links between hemicelluloses and other components. Besides, it also increases the porosity and reduce the crystalline of the cellulose. With alkaline pre-treatment, the carbohydrates can be accessible. However, alkaline pre-treatment is more suitable for biomass which contains small amount of lignocellulosic materials.

A cost effective pre-treatment process is required to minimise the production cost for bioethanol. There are several pre-treatment ways for microalgae, separated into physical, biological and chemical. Physical pre-treatment methods like pyrolysis where biomass is exposed to high temperature (Harun, 2011). Pyrolysis is a easy and direct process. However, it is not commercialise as pyrolysis require large amount of energy input which result in high cost. For biological method, it utilise an enzyme to cause degradation of biomass to soluble sugar. As for chemical method, acid or alkaline are used to convert carbohydrates into fermentable sugar (Lenihan, 2010). Utilisation of chemical is the most common method used as the energy input and chemical cost is low. An effective pre-treatment method can provides greater yield of fermentable sugar whereas the production of by-products that subsequently inhibits the fermentation or hydrolysis process can be avoided.

2.2.1 Acid hydrolysis

Acid hydrolysis is one of the convention pre-treatment method used to convert starch to glucose. Unlike lignocellulosic materials, pre-treatment using acid hydrolysis for microalgae is simple and straight forward. The general concept of acid hydrolysis involve breaking of cell wall and degradation of starch to fermentable sugar. The yield of fermentable sugar is higher and economical for acid hydrolysis compared to other methods (Castro, 2015). The rate of reaction is also faster and effective compared to other types of hydrolysis.

Acid pre-treatment process dissolves and degrades the hemicellulosic component in the biomass which allows the carbohydrates within the cell accessible (Wayman, 1996). Besides, acid also disassembles the cellulose into simple sugar which are fermentable. As microalgae do not consist lignocellulosic compound, mild acid treatment is sufficient to release the particular carbohydrates that is required for the fermentation process (Lam & Lee, 2015). Thus, the performance of using mild acid to pre-treat the microalgae were analysed in this study.

Based on cost effective consideration, acid hydrolysis of starch to glucose requires an optimum condition in order to maximise the productivity. Acid such as sulphuric acid, hydrochloric acid and phosphoric acid can be used to hydrolyse cellulose and starch of microalgae. According to Miranda (2012), sulphuric acid is one the efficient acid that can causes disruption of the cell and extract the sugar from microalgae. In Miranda (2012) research, hydrolysis of dried biomass from *Scenedesmus obliquus* using sulphuric acid at 1 M at 120 ^{o}C for 30 min is the optimum condition for the pre-treatment process. It extracts and converts the starch to soluble glucose prior to the fermentation process. Besides that, Harun et al (2010a) have studied on the production of bioethanol from microalgae under varying conditions. The highest yield of bioethanol takes place at dilute sulphuric acid with a concentration of 3% v/v at 160 ^{o}C for 15 min. This indicates that acid hydrolysis at optimum condition can produce a higher yield of fermentable sugar which increases the yield of bioethanol.

Besides sulphuric acid, phosphoric acid can be used as a acid medium for the hydrolysis of microalgae. Phosphoric acid is a weaker acid compared to sulphuric acid which is a strong acid. The degree of dissociation of phosphoric acid is weaker thus providing a less corrosive medium for the process equipments (Nair, 2015). Phosphoric acid is also less toxic compared to sulphuric acid, thus reducing the environmental impact. Besides, the neutralization of phosphoric acid with alkaline produces a phosphate compound which is a nutrient for microorganisms such as yeast (Lopez-Linares et al, 2013). The presence of the nutrients allow a higher growth rate for yeast where it increases the rate of fermentation. With phosphoric acid used for acid hydrolysis, it can avoid the contamination of sulphur in the feedstock as well.

From economical point of view, the use of acid for hydrolysis of microalgae provides a high fermentable sugar yield from the biomass. Thus, the use of enzymatic treatment is not necessary as it is far more expensive than acid treatment. However, acid hydrolysis parameters have to be properly controlled to avoid formation of side products which is toxic towards the yeast (Moxley, 2007). This toxic can inhibits the activity of yeast thus reducing the yield of bioethanol produced. This problem normally occurs in separate hydrolysis and fermentation (SHF). In SHF process, the hydrolysis takes place first before feeding the pre-treated solution to yeast for fermentation. Side products will be produced when the acid concentration used is too high or temperature is too high. Besides, the use of concentrated acid is limited as the cost for concentrated acid is high and the possibility of causing corrosion to the equipments increases (Sun & Cheng, 2002).

2.2.2 Enzymatic hydrolysis

Unlike acid hydrolysis, enzymatic action requires less energy where conversion of starch to glucose can be easily obtain. Fermentable sugars are produced from the used of enzyme to hydrolyse the biomass. Due to lower energy consumption, the cost is lower whereas corrosion of process equipments can be avoided. Generally, amyloglucosidase or α-amylase is used as the enzyme for the pre-treatment of microalgae with high starch content.

Amyloglucosidase is an enzyme which digest starch and release glucose within a short period (Warren, 2015). The product, glucose is then fed to yeast for fermentation to bioethanol. Amyloglucosidase is a commercial enzyme produced from a genetically modified strain of *Aspergillus* (Choi, 2010). Another enzyme used for pre-treatment of microalgae is α-amylase. α-amylase is derived from *Bacillus licheniformis*. It is capable of inducing a degradation on the glycoprotein in the cell wall of the microalgae (Imam, 1987). α-amylases reacts with the polysaccharide chain in the biomass where the polysaccharide chain will be broken into simple chain. The viscosity of the mixture also significantly reduced where the characteristic of blue solution by iodine is not observable (Kolusheva, 2007). Besides, α-amylases are

active and stable at lower pH, which allows the incubation step for both enzymes to perform at pH 5.

The optimum condition needed by the starch degrading enzyme is required for high productivity. The parameter that influence the rate of enzymatic activity are temperature, concentration of enzyme, pH of the mixture and the time for the reaction. A coordinated enzymatic hydrolysis utilising both amyloglucosidase and α-amylase can reduce the total energy required, higher reaction rate and reducing the impurities content (Mojovic, 2006).

Enzymatic treatment of microalgae are divided into two parts. First, the conversion of insoluble sugar to soluble sugar by α-amylase. This conversion process is also known as liquefaction. The optimum condition for liquefaction is set to be at pH 6, 0.005% concentration of enzyme, 90 ^{o}C for 30 min (Choi, 2010). Then, the complex sugar undergoes saccharification to simple sugar by amyloglucosidase enzyme. The rate of saccharification of starch is at optimum when the concentration of enzyme is 0.2%, temperature at 55 ^{o}C, pH 4.5 for 30 min (Choi, 2010). At these condition, the yield of glucose is at maximum which indicates that the enzymatic activity is at maximum as well.

However the rate of enzymatic activity is lower compared to acid hydrolysis. The lower rate result in longer treating time needed for the conversion of starch to glucose. Besides, the enzyme used for pre-treatment is expensive. Amyloglucosidase and α-amylase are costly and not economical for continuous purchase of new stock. Thus, the extraction of enzyme from the mixture is needed which will increase the operation cost when it is industrialise. The extracted enzyme can be recycled which reduce the need of replenishing new enzyme.

2.2.3 Ultrasonic homogenizer

There are various methods to physically disrupt the cell of microalgae to allows the carbohydrates within the cell to be accessible. Sonication is one of the method with lower operation cost as it operates at a lower temperature compared to other methods. It also prevents protein degradation due to high temperature. Chemical such as acid

or alkaline is not required by sonication process which remove the necessity of extraction of these chemical from the product after the treatment process. There are various equipments which is capable of creating an ultrasonic medium which disrupt the cell of microalgae. In general, increasing the energy input of the equipments increases the yield of desired component extracted from the biomass (Gerde, 2012).

Ultrasonic treatment is capable of causing cell disruption of the microalgae and releases the starch which can be used as a feedstock for bioethanol production. Cell disruption is necessary in order to increase the yield of starch and other desired component extracted from the microalgae (Johnson, 2008). Generally, the used of sonication environment in liquid will induce two types of main mechanism regarding how the microalgae cell may be altered. One of it is cavitations where the liquid medium will produce microbubbles which expand and implodes violently due to ultrasonic treatment. Through this mechanism, the shockwave is transferred to the cell which causes disruption to the cell structure (Montalbo-Lomboy, 2010). Another mechanism that occurs when using ultrasonic treatment is acoustic streaming. Acoustic streaming shows the mixing of the solution when it is treated. It is used to speed up the distribution of the ultrasound energy uniformly, convection flow of the liquid and dissipation of any heating that occurs (Khanal, 2007).

However, utilising sonication condition have to take the energy input and the product quality into account. Excessive energy input will result in a high operation cost which is not economical for large scale production (Gerde, 2012). Besides, prolonged exposure of biomass to ultrasonic medium can result in formation of free radicals which will result in quality drop or formation of undesired side-product. According to Gerde (2012), high amplitude accompany by short treating time have a lower chance of forming free radicals compared to low amplitude with long treating time.

2.3 Fermentation

Alcoholic fermentation is a metabolic process where pre-treated biomass was converted by ethanologen microbe to alcohol (McKendry, 2002). Yeast or bacteria

are normally used as a fermentation agent which converts fermentable sugar into bioethanol. Considerable amount of carbohydrates are stored within the cell of microalgae in the form of starch or cellulose. However, due to the cell structure of microalgae, pre-treatment have to be done to converts starch and cellulose to soluble sugar before feeding it to yeast. The absence of lignin in microalgae allows the pre-treatment and fermentation process to be simple and direct (Daroch, 2013). Pre-cultured of yeast stock is required in order to convert the inactive yeast to active yeast. Normally, yeast are cultivated in YDP broth or YDP agar where the pre-growth of yeast takes place. The pre-culture of yeast normally takes about 24 hr of treatment time. The cultivated yeast can then be added into the pre-treated microalgae for fermentation to take place.

Generally, there are 2 types of fermentation, (a) aerobic and (b) anaerobic (Suganya, 2016). The categorisation for aerobic and anaerobic fermentation is depending on the necessity of oxygen in the process. It is not necessary for fermentation to occur in an anaerobic environment. For most fermentation process of simple sugar, anaerobic fermentation takes place. Yeast is used to break down sugar to produce ethanol and carbon dioxide. Yeast consumes readily available sugar for growth. Carbohydrates such as starch and cellulose are one of the main content that is consume by yeast to produce bioethanol. However, starch and cellulose are not consumable by all types of yeast, thus pre-treatment of microalgae is needed depending on the type of yeast used.

Carbon dioxide are emitted during fermentation process. With the accumulation in amount of carbon dioxide in the medium, yeast activity will be reduced due to the increase in acidity of the medium caused by carbon dioxide. Thus, removal of carbon dioxide occasionally have to be done to ensure that the activity of yeast is not affected.

2.3.1 Fermentation configuration : separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF)

Generally, there are two conventional methods for the production of bioethanol from microalgae. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SFF).

SHF provides a guarantee performance in terms of bioethanol production. SHF is a two step process where the reaction occurs separately in a different reactor (Xiros, 2013). The hydrolysis and fermentation reaction are done separately. The main advantage of SHF is that each of the respective reaction can be carried out at its optimum condition. The optimum factors that influence the rate of reaction such as pH, temperature, reaction time and other factors are different for both reaction (Lam, 2015). Thus, carrying out the process independently will result in a higher productivity. Based on a study done by Ho (2013b), the production of ethanol from SHF is more effective where it is also possible to be used for large scale project to produce bioethanol from microalgae with high concentration of carbohydrate as feedstock.

However, the drawback for SHF is that the hydrolysis process utilise chemicals such as acid, alkaline or enzyme. During the hydrolysis process, the accumulation of the end product reduces the rate of reaction significantly. The presence of glucose and cellobiose will act as an inhibitors for the activities of cellulases, thus resulting in the decreasing rate of reaction rate. Besides, after the hydrolysis process, the neutralisation of acid or alkaline medium and the removal of by-product has to be done (Lam, 2015). Besides, the by product have to be removed from the mixture, otherwise the presence of by product will increase the viscosity of the mixture which will limit the mass transfer process (Ho, 2013a). As a result, these factors will affect the production rate of bioethanol. As for using enzyme as a hydrolysis agent, the extraction of enzyme has to be done occasionally. The is to minimise the enzyme lost during the treatment process.

As for SSF, the saccharification and fermentation takes places simultaneously in the same reactor (Hahn-Hagerdal, 2006). During the saccharification of starch to simple sugar in acid hydrolysis process, the increase in the production of glucose will gradually reduce the rate of hydrolysis reaction (Xiros, 2013). In SSF, the production

of glucose will directly be consumed by yeast. Thus, the inhibition by the accumulation of glucose can be avoided in SSF. SSF can provide a higher yield of bioethanol production (Lin, 2006). It also reduce the reaction time, reduce the amount of enzyme required and simplify the procedures for the fermentation process. Furthermore, the concentration of bioethanol produced is higher where foreign contamination can be reduced.

