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評估以石蓴水解物生產生質乙醇

Evaluation of Bioethanol Production from *Ulva lactuca* Hydroysate

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本論文係 馬丹利 君（學號R05642013）在國立臺灣大學生物科技研究所完成之碩士學位論文，於民國 107 年 7 月 2 日承下列考試委員審查通過及口試及格，特此證明

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Abstract

As the effects of climate change continues to plague the world as a result of our continual use of one of the chief contributor's 'fossil fuels', an alternative to this source of fuel should be developed. Bioethanol is the ideal substitute as it is made up from the fermentation of organic substrates. In this study, *Ulva lactuca* was used as a feedstock for bioethanol production using a combination of acidic pretreatment and enzymatic hydrolysis; followed by fermentation strategies to determine the effects of agitation, addition of nitrogen sources, substrate loading, temperature, pH on monocultures using *Kluyveromyces marxianus* K-21 (K-21) and *Pichia stipitis* BCRC 21777 (BCRC 21777) and a co-culture. The results of the agitation experiments revealed that static culture produced slightly higher ethanol (7.8±0.4 g/L) than agitations at 50 rpm and 150 rpm (6.10±0.7 g/L and 5.90±0.23 g/L) for K-21 and static culture, 50, 150 rpm for *Pichia stipitis* was 5.21±0.37 g/L, 4.90±0.19 g/L, and 4.80±0.35 g/L respectively. 10% substrate loading was found to be the optimum for both yeast K-21 and BCRC 21777 with ethanol concentrations of 10.60 ± 0.31 g/L and 6.8±0.2 g/L was obtained at optimized fermentation conditions of 35^{o}C, static culture and a pH of 6. For the result of the co-culture a total amount of 11.5±0.7 g/L bioethanol was produced. The results of this research proposes that *Ulva lactuca* could be used as a feedstock for bioethanol production.

Keywords:

Lignocellulosic material; *Ulva lactuca*; co-fermentation; *Kluyveromyces marxianus* K-21, *Pichia stipitis* BCRC 21777

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1. Introduction

Fossil fuel is a general term for buried combustible geologic deposits of organic materials, formed from decayed plants and animals that have been converted to crude oil, coal, natural gas, or heavy oils by exposure to heat and pressure in the earth's crust over hundreds of millions of years. Most carbon emissions in the atmosphere come from the burning of fossil fuels, such as coal, oil, and natural gas, which together supply an overwhelming share (more than 80%) of the world's commercial energy. Thus, stabilizing the climate implies reducing the emissions from fossil fuel combustion and switching to alternative forms of fuels.

Bioethanol is one renewable alternative fuels that could partially replace gasoline fuel and reduce greenhouse effect to earth. Bioethanol is could be made from crop such as corn or wheat (First generation biofuels), however, these sources are not sustainable due to competition between the growing population. This led to second generation biofuels which are made from lignocellulosic waste such as rice straw but this generation of biofuels has disadvantages such as costlier forms to derive sugary material, competition with agricultural plants for land and so on. This led to third generation biofuels, energy from marine algae (Trivedi et al., 2013). Marine algae were previously in the second generation biofuels category but, when it became apparent that algae are capable of much higher yields with lower resource inputs than other feedstock, many suggested that they be moved to their own category (Biofuel.org.uk, 2010).

Macroalgae contain little to no concentrations of lignin (Wi et al., 2009). Therefore, the conversion of carbohydrates stored in seaweeds into ethanol does not require delignification (Ge et al., 2011). Seaweeds are classified into three groups: green, brown and red. They contain various types of glucans, i.e., polysaccharides composed of glucose (Yaganisawa et al., 2013). The carbohydrates of seaweed are highly diverse; macroalgae mostly uses starch as a source of energy storage, however, some species may possess another compound such as ulvan in *Ulva* spp this portion may be about 2-29% of the algae dry weight. While cellulose and starch portions of this species of this species may contribute to the glucose (fermentable sugar) the ulvan fraction may not contribute to the formation of glucose (Korzen et al., 2015).

Siddhanta et al., (2013), reported a cellulose content of 20 wt% in *Chaetomorpha aerea*. Ventura et al., (1989) observed a cellulose and hemicellulose content of 20 wt% in *Ulva lactuca*. Conversely, based on cellulose and starch content of *Ulva* 31 wt% could be

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converted into fermentable sugars (Korzen et al., 2015). As a result of the major sugars being contained in ulvan is rhamnose, the major fraction of reducing sugars is obtained from cellulose and starch fractions.