However, the fermentation rate for SSF is difficult to optimise. The process control is difficult due to simultaneous reaction takes place in the same reactor, where the operating condition have to be adjust to suit both reaction (Gupta, 2010). Moreover, the process is more complicated due to presence of many components in it. The extraction of the enzyme and yeast is difficult as there are other chemical components presence within the mixture. Thus, large scale production using SSF method is yet to be industrialised (Olofsson, 2008). SSF process can be improved by reducing the contamination presence in the process, optimising a suitable process conditions, and uses yeast which is capable of converting pentose into bioethanol.

2.3.2 Yeast as fermentation microbe: *Saccharomyces cerevisiae* and *Saccharomyces diastaticus*

The fermentation of non-glucose sugars are more complicated and difficult. The reason behind this phenomenon is that the conversion of pentose sugar to ethanol by yeast is less efficient compared to the conversion of hexose sugar.

Saccharomyces cerevisiae is one of the most common yeast used for fermentation of biomass to bioethanol (Silva, 2016). *S.cerevisiae* is capable of producing a high yield of bioethanol by fermenting glucose, thus it is used in industrial scale. However, the raw material that is consumed by S.cerevisiae to produce bioethanol are limited to simple sugar such as glucose and sucrose (Rodrigues. et al, 2015).

Although being a desired end product, bioethanol itself can be an inhibitor for the cell growth of yeast, which reduces the yield of bioethanol (Izawa, 2010). However, *S.cerevisiae* has a higher tolerance towards inhibitors, resistance towards

ethanol, and fermenting at a higher rate. According to Dong study (2015), the results show that the ethanol tolerance for *S.cerevisiae* increases with the prolonged time of fermentation. Besides, the concentration of yeast increases will result in the increase of fermentation activity. With the increase in fermentation activity, the fermentation rate is also higher where the ethanol produce at a higher rate. However, when the amount of yeast exceed a certain point, the efficiency of ethanol production is reduced which indicates that the amount of yeast can become a limiting factor as well.

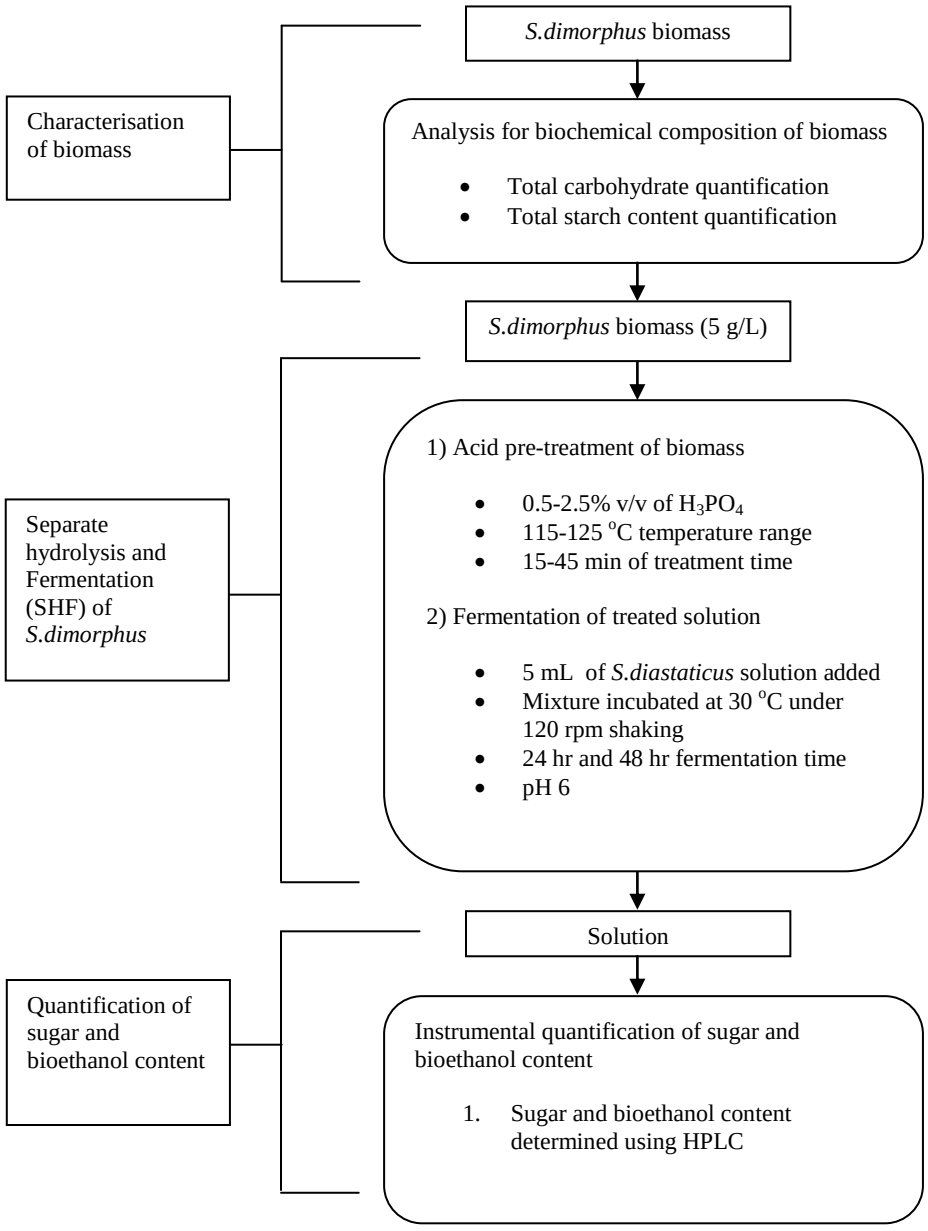
Based on a study done by Treebupachatsakul (2016), xylose is not fermentable by *S.cerevisiae* due to inability to use pentose sugars as food. The amount of non-fermentable sugar presence in the biomass will not contribute to the amount of bioethanol produced. Only fermentable sugar will influence the amount of ethanol produced in fermentation process. Due to the lack of certain enzyme such as β-glucosidase, the direct conversion of starch or cellulose to glucose is not available in *S.cerevisiae*. Thus, pre-treatment of biomass is essential before it can be fed to *S.cerevisiae* for fermentation (Treebupachatsakul, 2016). However, the coordination between *S.cerevisiae* and the necessary enzyme for the conversion of starch to glucose can be done. The integrated reaction is also known as simultaneous saccharification and fermentation (SSF). With SSF, the productivity and yield of bioethanol produced can be optimised whereas the operating procedure can be simplified.

S.diastaticus is an amylolytic yeast (Tesfaw, 2014). *S.diastaticus* is capable of utilising starch as a feedstock for fermentation and capable of direct conversion of starch to ethanol (BBSRC, 2016). Unlike *S.cerevisiae*, *S.diastaticus* is able to excrete glucoamylase enzyme extracellularly during fermentation process. Similar to amyloglucosidase, the enzyme selectively degrades starch to simple sugar which is then used by the yeast for fermentation to produce bioethanol. It is yet to be used as an industrial yeast for the fermentation of biomass to bioethanol. However, due to its capability of direct conversion of soluble starch to glucose, *S.diastaticus* is a yeast with high potential to be used as a fermenting yeast. Based on the research done by Pothiraj (2015), the starch saccharification potential of *S.diastaticus* is higher as the yield and total reaction time used by *S.diastaticus* for direct fermentation of cassave waste was superior compared to fungal.

CHAPTER 3

METHODOLOGY

3.1 Research flow chart



3.2 Materials and chemicals

3.2.1 Chemicals used in this study

Table 3.1: Chemicals Used in this Study

Chemicals	Purity	Source
Amyloglucosidase aqueous solution	≥300 U/mL	Sigma- Aldrich, USA
Dextrose, $C_6H_{12}O_6$	99.0	Sigma- Aldrich, USA
Ethanol, C_2H_5OH	99.9%	HPLC grade
Glacial acetic acid	≥99.8%	Merck
Hydrochloric acid, HCl	37%	Fisher Scientific, UK
Peptone	-	Sigma- Aldrich, USA
Phosphoric acid, H_3PO_4	85%	RCI Labscan
Sodium acetate	≥99.0%	Fisher Scientific, UK
Sodium Hydroxide, NaOH	≥99%	Merck
Sodium nitrate, $NaNO_3$	99+%	Acros Organics
Starch from rice	-	Sigma- Aldrich, USA
Sulfuric acid, H_2SO_4	97.0%	Fisher Scientific, UK
Yeast Extract	-	Sigma- Aldrich, USA

3.2.2(a) *Saccharomyces diastaticus*

A pure culture of *Saccharomyces diastaticus* (ATCC 28338) was purchased from American Type Culture Collection (ATCC).

3.2.2(b) *Scenedesmus dimorphus*

Dried biomass of *S.dimorphus* (UTEX 1237, University of Texas) was obtained from School of Chemical Engineering, Universiti Sains Malaysia.

3.3 Cultivation of *Saccharomyces diastaticus* as stock culture

Yeast extract peptone dextrose (YPD) broth is used as a cultivation medium for *S.diastaticus*. YPD broth with the composition of 3 g/L of yeast extract, 5 g/L peptone, and 10 g/L glucose dissolved in distilled water was used to cultivate *S.diastaticus*. 300 mL of YPD broth was used to cultivate *S.diastaticus*. 0.9 g of yeast extract, 1.5 g of peptone and 3 g of glucose were dissolved in 300 mL of distilled water. The mixture was then stirred for 30 min using a magnetic stirrer. After stirring, the mixture was autoclave at 121 ^{o}C for 20 min at pressurized environment. After 20 min, the sterilized broth was removed and allowed it to room temperature. 1 mL of pre-cultivated *S.diastaticus* was inoculated into the flask containing the prepared liquid medium. The mixture was shake with a orbital shaker for 48 hr at 30 ^{o}C at 120 rpm. After 48 hr, the cultivated *S.diastaticus* was ready for the fermentation process.

3.4 Biomass characterization

3.4.1 Total carbohydrate content

A modified method based on National Renewable Energy Laboratory was used to quantify the carbohydrate content in the biomass. A two step hydrolysis was used as the hydrolysis method for the biomass where the hydrolysis agent used is sulphuric acid. A total mass of 300 mg of *S.dimorphus* biomass and dextrose was added into each test tube respectively where 3 mL of 72 wt% of H_2SO_4 was added after the addition of biomass. The mixture was placed on a water bath set at 30 ^{o}C for 1 hr while continuous stirred. After 1 hr, the sample was transferred over to a sample bottle where 84 mL of deionised water was then added into the sample bottle containing the sample. The bottle was placed in an autoclave machine for 1 hr at 121 ^{o}C. After the sterilizing, the sample was cooled and neutralised with NaOH to about pH 3. The mixture was then centrifuged at 3000 rpm for 30 min and the top layer were stored in a sample bottle in fridge for HPLC analysis later on.

3.4.2 Total starch content

A modified method based on National Renewable Energy Laboratory was used to quantify the starch content in the biomass. Amyloglucosidase was used as the hydrolysis enzyme for the biomass (Ehrman, 1996). 100 mg of dry *S.dimorphus* was added into a boiling tube followed by 5 mL of distilled water. After that, 1 mL of 2 N NaOH was added and the mixture was stirred in a preheated water bath at 90 ^{o}C. 1 mL of 2 N HCl was added to neutralise the solution after 20 min. The solution is then cooled to temperature below 50 ^{o}C. Then, 2 mL of acetate buffer at pH 4.5 was added. After that, 0.5 mL of amyloglucosidase (60 units of activity per mililiter) was added to the solution. The solution was stirred at 40 ^{o}C for approximately 60 min. The solids were removed via centrifugation at 3000 rpm for 15 min. The remaining hydrolysed sample was analysed with HPLC.

3.5 Acid pre-treatment of biomass

Pre-treatment of microalgae at various conditions was investigated using phosphoric acid as the hydrolysis agent. The pre-treatment of biomass was carried out in a 250 mL of Scott bottle. The amount of biomass is fixed as a constant variable at a concentration of 20 g/L. The phosphoric acid solution was prepared by diluting concentrated phosphoric acid with distilled water to obtain acid concentration of 0.5% to 2.5% v/v.

Pre-treatment time for the hydrolysis of *S.dimorphus* was investigated at 15 min, 30 min and 45 min. The reaction temperature were set at 115 ^{o}C, 120 ^{o}C and 125 ^{o}C. The conditions of the pre-treatment were tabulated in Table 3.2.

One gram of *S.dimorphus* was added into a reaction bottle and sealed. 50 mL of the respective concentration of phosphoric acid were added into the reactor bottle containing the biomass. A magnetic stirrer was added into the bottle and the mixture was stirred for 30 min on a magnetic stir plate. After 30 min, the mixture was placed in an autoclave machine with the respective reaction temperature and treatment time. Upon the completion of acid pre-treatment, the mixture were placed on a magnetic stir plate and stirred for 30 min at constant speed.

After that, the samples were centrifuged at 3000 rpm for 30 min. The liquid solution collected were separated from the solid. It is then subjected to subsequent fermentation.

Table 3.2: Reaction Condition for Acid Pre-treatment of Microalgae

No.	Acid Conc. % (v/v)	Temperature (^{o}C)	Time (min)
1	0.5	120	30
2	1.0	120	30
3	1.5	120	30
4	1.5	120	15
5	1.5	120	45
6	1.5	115	30
7	1.5	125	30
8	2.0	120	30
9	2.5	120	30

3.6 Fermentation

S.diastaticus was pre-cultured in YDP medium. The cultivated *S.diastaticus* was added into the treated solution for the conversion of fermentable sugar to bioethanol. 30 mL of the pre-treated solution were added into a conical flask. It is then diluted with 10 mL of distilled water. 3 g/L of yeast extract (0.12 g) and 5 g/L of peptone (0.2 g) were added into the solution. Then, the pH of the solution were adjusted to pH 6 using 2 N of NaOH. The mixture was swirled until the components added were fully dissolved in the solution. The mixture was sterilized with an autoclave machine at 121 ^{o}C for 20 min. After sterilizing, the mixture was allowed to cool to room temperature. 5 mL of pre-cultured *S.diastaticus* was placed in a centrifuge tube and centrifuged at 3000 rpm for 30 min. The supernatant was removed. 5 mL of deionised water were added into the yeast cell which is ready as inoculums for fermentation. The yeast solution were added into the sterilized solution and placed in an orbital shaker at 30 ^{o}C and 120 rpm. Sample of 1 mL was taken from fermentation broth after 24 hr and 48 hr of fermentation time. The samples were subjected to HPLC analysis for the sugar content and bioethanol content quantification.

The maximum theoretical yield of bioethanol is at 51.1%. It is limited by the nature of fermentation where equation (1) was used to determine the yield of bioethanol based on the total sugar available while taking the maximum theoretical yield into account.

$$Bioethanol\ Yield, \% = \frac{Ethanol\ (g)}{Total\ Sugar\ (g) \times 0.511} \times 100\% \qquad (1)$$

3.7 Instrumental analysis method

Sugar content measurement and bioethanol content determination were carried out with high performance liquid chromatography (Agilent series 1200) system equipped with refractive index detector (Aminex-HPX-87H, 300 x 7.8 mm column). The column was placed in an oven set at 50 ^{o}C. A total molarity of 0.005 M sulphuric acid was used as the mobile phase and the flow rate was set at 0.6 mL/min. Calibration curve were constructed using standard glucose and ethanol. Quantification of glucose content and bioethanol content were determined based on the calibration line.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of microalgae biomass

Total carbohydrate of microalgae biomass were determined based on the modified method from National Renewable Energy Laboratory (Ruiz & Ehrman, 2010).

For the determination of total carbohydrate content in *S.dimorphus*, a two step hydrolysis was carried out on 0.3 g of biomass using sulphuric acid as the hydrolysis agent. Complex carbohydrates such as cellulose, starch and other polysaccharides was hydrolysed to simple sugar. Sulphuric acid also serve to convert the insoluble sugar into soluble sugar which is essential to ease the extraction of the carbohydrate content within the cell. The carbohydrates content that are presence in the biomass are mainly starch, glucose, galactose, sucrose and mannose.

Based on Fig 4.1, the result from HPLC indicates the presence of glucose based on a peak at residence time of 9.807 min. The amount of glucose calculated is based on the glucose calibration standard as shown in Appendix D. Another peak is presence at time 7.274 min, which is identified to be maltotriose. Based on the result of analysis, the total glucose produced from *S.dimorphus* for a complete hydrolysis is approximately 44±0.5% w/w (Table 4.1).

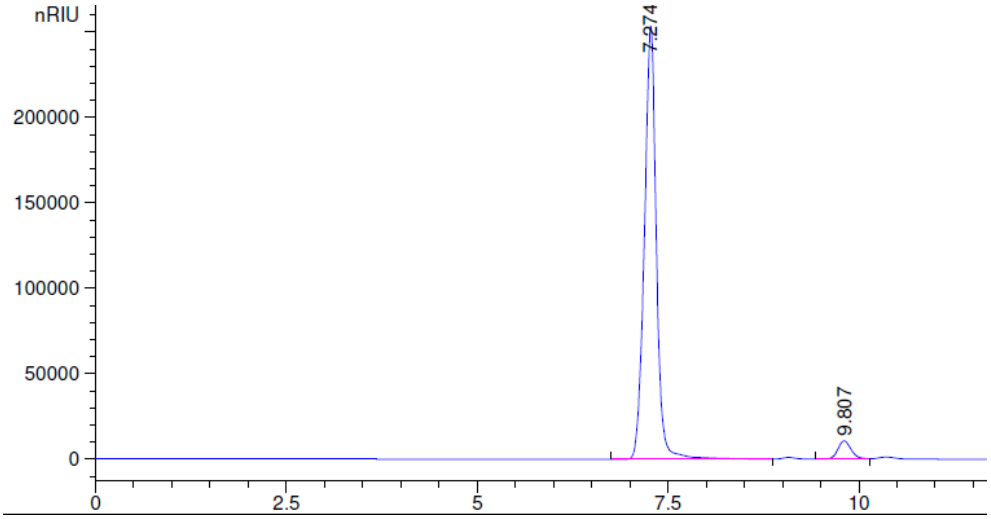


Figure 4.1: HPLC analysis for H_2SO_4 treated microalgae biomass

As for the total starch quantification, it is carried out based on a modified method from National Renewable Energy Laboratory which uses amyloglucosidase as the enzyme (Ehrman, 1996). Amyloglucosidase catalyze the hydrolysis of starch to glucose. The weight of biomass used in this analysis is 0.1 g. The hydrolysed biomass were analysed for its total starch content using HPLC column as the analytical tool. Based on Fig 4.2, the peak presence at 9.804 min indicates that the presence of glucose was found in the treated sample .

The amount of glucose produced is equals to the amount of starch presence in the biomass as amyloglucosidase selectively converts starch to glucose. The total starch content of *S.dimorphus* were calculated at around 28±0.5% w/w (Table 4.1). Based on Fig 4.2, maltotriose and galactose was found at time 7.215 min and 10.531 min respectively. Galactose is also a type of carbohydrates which could contribute to the amount of bioethanol produced (Steward & Russell, 1987). Maltotriose is a trisaccharide consisting of three glucose molecules linked with α-1,4 glycosidic bonds. This compound is commonly produced from the hydrolysis of starch through the use of digestive enzyme such as amyloglucosidase (Pfisterer & Wagner, 1975).

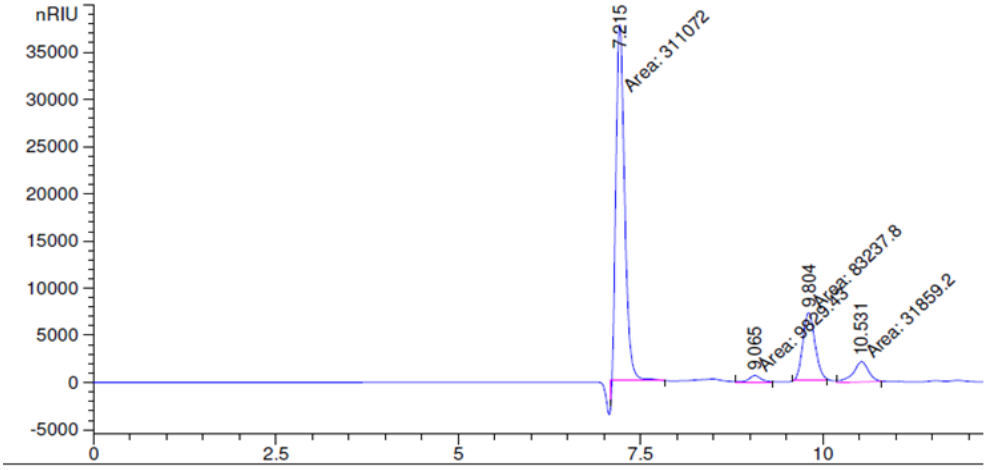


Figure 4.2: HPLC analysis of microalgae biomass that treated by amyloglucosidase enzyme

Table 4.1: Biochemical Composition of *Scenedesmus Dimorphus*

	Composition of biomass in dry basis, w/w (%)	
	Total glucose obtained from biomass	Total starch obtained from biomass
Dried biomass	44±0.5	28±0.5

Based on the results obtained, *S.dimorphus* contain significant amount of carbohydrates that are potential for subsequent fermentation process to produce bioethanol.

4.2 Performance of mild acid pre-treatment on microalgae

4.2.1 Effect of acid concentration on the total glucose produced

Acid is one of the widely used chemical that converts insoluble and complex polysaccharide into soluble simple sugar. Thus, the concentration of the acid in pre-treatment step plays an important role in manipulating the amount of glucose produced. Based on Fig 4.3, it indicates that an increase in acid concentration

increases the glucose produced. For the lowest acid concentration at 0.5% v/v, the yield of glucose produced is the lowest, at about 27 mg of glucose. Whereas for the highest acid concentration used (2.5% v/v), the amount of glucose produced is the highest among the concentration used, at approximately 90 mg.

From Fig 4.3, the relationship between the total glucose produced and acid concentration is directly proportional within the range of 0.5% v/v to 2.5% v/v of phosphoric acid. In another words, an increases in acid concentration could enhance the degree of hydrolysis, thus producing higher amount of simple sugar. The main reason is that at higher acid concentration, the hydrolysis reaction can occur more rapidly as there are more interaction between the microalgae cell and phosphoric acid. Thus resulting in a higher degree of degradation on cell wall and starch eventually increase the amount of simple sugar. A concentration beyond 2.5% v/v might result in a greater amount of glucose produced.

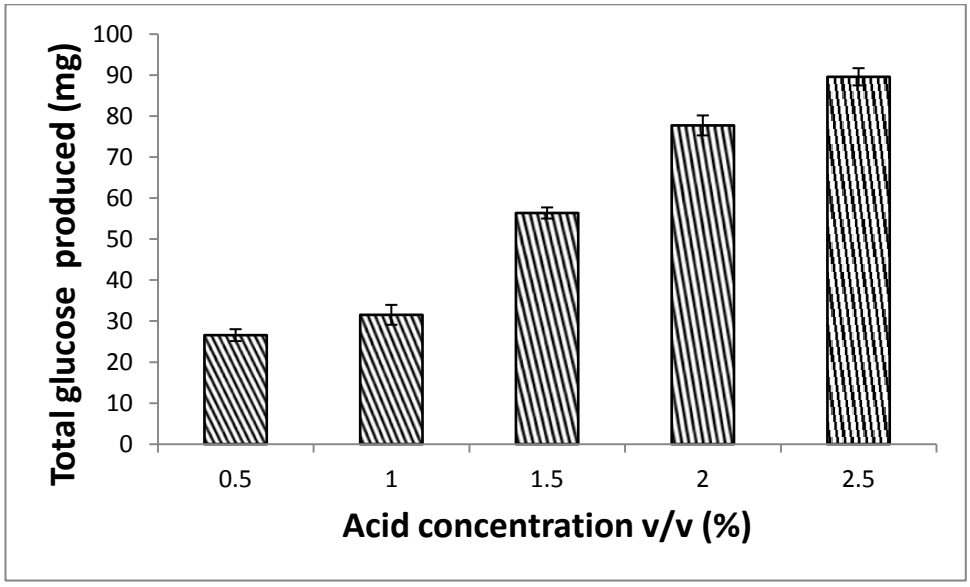


Figure 4.3: Total glucose produced from acid pre-treatment with different acid concentration by maintaining pre-treatment temperature and reaction time at 120 ^{o}C and 30 min respectively

However, up to a certain point, the increase in acid concentration will not significantly increase the total glucose produced (Harun & Danquah, 2011). Excessive acid could cause the degradation of sugar to inhibitors such as furfural and

5-hydroxymethylfurfural (Yazdani et al, 2011). Therefore a suitable acid concentration has to be selected in order to maximise the yield and minimise the degradation of carbohydrates. Based on the result, 2.5% v/v is the most suitable acid concentration as it provides the highest yield of glucose.

From Fig 4.4, the analysis using HPLC for the sample of 2.5% v/v phosphoric acid, various carbohydrates were indicated in the chromatogram besides glucose. The peak obtained were compared with the standard done by Bio Rad Laboratories to identify the respective components that are presence in the sample (Bio Rad Laboratories, 1994). The presence of significant amount of glucose was indicated due to a peak at time 9.802 min. A small amount of galactose was detected in the sample which is a peak at 10.362 min.

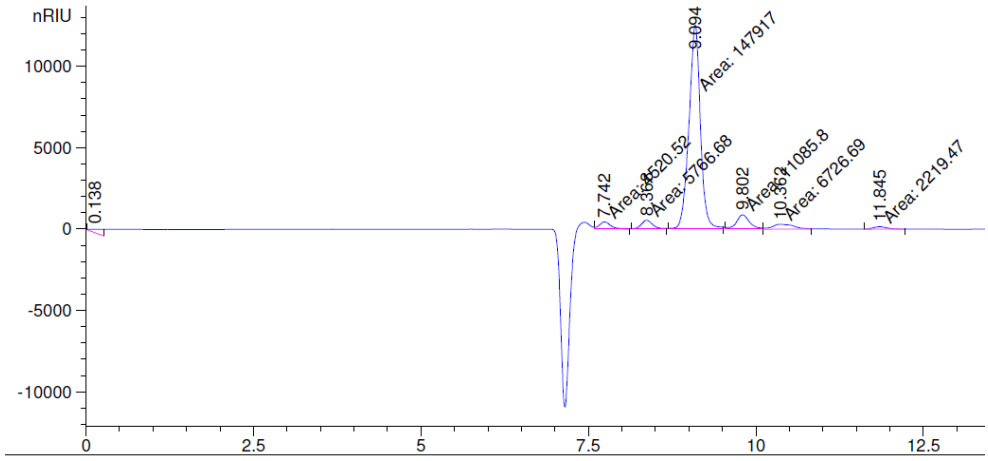


Figure 4.4: HPLC analysis for 2.5% v/v phosphoric acid treated microalgae biomass treated at 120 ^{o}C for 30 min

The mixture of galactose and glucose were formed from the hydrolysis of starch and cellulose within the microalgae biomass. The presence of these sugars contribute to the amount of bioethanol produce (Steward & Russell, 1987). Fermentable sugar such as glucose is one of the favourite carbohydrate to produce bioethanol. Besides glucose, other carbohydrates like galactose are also capable of being utilized by yeast, *S.diastaticus* for fermentation process (Gilliland et al, 1966).