In ethanol production the concentration of reducing sugars is very important, this depends on which approaches that are undertaken to maximize the amounts of reducing sugars such as acidic or enzymatic hydrolysis. Another factor that is important is which fermentation approach could be utilized. Therefore, the objective of this experiment is to produce bioethanol from *Ulva lactuca* using different hydrolysis and fermentation approaches.

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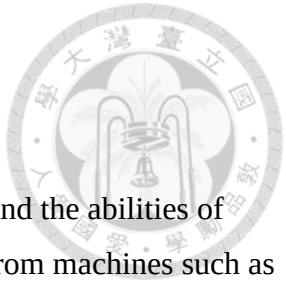
2. Literature Review



2.1 Environmental Issues Related to Fossil Fuels

In the beginning human beings depended mostly on their abilities and the abilities of their livestock. The extra source of energy they obtained was acquired from machines such as a wind mill and a water wheel. However, in the mid-18th to 19th century the industrial revolution took place. This is the period where man began making use of fossil fuels; fossil fuels are made from the remains of prehistoric organisms that have been laid beneath the earth's surface for millions of years (History.com Staff, 2009).

The rising temperatures may also have a negative impact on the sea surface temperature. The temperature of the sea varies from different points, warmer near the equator and colder near the Arctic and Antarctic regions (Fuentes et al., 2016). As the ocean continues absorbing more heat the circulation patterns changing and the normal systems of transporting warm and cold water also changes. These changes may have changes may contribute to a modification to marine ecosystems in many ways. For examples, discrepancies in ocean temperature can disturb species of plants, animals, and microbes present in a location, modify migration and breeding patterns, threaten sensitive ocean life such as corals, and alter the frequency and intensity of harmful algal blooms such as red tide, (EPA, 2016).

The effects of the rising temperatures may also have an impact on the wildlife as mentioned by the Intergovernmental Panel on Climate Change (IPCC, 2001). The general characteristic of each animal and their physiology that make them suitable for a particular environment make them effective indicators that climate change is taking place (Holt et al., 2010). Many already commonly identified patterns such as poleward movement, shifts to a higher elevation, change in breeding times are taking place globally. One example, of such indicator takes place in the Sundarbans, the only remaining habitat of Bengal tigers (*Panthera tigris tigris*) in Bangladesh, is projected to decrease considerably in size as a result of rising sea levels; estimations are a loss of 18% of the land by 2050 and as much as 34% by 2100. For tigers and the many other species that inhabit these forested wetland habitats, migration to higher ground probably would be blocked by human habitation of adjacent lands, (IPCC, 2001).

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2.2 Economic Issues Relating to Fossil Fuels

The industrial revolution began in the mid eighteenth century and as a result of this provided humans with capabilities beyond that of animals and human powers. The era known as the Industrial Revolution was a period in which fundamental changes occurred in agriculture, textile and metal manufacture, transportation, economic policies and the social structure (History.com Staff, 2009). The industrial revolution led to machines that can perform tasks in the place of human hands or animals, led to the invention of locomotives, in summary it eased the burden of man. All this could not have happened if were not for our fossil fuel sources. The world population is growing, today the total population is about 7 billion, another 2 billion could be added to that amount by 2050, another 1 billion added which could mean that in 2100 the earth might have a total of 10 billion people, according to the International Energy Agency (IEA, 2011), based in Paris has projected that the world's energy demand will increase from about 12 billion tonne oil equivalents (t.o.e) in 2009 to either 18 billion t.o.e. or 17 billion t.o.e. by 2035 under their 'current policies' or 'new policies' scenarios, respectively2. Carbon-dioxide emissions are expected to increase from 29 gigatonnes per year to 43 Gt yr−1 or 36 Gt yr−1 under the current and new policies, respectively (Chu, 2012).

Fossil fuels such as coal, oil, and natural gas account for 81% of global consumption in the year 2010. The amount of fossil fuels in the earth is diminishing and will continue to decrease as a result of the surging demand of food, feed and energy for the increasing global population, (Rahman et al., 2014). In order to meet future energy demands and also to reduce the amount of CO_2 emissions entering into the atmosphere another alternative to this source should be invested such as ‘Bioenergy’.