According to the result obtained, the presence of simple sugar after the pre-treatment process indicates that phosphoric acid is capable to decompose

carbohydrates and convert them into soluble sugar even though phosphoric acid is a mild acid. However, the glucose produced using concentrated sulphuric acid to pre-treat the microalgae biomass is about 440 mg. By comparing with the highest glucose produced from phosphoric acid pre-treatment was only about 111 mg. It can be deduce that the amount of glucose produced from mild phosphoric acid is lesser compared to the use of concentrated sulphuric acid. However, the production of inhibitors can be avoided with the use of mild phosphoric acid.

4.2.2 Effect of pre-treatment temperature on the total glucose produced

The reaction temperature is one of the main factors that determine the amount of glucose produced. In this study, a range from 115 ^{o}C to 125 ^{o}C was used to pre-treat the microalgae where the acid concentration were maintained at 1.5% v/v and pre-treatment time at 30 min. Based on Fig 4.5, it indicates the relationship between total glucose produced is directly proportional to the pre-treatment temperature. At the lowest temperature 115 ^{o}C, the amount of glucose produced is the lowest among the temperature range which is 46 mg. As for the highest temperature at 125 ^{o}C, the yield of glucose is the highest at around 111 mg.

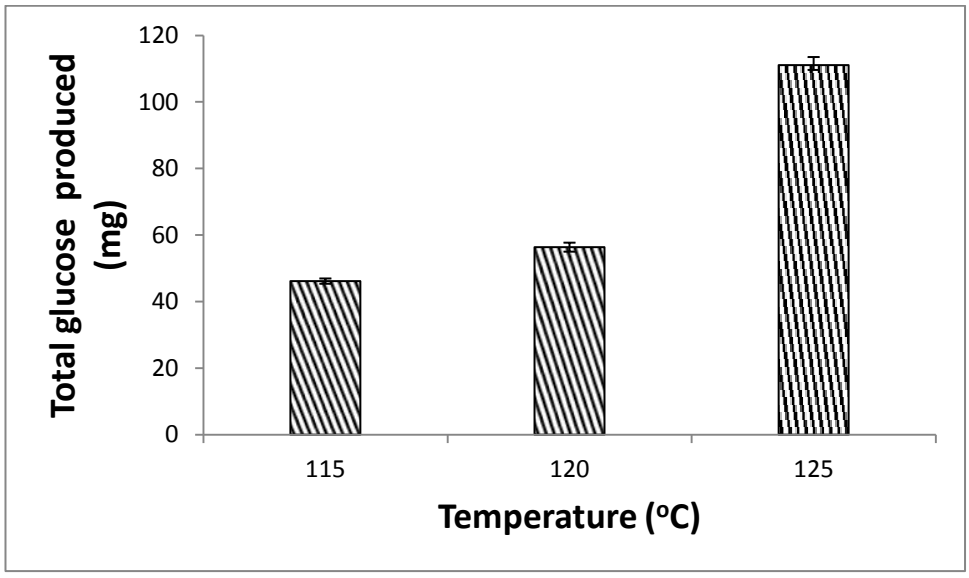


Figure 4.5: Total glucose produced from acid pre-treatment at different temperature by maintaining acid concentration and reaction time at 1.5% v/v and 45 min respectively

The HPLC chromatogram analysis of microalgae biomass treated with 1.5% v/v phosphoric acid at 125 ^{o}C for 30 min are as shown in Fig 4.6. Based on Fig 4.6, it is shown that the types of carbohydrate presence in the mixture is similar to Fig 4.4. Glucose and galactose were identified based on the HPLC chromatogram. The mixture of sugar produced were due to the partial hydrolysis of microalgae biomass using mild phosphoric acid as the hydrolysis agent. The conversion of starch and cellulose to simple sugar was enhanced by increasing reaction temperature. By increasing the temperature, the reaction constant increases. With the increase in reaction constant, the rate of hydrolysis of the carbohydrate will increase. Besides, at higher temperature, the degradation of the cell wall occurs more rapidly. The rapid degradation of cell wall allows the complex sugar to be exposed to the acid environment at a higher rate which resulted higher degree of degradation.

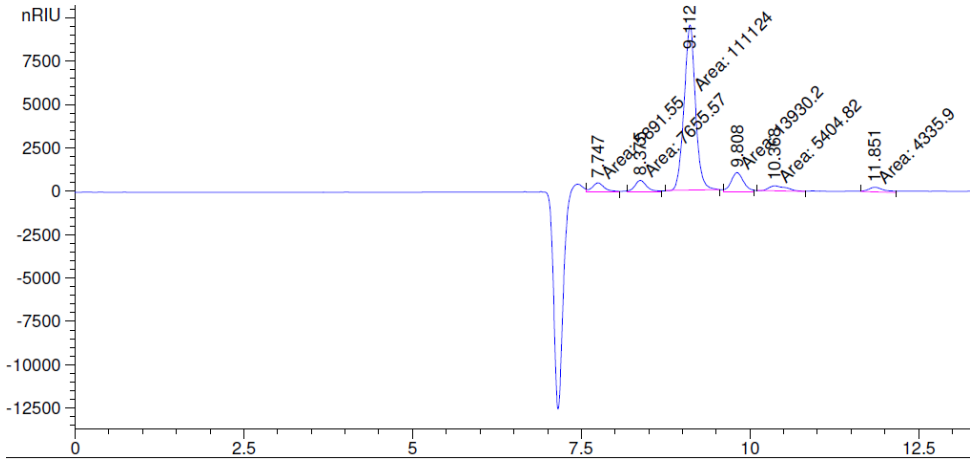


Figure 4.6: HPLC analysis for 1.5% v/v phosphoric acid treated microalgae biomass at 125 ^{o}C for 30 min

However, a further increase in temperature may not necessary result in a higher amount of glucose produced. According to Harun and Danquah (2011b), the study reported that an increase in temperature and reaction time beyond the optimum point will result in the reduction in the bioethanol yield. An extreme temperature used will causes the cell wall to degrade rapidly and the rate of reaction to be high. However, the glucose produced will be degraded as well due to high temperature (Rodrigues. et al, 2015). At extreme condition, the degraded carbohydrates such as furfural, aldehydes, vanillin and 5-hydroxymethylfurfural acts as an inhibitor which

inhibits the fermentation process (Lam & Lee, 2015). These inhibitors reduced the fermentation rate by inhibiting the conversion of fermentable sugar to bioethanol. As a result, the yield of bioethanol will be reduced. Thus, a proper selection of temperature for the pre-treatment have to be done to prevent the degradation of desired product as well as to reduce the energy consumption in providing the high temperature environment.

4.2.3 Effect of pre-treatment time on the total glucose produced

The pre-treatment time significantly affects the total glucose produced from the pre-treatment of biomass. Based on Fig 4.7, the increase in reaction time will result in an increase in the total glucose produced. The shortest pre-treatment time which is 15 min produced the lowest yield of glucose at about 50 mg. Short pre-treatment time do not provide sufficient time for the hydrolysis to occur, therefore less glucose is produced. As for 45 min pre-treatment time, the yield of glucose is significantly higher compared to 30 min and 15 min with the weight of 94 mg.

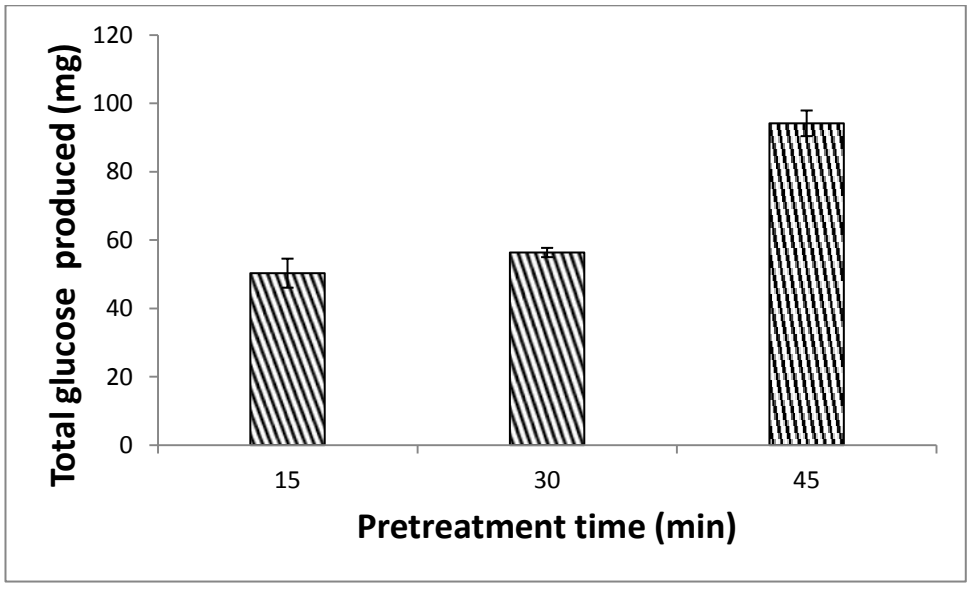


Figure 4.7: Total glucose produced from acid pre-treatment for different pre-treatment time by maintaining acid concentration and reaction temperature at 1.5% v/v and 120 ^{o}C respectively

A certain amount of time is required to cause the degradation of the cell wall before the decomposition of starch can take place. Thus, a longer pre-treatment time provides sufficient time needed for the degradation of the cell wall. In addition, longer residence time allows a higher degree of conversion of starch to simple sugar. In general, a longer pre-treatment time for the pre-treatment of microalgae biomass produces a higher amount of glucose.

From Fig 4.8, the trend is similar to the other condition for the acid pre-treatment of *S.dimorphus*. The presence of glucose was indicated by the peak at 9.813 min whereas for galactose, it is indicated by the peak at 10.524 min (Bio Rad Laboratories, 1994). At longer pre-treatment time, there are potential of decomposition carbohydrates to undesired product. However, by referring to the amount of carbohydrates indicated in Fig 4.8, the decomposition of carbohydrates have yet to occur in 45 min of pre-treatment.

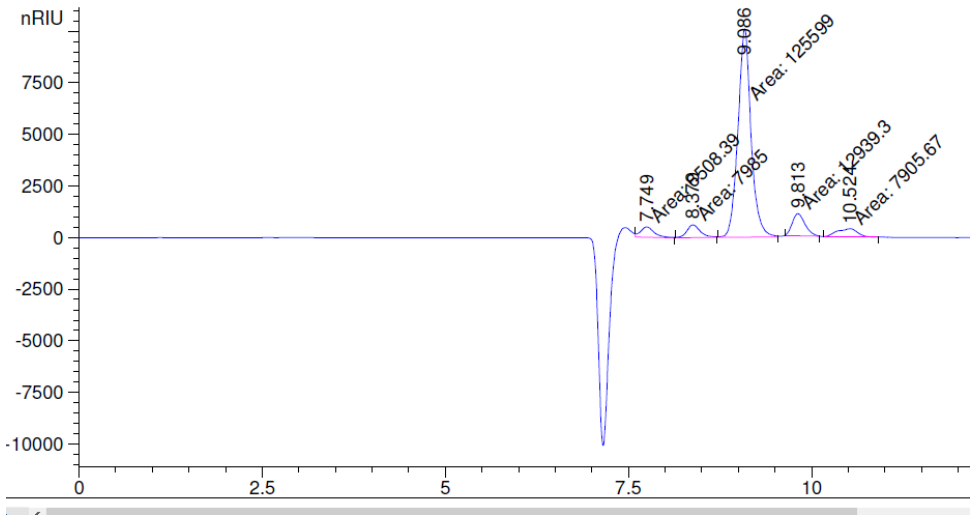


Figure 4.8: HPLC analysis for 1.5% v/v phosphoric acid treated microalgae biomass at 120 ^{o}C for 45 min

However, if the reaction time is increase further, it might not provide a significant increase in the glucose produced. According to Saha et al (2005) study, the increase in pre-treatment duration up to 60 min does not result in a higher degree of hydrolysis of wheat straw. In a matter of fact, bioethanol yield might reduced due to the degradation of carbohydrates which is no longer consumable by yeast. Besides, a longer pre-treatment time means a longer cycle time. A longer pre-treatment may

result in a high amount of glucose produced, but the production rate will be reduced. On the other hand, a short cycle time produce a high production rate, but the yield of glucose will be low. Therefore a suitable time for the pre-treatment is required to maximised the yield of desired product, minimised the cycle time and carry out the pre-treatment process at economical rate.

4.3 Fermentation for 24 hr

4.3.1 Effect of acid concentration on the total bioethanol produced

Based on Fig 4.9, the trend of total bioethanol produced increases with the increases in acid concentration. The lowest amount of bioethanol was found at concentration of 0.5% v/v where the amount of bioethanol produced and conversion achieved are 36 mg and 26.5% respectively. The conversion calculated were based on the total carbohydrates content within *S.dimorphus* biomass. The highest amount of bioethanol was produced at 2.5% v/v of phosphoric acid. The amount of bioethanol produced is approximately 128 mg whereas the conversion achieved based on equation 1 (Chapter 3) is 94%. On dry weight basis, the bioethanol yield is about 0.128 g bioethanol/ g biomass.

The reason for the increased in amount of bioethanol produced is due to the presence of higher amount of fermentable sugar. The trend observed in Fig 4.9 can be explained through the glucose content from the pre-treatment of microalgae. By referring to Fig 4.3, it can be observed that the glucose content increases with increase in acid concentration. Glucose is one of the favourite carbohydrates that can be easily utilized by yeast for fermentation. Thus, the amount of bioethanol produced increases with acid concentration due to the increase in glucose presence.

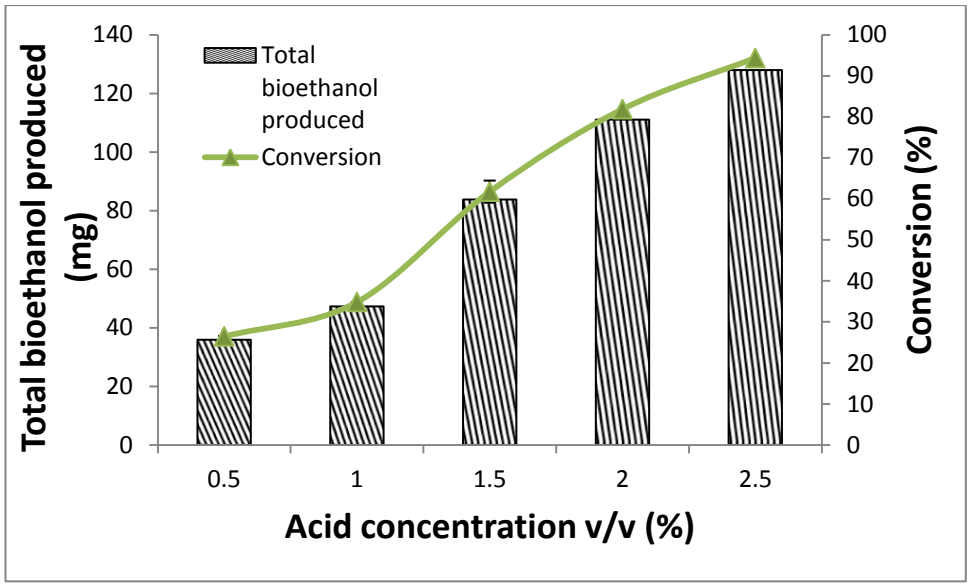


Figure 4.9: Total bioethanol produced and conversion achieved with different acid concentration by maintaining pre-treatment temperature and reaction time at 120 ^{o}C and 30 min (fermentation time 24 hr)

Besides glucose, there are also other carbohydrates that are being utilized for bioethanol production as well. Referring to Fig 4.10 and compared to Fig 4.4, it can be observed that the amount of galactose and glucose decreased significantly. The bioethanol produced were indicated by the peak at time 22.213 min (Bio Rad Laboratories, 1994). An assumption can be made that galactose was also being utilize by *S.diastaticus* for fermentation aside from glucose. Thus, the products from partial hydrolysis of microalgae can also be used for fermentation process depending on the type of yeast used. The peak at 9.913 min which is the product from the hydrolysis of microalgae biomass remains constant as well after 24 hr of fermentation.

For *S.cerevisiae*, it is highly depending on glucose for determining the bioethanol yield as this species only consumes simple sugar such as glucose. The bioethanol yield is based on the total glucose presence in the sample. However in this study, *S.diastaticus* was used as the yeast to replace *S.cerevisiae* for fermentation process. Based on the consumption of glucose and galactose, it can be deduced that *S.diastaticus* is a type of yeast that can consumes various type of simple sugar as food source. It is proven by the observation in the reduction of galactose and glucose

from the HPLC chromatogram shown in Fig 4.10 after 24 hr of fermentation. *S.diastaticus* is a type of yeast that capable of fermenting starch (Gilliland et al, 1966). It is proven that *S.diastaticus* can ferment starch to bioethanol because the amount of bioethanol produced is higher compared to the available glucose in the pre-treated sample. Therefore, the yield of bioethanol will be based on the total carbohydrates presence in the biomass instead of based on the glucose content.

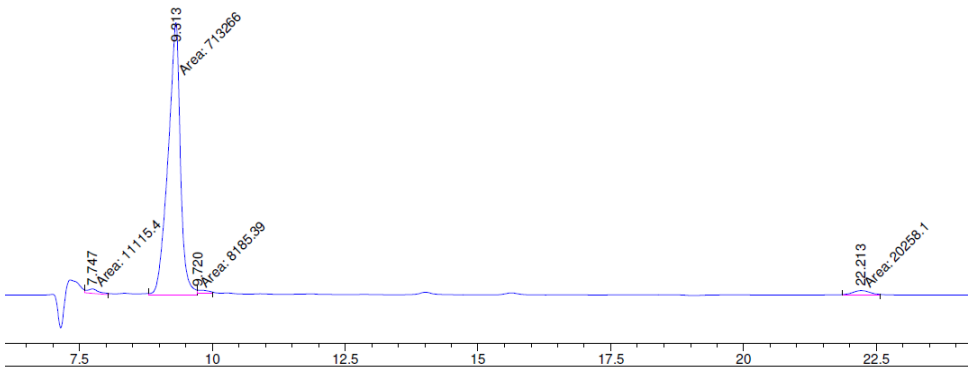


Figure 4.10: HPLC analysis for 24 hr of fermentation of pre-treated microalgae biomass with 2.5% v/v phosphoric acid at 120 ^{o}C for 30 min

The main purpose of phosphoric acid in this study is to convert the insoluble sugar to soluble sugar while at the mean time, partially hydrolysed complex sugar to produce simple sugar. By coordinating with yeast, *Saccharomyces diastaticus*, the bioethanol yield produced is high and contestable with yield produced from sulphuric acid pre-treatment. Besides, the use of mild acid concentration is more environmental friendly and the corrosion of the process equipments will be reduced.

4.3.2 Effect of pre-treatment temperature on the total bioethanol produced

Similar to the trend for different acid concentration, the trend for the total bioethanol produced increases with the increase in pre-treatment temperature as observed in Fig 4.11. The minimum bioethanol produced at 115 ^{o}C is about 59 mg whereas the conversion achieved is around 44%. At treatment temperature of 125 ^{o}C, the

bioethanol produced is the highest which is approximately 126 mg and the conversion is 93%.

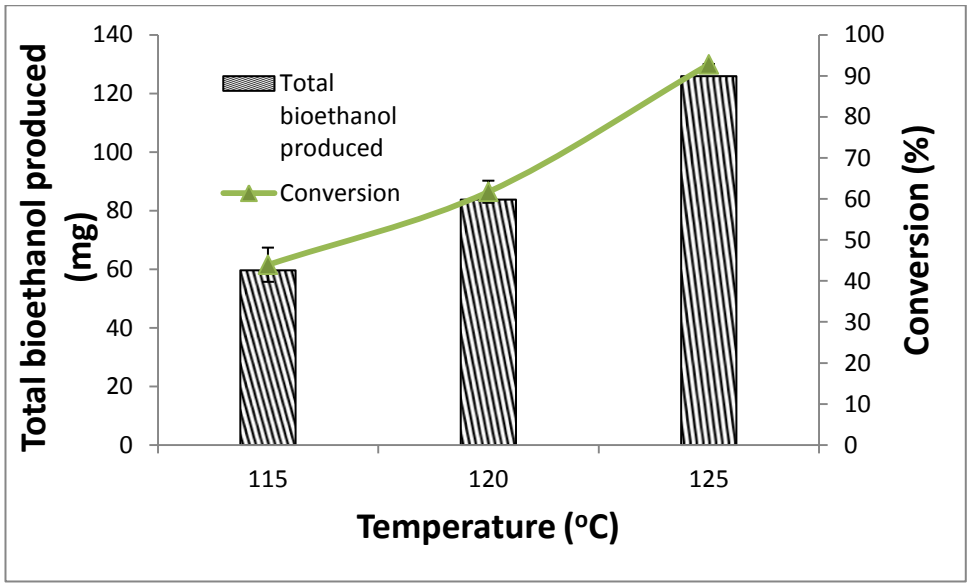


Figure 4.11: Total bioethanol produced and conversion achieved at different temperature by maintaining acid concentration and reaction time at 1.5 % v/v and 30 min respectively (fermentation time 24 hr)

The reason for the increase in the amount of bioethanol produced is the same with the effect of acid concentration. The total glucose and fermentable sugar content presence is greater at higher temperature. The higher degree of hydrolysis achieved at elevated temperature resulted in greater amount of fermentable sugar produced which increases the bioethanol produced.

By referring to Fig 4.12, the amount of galactose and glucose as observed at time 9.8 min and 10.37 min in the HPLC chromatogram respectively decreases significantly compared to Fig 4.6. It indicates that these carbohydrates were being utilized by *S.diastaticus* for fermentation which resulted in high amount of bioethanol produced.

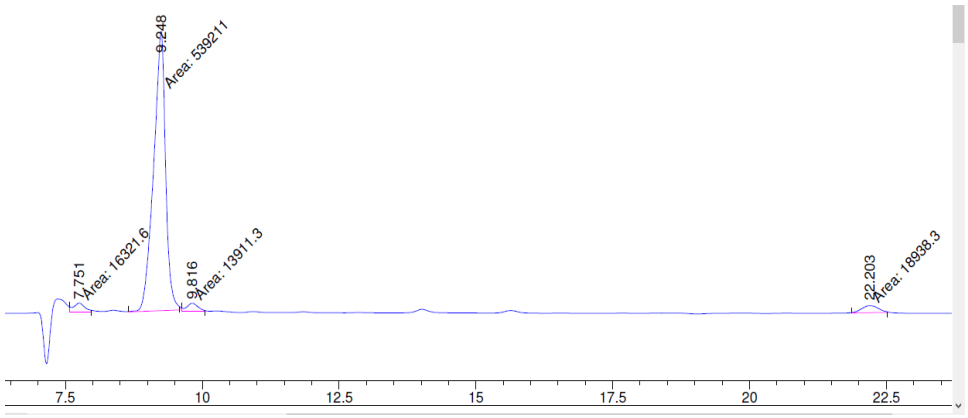


Figure 4.12: HPLC analysis for 24 hr of fermentation of pre-treated microalgae biomass with 1.5% v/v phosphoric acid at 125 ^{o}C for 30 min

Based on the temperature factor, the increase in temperature indicates a higher degree of hydrolysis or decomposition of carbohydrates achieved. The amount of insoluble sugar converted to soluble sugar increases as well. Thus, resulting in higher amount of fermentable sugar presence in the medium for yeast to consume. As a result, the amount of bioethanol produced increase. The high yield of bioethanol produced further proof that 125 ^{o}C does not causes any degradation of the carbohydrates to undesired products. Besides, the use of phosphoric acid to pre-treat *S.dimorphus* biomass at lower temperature environment indicates that less energy is required for the reaction, thus saving cost. Thus, the use of mild temperature is also suitable for the pre-treatment of microalgae while producing high yield of bioethanol.

4.3.3 Effect of pre-treatment time on the total bioethanol produced

Based on the Fig 4.13, the total bioethanol produced increases with the increase in pre-treatment time. The lowest bioethanol produced is about 49 mg where the conversion is 36% for 15 min of acid pre-treatment. As for the highest amount of bioethanol produced, the amount is about 118 mg and the conversion achieved is 94%.

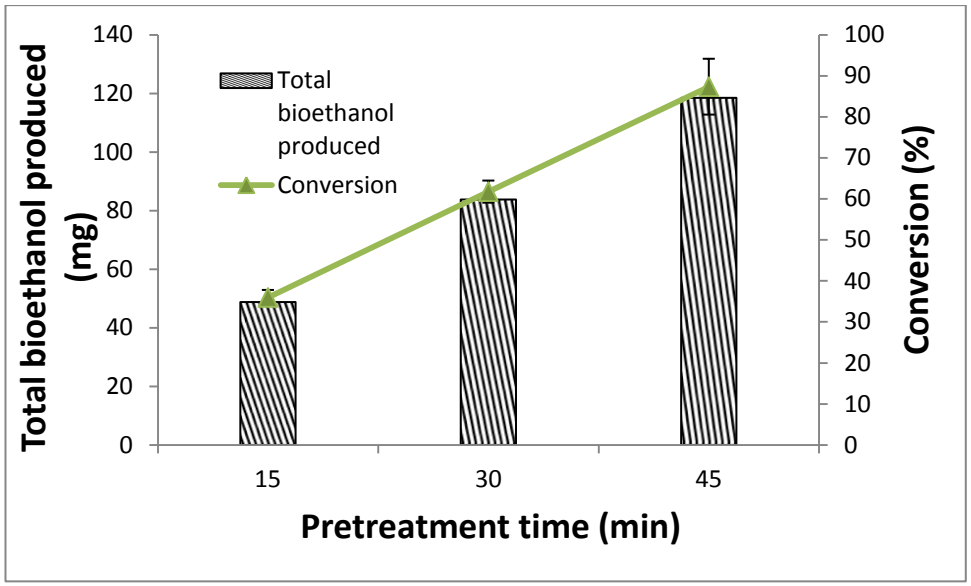


Figure 4.13: Total bioethanol produced and conversion achieved for different time by maintaining acid concentration and pre-treatment temperature at 1.5 % v/v and 120 ^{o}C (fermentation time 24 hr)

The bioethanol content increases with prolonged pre-treatment time due to the increases of glucose content amount. The increase in fermentable sugar resulted in higher conversion of fermentation, thus yield higher amount of bioethanol. By comparing the HPLC chromatogram of Fig 4.8 with Fig 4.14, it indicates a significant reduction in the glucose and galactose content. The peak at 22.205 min shown in Fig 4.14 indicates the production of bioethanol after 24 hr of fermentation.