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2.3 Bioenergy

Bioenergy can be defined as energy derived from the conversion of biomass to fuel, or processed liquids and gases. It is the single largest renewable resource on the planet providing 10% of world primary energy supply (Demirbas and Demirbas (2010). The use of biomass as a renewable source for energy and chemical purposes and as an emission mitigation measure has received much attention. From an energy perspective, the use of biomass is attractive given its potentially low greenhouse gas emissions and the ability to relatively easily replace fossil fuels in many parts of the energy system. Biomass-based energy carriers (bioenergy) can be used in transport, as heating or cooking fuels in households, or for conversion into electricity. Biomass can also replace fossil fuels in non-energy purposes as a feedstock for the production of bulk chemicals, (Daioglou et al., 2014). Currently, 19% of the global energy demand is being met through the renewable sources, where biomass contributes up to 9%, with an increasing rate of 2.5% per year, (Ahmad et al., 2017).

Table 1. Bioenergy (biomass) sources.

Feedstock	Conversion	End Product
Solid biomass (wood chips, straw)	Combustion, pyrolysis, gasification	Heat, fuel, bio oil
Wet biomass (manure, organic waste)	Digestion	Biogas
Oil crops (rape seed, sunflower)	Extraction and esterification	Biodiesel
Sugars and starches (sugar beets, wheat)	Hydrolysis and fermentation	Bioethanol

(EIA, 2018)

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2.3.1 Biodiesel

Biodiesel is a domestically produced, renewable fuel that can be manufactured from vegetable oils, animal fats, or recycled restaurant grease for use in diesel vehicles. Biodiesel's physical properties are similar to those of petroleum diesel, but it is a cleaner-burning alternative (Alternative Fuels Data Center, 2018). As a result of its environmental benefits and the increase in petroleum price, a rapid increase in biodiesel production is observed. The global biodiesel production currently around 6 billion liters/year and represents 10% of the entire biofuel production. It is mainly produced by transesterification of edible oils such as those from rapeseed, soybean, sunflower, and palm, thus leading to conflict with food supply. More oil supply will be necessary to meet the growing demand for biodiesel production, where particular attention should be paid to various feedstocks as a potential alternative to edible oils, (Hama, 2013). Some alternative oils may be obtained from microbial and waste cooking oil using chemical and enzymatic processes, (Wang et al., 2017).

Table 2. Sources of Biodiesel

Vegetable oils	Non-edible oils	Animal Fats	Other Sources
Soybeans	Almond	Lard	Bacteria
Rapeseed	*Abutilon muticum*	Tallow	Algae
Canola	*Andiroba*	Poultry Fat	Fungi
Safflower	*Babassu*	Fish oil	Micro algae
Barley	*Brassica carinata*		Tarpenes
Coconut	*B. napus*		Latexes
Copra	*Camelina*		Cooking Oil (Yellow Grease)
Cotton seed	*Cumaru*		Microalgae (*Chlorella vulgaris*)
Groundnut	Cynara cardunculu		
Oat	*Jatrophacurcas*		
Rice	*Jatropha nana*		
Sorghum	Jojoba oil		
Wheat	*Pongamiaglabra*		
Winter rapeseed oil	Laurel		

(Arshad et al., 2018)

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2.3.2 Biogas

Biogas is produced by processing residual waste from livestock (dung, manure and uneaten food), food production (fruit and vegetable waste, residues from meat, fish and dairy processing, brewery waste, food waste and much more) and effluents from industrial as well as municipal wastewater treatment plants (Surendra et al., 2014). Energy from biogas is only associated with methane. Biogas is a renewable, high quality fuel, which can be utilized for various energy services such as heating, combined heat and power (CHP), or a vehicle fuel instead of using fossil fuel but it needs to be purified before usage to remove some constituents fatal for the engine (H_2S, H_2O), (Senghor et al., 2017).

It offers alternative fuel, high-quality fertilizer as a by-product, electricity, heat, complete waste recycling, greenhouse gas reduction and environmental protection from pollutants. Biogas systems convert organic household waste or manure into gas for cooking and lighting. These wastes like (rice, ugali), vegetables (tomatoes, cabbage), peels of potatoes and fruit, excreta can be converted to energy instead of disposing of them. Waste disposal and storage attracts insects and pests (Achinas et al., 2017). Biogas helps in management of waste and contributes to improved hygiene in rural areas. The raw material used in biogas production is cheap and it also generates income making it an economically viable option for conversion of biomass. Biogas is also generated using animal waste, which is available in large quantities and is almost free (Nahar et al., 2017).