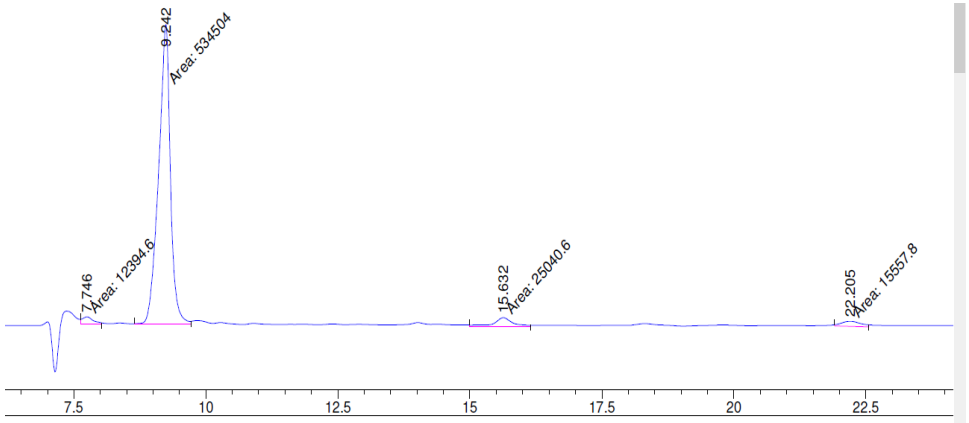


Figure 4.14: HPLC analysis for 24 hr of fermentation of pre-treated microalgae biomass with 1.5% v/v phosphoric acid at 120 ^{o}C for 45 min

As discuss previously, the increase in pre-treatment time allows more conversion of starch to glucose. Thus, the amount of fermentable sugar that available for conversion is high. As a result, the yield of bioethanol produced is high. However, when a longer reaction time is set, the degradation of carbohydrates to unwanted product will occur. Based on the bioethanol yield obtained, 45 min of pre-treatment time is yet to cause the degradation to carbohydrates.

It is well-known that the use of sulphuric acid to pre-treat microalgae will produce inhibitors especially at high temperature and prolonged reaction time which is capable of inhibiting the fermentation process (Bensah & Mensah, 2013). However, based on the bioethanol yield achieved, it can be deduced that production of inhibitors does not occurred. The absence of inhibitors allows high conversion to be achieved during the fermentation process (Ho et al, 2013). Therefore, phosphoric acid hydrolysis is suitable for the pre-treatment of microalgae with a suitable reaction time set in order to produce high amount of bioethanol.

4.4 Fermentation for 48 hr

The fermentation process is allowed to proceed for 48 hr and the respective bioethanol content were measured. Based on the comparison of 48 hr and 24 hr of fermentation as shown in Fig 4.15, Fig 4.16 and Fig 4.17, the bioethanol content were generally reduced significantly in amount. The main reason for the significant reduction in bioethanol content is that *S.diastaticus* started to utilize bioethanol as its carbon source in order to sustain its life. The reduction in bioethanol suggested that the depletion of glucose in the medium causes the starvation of the yeast which direct the yeast to consume bioethanol as its food source in order to sustain its growth.

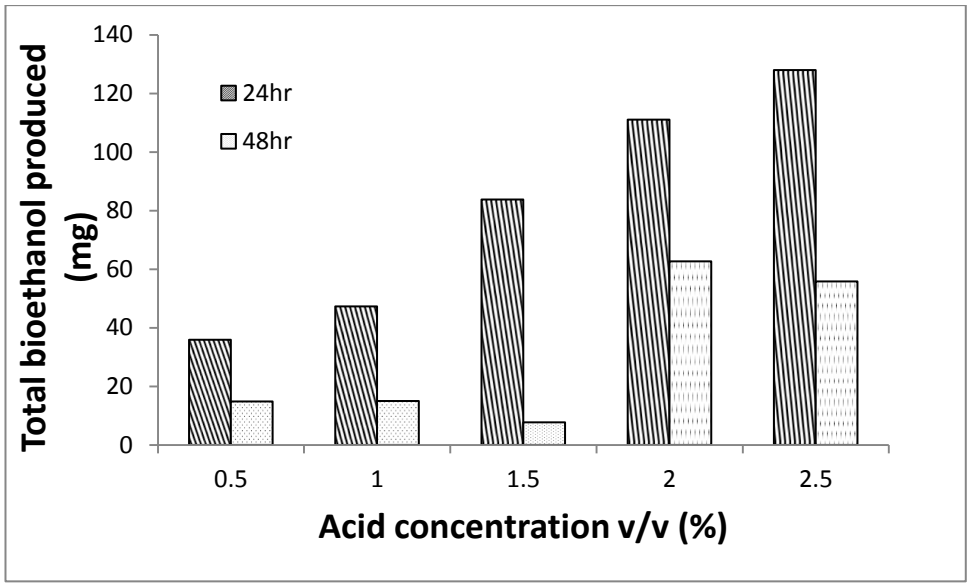


Figure 4.15: Comparison of total bioethanol produced with different acid concentration for 24 hr and 48 hr of fermentation

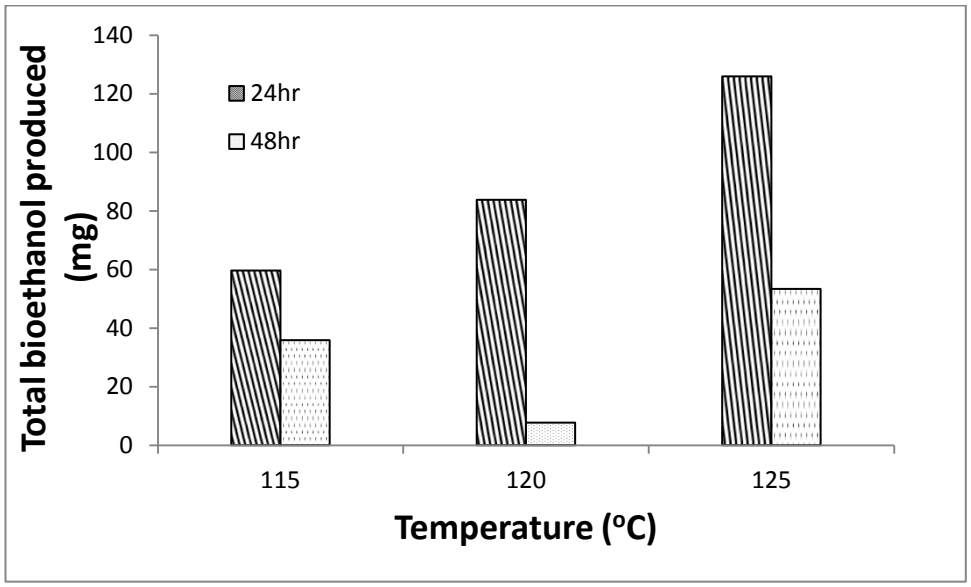


Figure 4.16: Comparison of total bioethanol produced at different temperature for 24 hr and 48 hr of fermentation

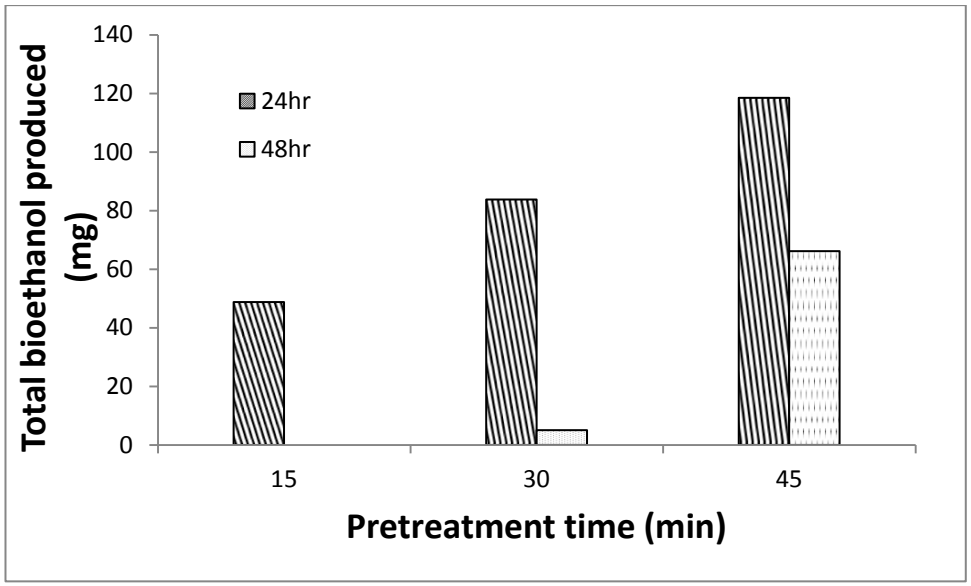


Figure 4.17: Comparison of total bioethanol produced at different time for 24 hr and 48 hr of fermentation

At the beginning of fermentation process, glucose and other fermentable sugar were rapidly consumed by the yeast to produce bioethanol. Up to a point, the fermentable sugar presence in the mixture is very low in content. In order for the biological growth of the yeast to be sustained, *S.diastaticus* started to consume bioethanol as its food source, thus explaining the decrease in the amount of bioethanol at 48hr of fermentation compared to 24 hr. This phenomenon was also appeared in Harun and Danquah (2011) studies, where the bioethanol content were in a reducing trend after 30 hr of fermentation.

From the result obtained, it can be concluded that *S.diastaticus* is a type of yeast which can utilise bioethanol as carbon source as well. Thus, if the fermentation were set to be beyond the optimum duration, the bioethanol will be consumed and result in a decrease in bioethanol yield.

Based on Fig 4.18, it indicates that glucose and galactose were no longer found in the sample. Besides, by comparing Fig 4.14 and Fig 4.18 at time 22.2 min, it shows that the amount of bioethanol presence in the sample have decreased significantly as well. This shows that *S.diastaticus* is capable of consuming bioethanol as well as a food source when the medium have limited carbon source.

Therefore, a suitable fermentation time should be set in order to prevent bioethanol from being consumed by the yeast.

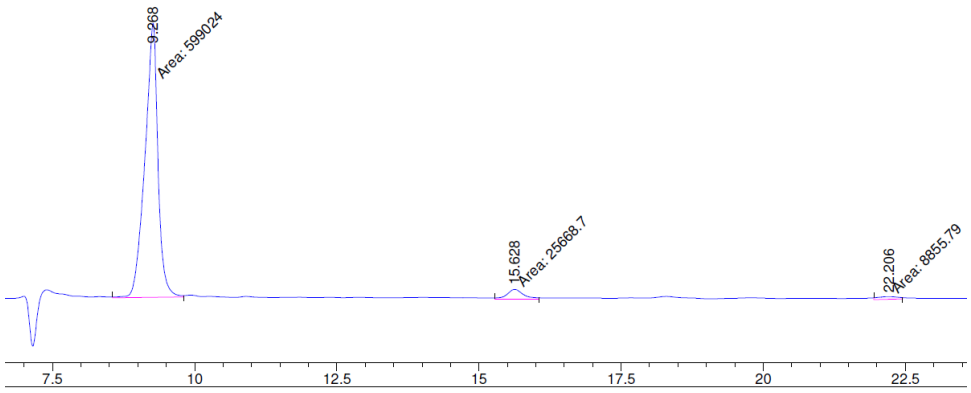


Figure 4.18: HPLC analysis for 48 hr of fermentation of pre-treated microalgae biomass with 1.5% v/v phosphoric acid at 120 ^{o}C for 45 min

4.5 Comparison of the method used in this study for bioethanol production from microalgae with other studies

The highest yield of total glucose produced from the pre-treatment of *S.dimorphus* is 111 mg with the use of phosphoric acid concentration of 1.5% v/v, treated at 125 ^{o}C for 30 min. The conversion achieved based on this parameter is approximately 42%.

Based on Table 4.2, chemical hydrolysis using sulphuric acid and enzymatic hydrolysis using α-amylase can achieve high conversion of complex sugar to simple sugar during the pre-treatment. It is well known that sulphuric acid is a strong acid can cause rapid degradation of cell wall and high degree of hydrolysis of carbohydrates to produce large amount of glucose. However, the drawback of sulphuric acid is that the production of inhibitors will affect the bioethanol yield.

Table 4.2: Comparison of Conversion Achieved via Different Hydrolysis Methods for the Pre-treatment of Microalgae in Various Studies

Hydrolysis method	Microalgae	Glucose Conversion (%)	Bioethanol Yield	Reference
Chemical				
H_2SO_4	*Scenedesmus obliquus*	71-97	-	Miranda et al (2012)
H_2SO_4	*Scenedesmus bijugatus*	84	0.158g bioethanol/ g dry biomass	Ashokkumar et al (2015)
H_2SO_4	*C. vulgaris*	95	11.66g/L	Ho et al (2013b)
H_3PO_4	*Scenedesmus dimorphus*	42	0.126g bioethanol/ g dry biomass	This study
Enzymatic				
α-amylase	*Chlamydomonas reinhardtii*	94	11.73g/L	Choi et al (2010)

In this study, phosphoric acid is chosen as the chemical used to hydrolyse the *S.dimorphus*. Based on the conversion achieved, it can be deduced that the degree of hydrolysis is comparatively lower than other methods. However, the cellulose have been broken down as some of the carbohydrate content within the cell is converted to glucose. Based on the glucose content, it can be conclude that mild phosphoric acid concentration could only partially hydrolysed the microalgae.

However, in this study *Saccharomyces diastaticus* was used as the fermentation yeast. The highest bioethanol yield produced is at about 94% based on equation (1). The high bioethanol yield achieved is due to the production of fermentable sugar by phosphoric acid pre-treatment which is consumable by *S.diastaticus*. The high yield of bioethanol produced indicated that *S.diastaticus* is able to provide high conversion rate of fermentable sugar to bioethanol. Thus, *S.diastaticus* is a highly potential yeast as it can consumes complex sugar during fermentation to produce bioethanol. The coordination with mild acid pre-treatment can result in high yield of bioethanol with the use of *S.diastaticus*.

Besides, another advantageous properties of using phosphoric acid is that when it is neutralised with sodium hydroxide, it produces sodium phosphate which is a type of nutrient for yeast. Thus it boost up the growth rate of *S.diastaticus* which will result in a higher fermentation rate. The use of mild acid to pre-treat microalgae is more environmental friendly as it produce less corrosive waste, whereas the life-cycle of process equipments are extended due to lower risk of corrosion. The cost of using lower concentration acid is cheaper as well.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

S.dimorphus consist of high amount of carbohydrates content which is one of the potential biomass for the production of bioethanol. The total starch content of *S.dimorphus* is about 28±0.5% w/w on dry basis. The total carbohydrate content of *S.dimorphus* is about 44±0.5% w/w on dry basis.

The effect of acid concentration, pre-treatment temperature and pre-treatment time were studied to analysed the effect on the acid pre-treatment of *S.dimorphus*. For an increase in phosphoric acid concentration from 0.5% v/v to 2.5% v/v, the amount of glucose and fermentable sugar produced increases. The highest amount of glucose produced is 90 mg when 2.5% v/v of phosphoric acid is used. For the trend of pre-treatment temperature, the total glucose produced increases with the pre-treatment temperature. The highest amount of glucose is 111 mg when *S.dimorphus* was pre-treated at 125 ^{o}C with 1.5% v/v of phosphoric acid for 30 min.

Whereas for the pre-treatment time, the longer the reaction time, the degree of hydrolysis achieved is greater. The highest glucose produced was at the pre-treatment time of 45 min where the amount is approximately 94 mg. Mild phosphoric acid is capable of producing partial hydrolysis of carbohydrates and the degradation of the cell wall. In addition, it has various advantageous properties such as less toxic and less corrosive compared to sulphuric acid. Thus, phosphoric acid is a potential acid which can be used for the pre-treatment of microalgae.

In this study, *S.diastaticus* instead of the conventional yeast *S.cerevisiae* was used as fermentation microbe. The yield of bioethanol produced from the pre-treated

microalgae is considerably high. Based on the results and data analysed, *S.diastaticus* can utilize complex sugar as well during the fermentation process.

The highest amount of bioethanol produced is at 2.5% v/v phosphoric acid treated at 120 ^{o}C for 30 min. The amount of bioethanol produced is around 128 mg whereas the conversion achieved based on equation (1) is 94%. On dry weight basis, the bioethanol yield is about 0.128 g bioethanol/ g *S.dimorphus*.

The high bioethanol yield produced in this study indicates that *S.diastaticus* is potential as fermentation microbe for the fermentation process. The coordination with mild acid pre-treatment process can avoid the need of concentrated acid and high temperature reaction which has the risk in forming inhibitors.

5.2 Recommendation

The need of the study on the effective production of bioethanol from microalgae is necessary as microalgae is capable of replacing crops as a raw material. Besides, bioethanol is a clean fuel which is being used in the various country in order to reduce the reliance on crude oil and mitigates global warming. Thus, a cost effective method to produce bioethanol from microalgae is a vital information to produce bioethanol at an economical scale.

Scenedesmus dimorphus is a type of microalgae which has high potential to be used as the raw material for the production of bioethanol because it contain high amount of carbohydrates within the cell. The carbohydrate can be utilized for the production of bioethanol which helps to reduce the global pollution. A more detailed study on the cell structure and nature of *S.dimorphus* will further determine whether it is suitable for bioethanol production in industrial scale. Besides, the cultivation of this species needs to be studied as well.

Aside from the characterization on the biomass, the determination of an optimum condition for the pre-treatment of microalgae is essential in order to industrialised this process. Further studies needed to be conducted based on various factor to aid the proper selection of an optimum condition for the pre-treatment of

microalgae. A suitable acid concentration, optimum temperature and sufficient reaction time will provide a high yield of desired products whereby the cost for the pre-treatment process can be optimized as well. The study on the effectiveness of phosphoric acid can also be done by conducting further studies on the end product produced from the acid pre-treatment.

The capability of *S.diastaticus* need to be analysed further to determine the amount and type of complex sugar that the yeast can consume. The determination of whether there is any presence of side products produced during the fermentation process has to be done as well. In addition, *S.diastaticus* is able to consume bioethanol when the carbon source in the medium is very low. Thus, the rate of fermentation by *S.diastaticus* needs to be determined in order to have a proper set of fermentation duration to minimize the consumption of bioethanol. The limitation of *S.diastaticus* such as chemical resistance, temperature resistance, pH resistance and others need to be determined as well before it is able to be used as a industrial yeast for fermentation process. In summary, further research is a must to establish a cost effective and sustainable technology in production of bioethanol from microalgae biomass.

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APPENDICES

APPENDIX A : HPLC Chromatography

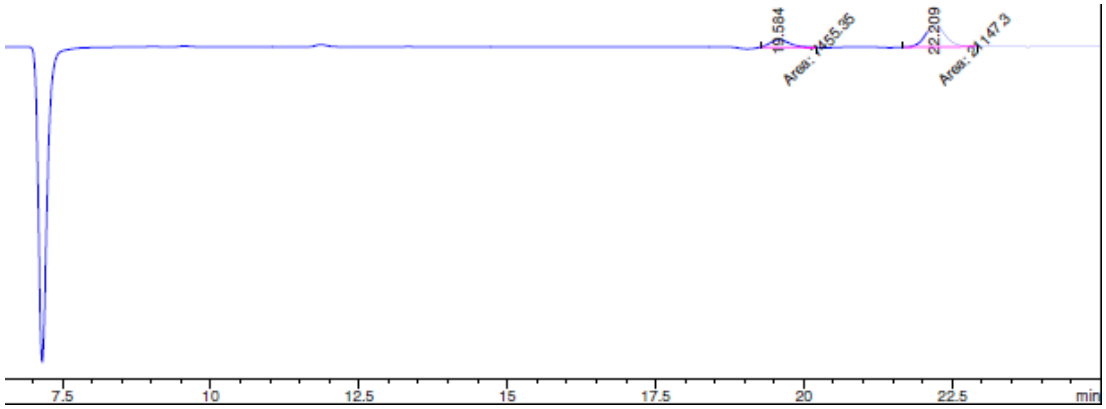

Figure A.1: HPLC chromatography of ethanol standard at concentration of 0.6 mg/mL

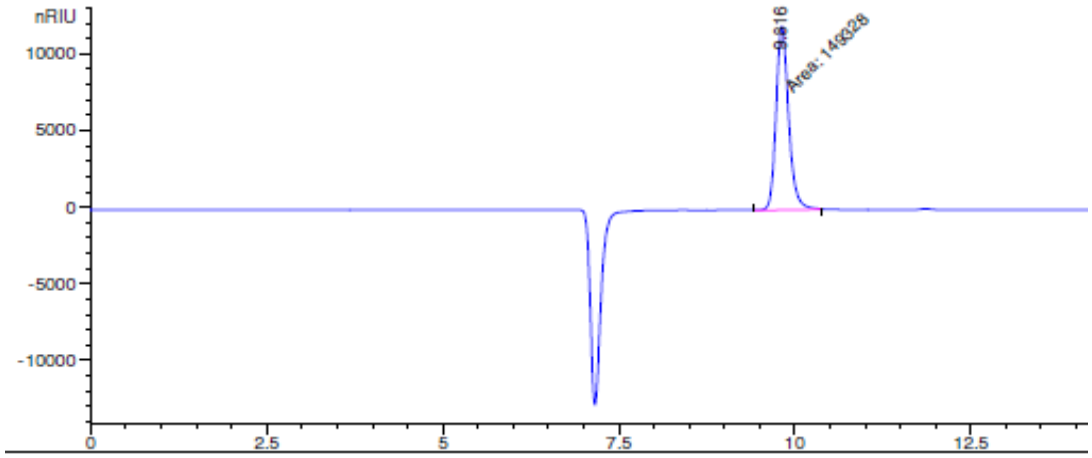

Figure A.2: HPLC chromatography of glucose standard at concentration of 0.6 mg/mL

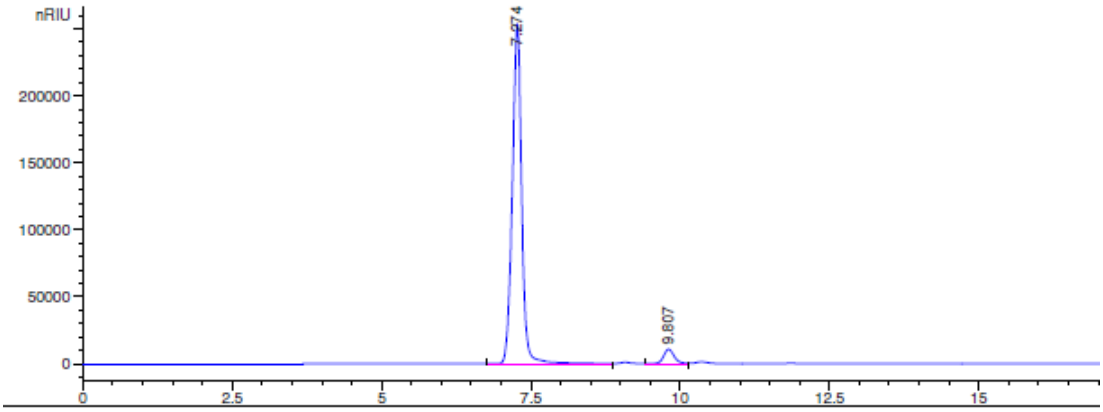

Figure A.3: HPLC chromatography of biomass treated with H_2SO_4 for the total carbohydrates quantification

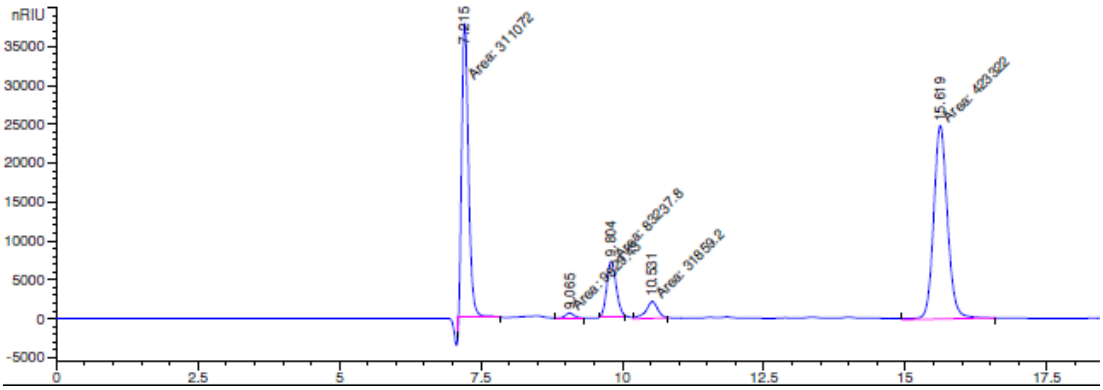

Figure A.4: HPLC chromatography of the biomass treated with amyloglucosidase for the total starch quantification