Table 3. Various sources of biogas

Animal Sources	Plant sources
Horse manure	Pig slurry
Fat	Maize silage
Sewage	Fruit wastes
Food waste (disinfected)	Sewage
Chicken litter	Food waste (disinfected)
Cattle dung	Municipal solid waste
Municipal solid waste	Sugar beet

(Achinas et al., 2017)

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2.3.3 Bioethanol

Bioethanol is a natural fuel that is made from sugar and starchy crops. Ethanol also known as ethyl alcohol or bioethanol is a colourless, flammable, volatile liquid with a strong odour. Its chemical formula is C_2H_6O, which may also be written as C_2H_5-OH, its molar mass is 46.07 g and its melting and boiling point is as follows; 114.1ºC and 78.5ºC (Vohra et al., 2014). It is a renewable and sustainable liquid fuel that has potential in facing today's global energy crisis and greenhouse gas pollution that is affecting global temperature and air quality. In the period of 2011, bioethanol production was stated to be 100 billion litres and was expected to increase up to 3-7% in the years 2012-2015 (Aditiya et al., 2016).

Table 4. Major sources for bioethanol production

Sugary materials	Starchy materials	Cellulosic materials
Sugar cane	Grains (maize, wheat)	Wood
Sugar beet	Root crops (cassava, potato)	Agricultural residues (straws, stover, bagasse)
Sweet sorghum		Munciple solid waste
Cheese whey		Waste paper, Paper pulp
Fruits		

(Zabed et al., 2017)

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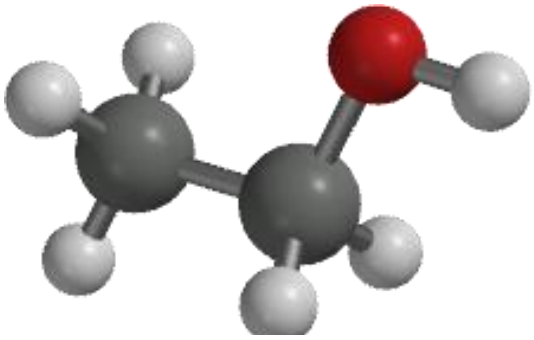

(http://www.chem.ucla.edu)

Figure 1. The Ball and spoke model for ethanol (CH_3CH_2OH)

2.4 Production of Bioethanol

Ethanol is the most dominant biofuel and global production shows an upward trend over the past 25 years with a sharp increase from the year 2000. Worldwide capacity in 2005 and 2006 was about 45 and 49 billion liters per year (Talebnia et al., 2010). The main lead of bioethanol as compared with conventional fuel is its biodegradability, less toxicity, reduction of greenhouse gas emission and use of renewable and ever-present biomass as primary substrate. The USA and Brazil accounts for 87.1% of bioethanol production in 2011 as estimated by Renewable Fuel Association (RFA), (2012).

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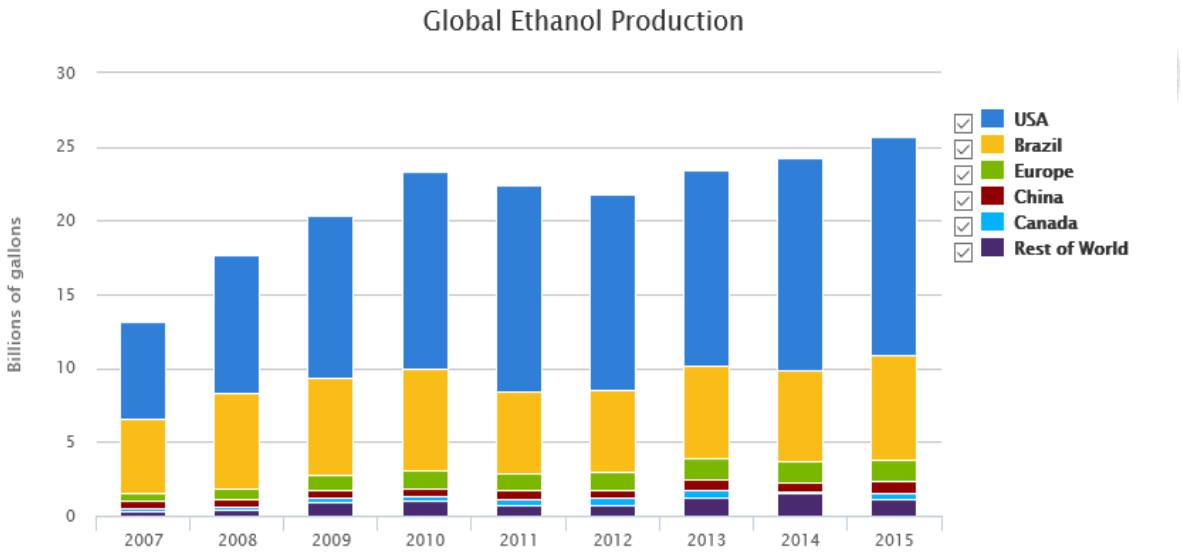

(Renewable Fuels Association, 2016)

Figure 2. This chart shows global ethanol production by country or region, from 2007 to 2015. Global production peaked in 2015 after a dip in 2012 and 2013. The United States is the world's largest producer of ethanol, having produced nearly 15 billion gallons in 2015 alone. The vast majority of U.S. ethanol is produced from corn, while Brazil primarily uses sugar.