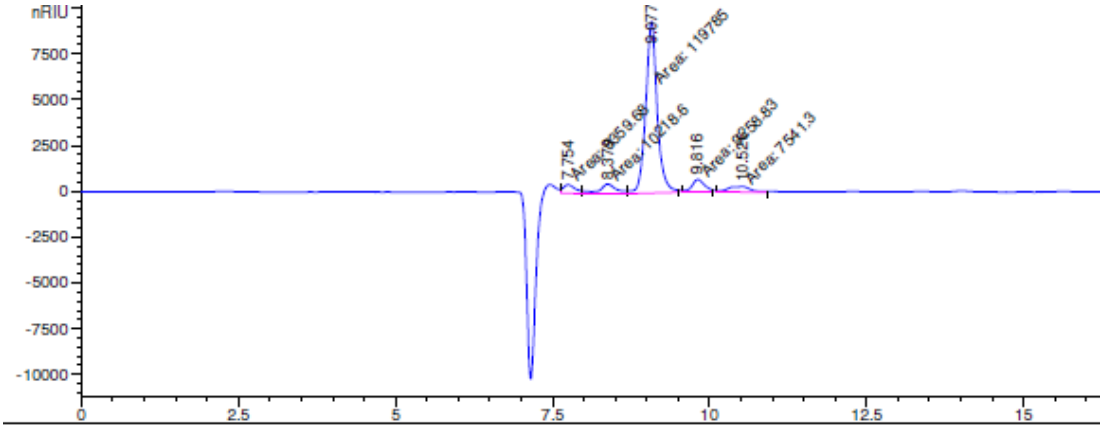

Figure A.5: HPLC chromatography of the biomass treated with 1.5% v/v phosphoric acid at 120 ^{o}C for 30 min

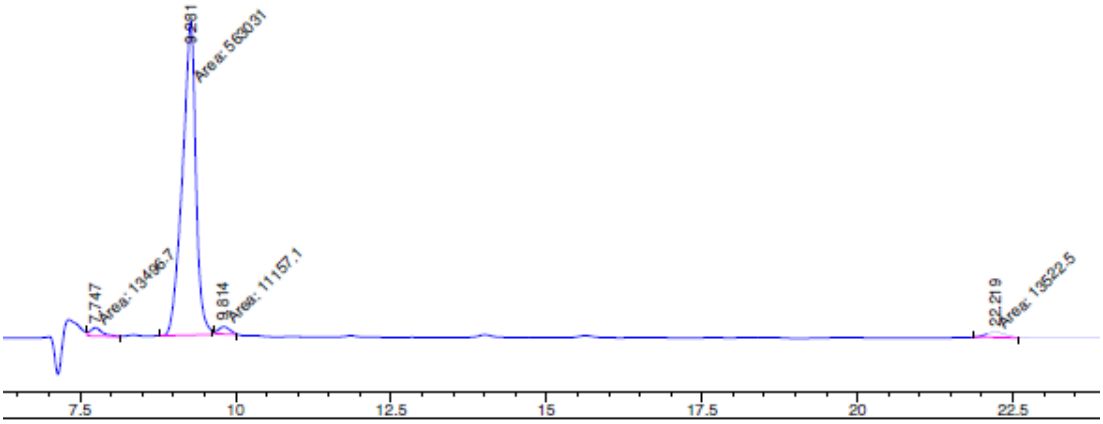

Figure A.6: HPLC chromatography for 24 hr of fermentation of biomass treated with 1.5% v/v phosphoric acid at 120 ^{o}C for 30 min

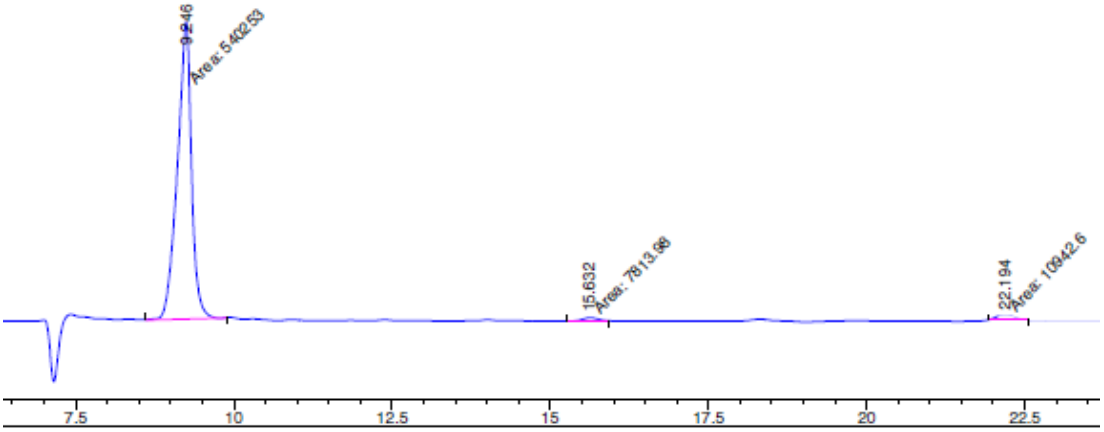

Figure A.7: HPLC chromatography for 48 hr of fermentation of biomass treated with 1.5% v/v phosphoric acid at 120 ^{o}C for 30 min

APPENDIX B : Graph of the standards for ethanol and glucose

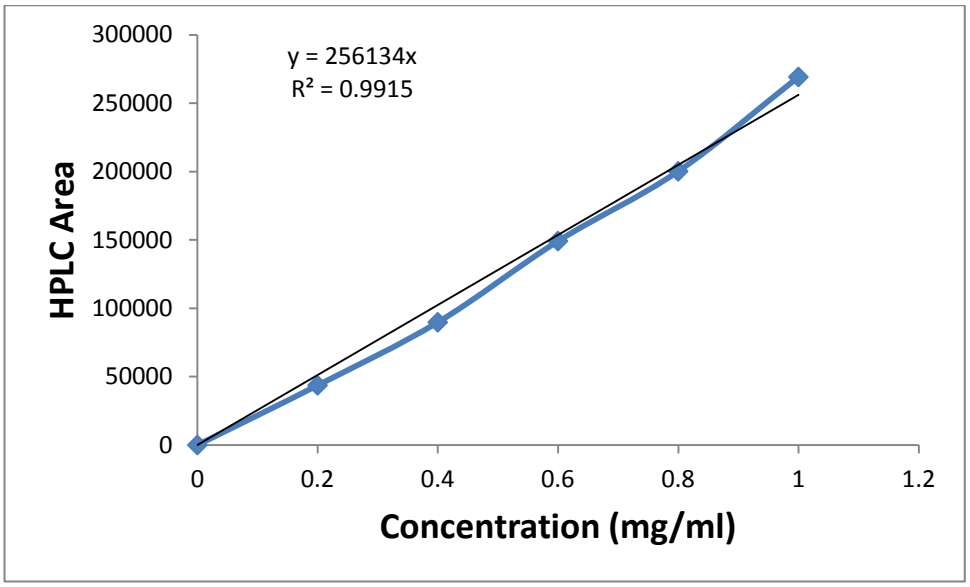

Figure A.8: HPLC area versus the concentration of glucose

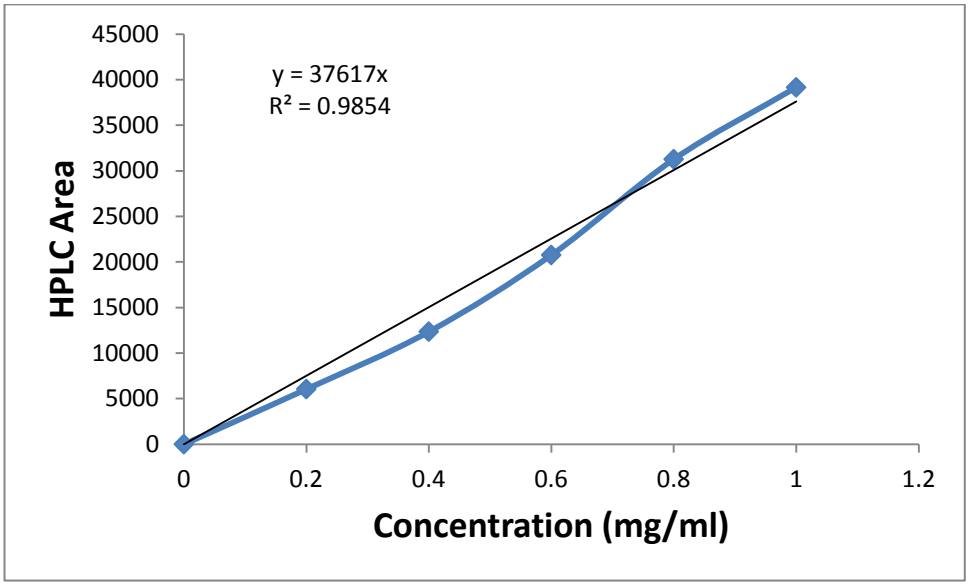

Figure A.9: HPLC area versus the concentration of ethanol

Appendix C : Sample calculation for total starch content

$Biomass\ sample\ weight = 0.1g$

$Gradient\ of\ glucose\ standard = 256134$

$$Concentration\ of\ glucose = \frac{Area\ from\ HPLC}{256134}$$

$Area\ from\ HPLC = 93148.7$

$$Concentration\ of\ glucose = \frac{93148.7}{256134} = 0.364mg/ml$$

$Initial\ concentration\ of\ glucose\ before\ diluting$

$$= 0.364 * \frac{10mL}{1mL} = 3.64mg/ml$$

$Total\ glucose\ content$

$= concentration * total\ volume$

$$= \frac{3.64mg}{ml} * 11.5ml = 41.82mg$$

$Average\ glucose\ content\ in\ amyloglucosidase = 11.99mg$

$Total\ glucose\ content\ of\ biomass$

$= Total\ glucose\ content - Average\ glucose\ content\ in\ amyloglucosidase$

$Total\ glucose\ content\ of\ biomass = 29.83mg$

$Total\ starch\ content = 29.83mg$

$Average\ of\ run\ A\ \&\ run\ B\ starch\ content = 27.8mg$

$Total\ starch\ content\ in\ biomass \approx 28\ wt\%$

Appendix D : Sample calculation for total carbohydrate content

$$Biomass\ sample\ weight = 0.3g$$

$$Gradient\ of\ glucose\ standard = 256134$$

$$Concentration\ of\ glucose = \frac{Area\ from\ HPLC}{256134}$$

$$Area\ from\ HPLC = 128324$$

$$Concentration\ of\ glucose = \frac{128324}{256134} = 0.501\ mg/ml$$

$$Initial\ concentration\ of\ glucose\ before\ diluting$$

$$= 0.501 * \frac{10mL}{5mL} = 1.002\ mg/ml$$

$$Total\ glucose\ content = concentration * total\ volume$$

$$= \frac{1.002\ mg}{ml} * 87ml = 87.17\ mg$$

$$Conversion\ of\ glucose \approx 68\%$$

$$Actual\ total\ glucose\ content = \frac{87.17}{0.68} = 128.2\ mg$$

$$Average\ of\ run\ A\ \&\ run\ B\ carbohydrate\ content\ from\ biomass$$

$$= 132.72\ mg$$

$$Total\ carbohydrate\ content\ in\ biomass \approx \frac{132.72}{300} * 100\% \approx 44\%$$

Appendix E : Sample calculation for glucose produced from acid pre-treatment

Glucose content determination for microalgae biomass pre-treated with 1.5% v/v phosphoric acid at 120^{o}C for 30 min

$$Biomass\ sample\ weight = 1\ g$$

$$Gradient\ of\ glucose\ standard = 256134$$

$$Concentration\ of\ glucose = \frac{Area\ from\ HPLC}{256134}$$

$$Area\ from\ HPLC = 7040.42$$

$$Concentration\ of\ glucose = \frac{7040.42}{256134} = 0.0275\ mg/ml$$

$$Initial\ concentration\ of\ glucose\ before\ diluting$$

$$= \ 0.501 * \frac{4.1mL}{0.1mL} = 1.127\ mg/ml$$

$$Total\ glucose\ content$$

$$= concentration * total\ volume$$

$$= \frac{1.127\ mg}{ml} * 50ml = \ 56.35\ mg$$

Appendix F : Sample calculation for bioethanol produced from 24 hr fermentation of acid pre-treated microalgae biomass

Bioethanol content determination for microalgae biomass pre-treated with 1.5% v/v phosphoric acid at 120^{o}C for 30 min

$Biomass\ sample\ weight = 1\ g$

$Gradient\ of\ bioethanol\ standard = 37617$

$$Concentration\ of\ bioethanol = \frac{Area\ from\ HPLC}{37617}$$

$Area\ from\ HPLC = 13409$

$$Concentration\ of\ bioethanol = \frac{13409}{37617} = 0.356\ mg/ml$$

$Initial\ concentration\ of\ bioethanol\ before\ diluting$

$$= 0.356 * \frac{5mL}{1mL} = 1.78\ mg/ml$$

$Total\ bioethanol\ content$

$= concentration * total\ volume$

$$= \frac{1.78\ mg}{ml} * 47ml = 83.77\ mg$$

$Total\ carbohydrate\ content\ in\ biomass = 442mg$

$30mL\ out\ of\ 50mL\ are\ extracted\ from\ sampel\ for\ fermentation$

$$Total\ carbohydrate\ content\ in\ 30mL = \frac{30}{50} * 442 = 265.2mg$$

$Bioethanol\ yield$

$$= \frac{bioethanol\ content}{0.511 * total\ sugar} * 100\%$$

$$= \frac{83.77}{0.511 * 265.2} * 100\% = 61.8\ \%$$