2.4.1 Feedstock for Ethanol Production

Bioethanol can be produced from many kinds of feedstock including sugar containing feed stock, starchy and lignocellulosic feedstock (Rulli et al., 2016). It is projected that by 2050, liquid biofuels such as bioethanol is forecasted to be on top of the ‘biofuel ladder’ due to their efficiency in replacing gasoline for the transportation sector. The ease of availability of feedstock with respect to its geographical distribution plays an important role in the development and commercialization of bioethanol. the search of a suitable feedstock for bioethanol has led to the rise of three generations so far namely first generation derived from edible crops, second generation from non-edible crops and third generation from the algal feedstock (Jambo et al., 2016).

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2.4.2 Utilizing Corn for Bioethanol Production

In US, the main candidate for ethanol production is maize (corn). The potential for ethanol from maize lies not only in converting the grain to ethanol, but also in applying cellulose conversion technology to the pericarp that covers the grain. Cellulose conversion technology (consists of pretreatment and hydrolysis) forecasts to production of ethanol not only to the grain of the corn but also to other parts of the plant (Li et al., 2010). Corn grain possess high percentages of starch that may be converted to monosaccharides upon pretreatment and hydrolysis. The cob of corn contains glucan which is also present in, the stalk, and the leaves, but in a different form, i.e., cellulose, and at lower amounts compared to corn grain. The other major structural polysaccharide is hemicellulose, which is predominantly xylan (Schwietzke et al., 2009).

Table 5. Displays the composition of three main parts of the corn grain, cob, and stover

Type of material	Grain[a]	Cob[b]	Stover[c]
Starch	71.7	n/m	n/m
Cellulose	2.4	42	36
Hemicellulose	5.5	33	26
Protein	10.3	n/m	5
Oil	4.3	n/m	n/m
Lignin	0.2	18	19
Ash	1.4	1.5	12
Other	4.2	5.5	2
Total	100	100	100
maximum yield of monosaccharides (Ib/ton, 100% efficiency)	1778	1684	1392
calculated best case ethanol yield (gal/ton, 100% efficiency)	135	128	105
dry weight (%)	52.4	-9.5	47.6
dry weight (kg/acres)	4000	-725	3630

(Schwietzke et al., 2009)

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2.4.3 Utilizing Sugarcane as a Feedstock for Bioethanol Production

Brazil is the biggest producer of sugarcane in the world. In the 2012/13 harvest, for example, it was estimated that more than 602 million tons of sugarcane will be processed by the Brazilian sugar-alcohol mills (Canilha et al., 2012). The sugarcane is basically consisted of stem and straw. The sugarcane straw (or trash) is divided in three principal components, that is, fresh leaves, dry leaves, and tops. The sugarcane stem is milled to obtain the cane juice, which is subsequent used for sugar (sucrose) or alcohol (ethanol) production. The residual fraction from the sugarcane stem milling is named bagasse. Sugarcane bagasse and straw are normally burned in industries to supply all the energy required in the process. If, instead, both were used for ethanol production, much more ethanol would be produced from each hectare of sugarcane processed (Chandel et al., 2011).

SB and SS are chemically composed of cellulose, hemicellulose and lignin. Cellulose, and hemicellulose fractions are composed of mixture of carbohydrates polymers. A number of different strategies have been envisioned to convert the polysaccharides into fermentable sugars (Kumar and Sharma, 2017). One of them, the hemicellulose fraction can be hydrolyzed with dilute acids followed by cellulose hydrolysis with enzymes. The cellulosic fraction is solid rich in glucose, and hemicellulosic fraction is liquid rich in xylose, glucose, and arabinose, where both (solid and liquid) can be fermented to produce ethanol (Canilha et al., 2012).

2.4.4 Producer Microorganism of Bioethanol

Microorganisms obtain energy by breaking down carbon sources into by products such as; carbon dioxide, lactic acid, cellulose and ethanol. Ethanol may be produced by bacteria, olds and yeast. The core metabolic pathway involved in the ethanol fermentation is Embden–Meyerhof–Parnas or EMP pathway or much more simply glycolysis, this is the process through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced (Ruiz et al., 2012). In aerobic conditions the pyruvate will be further hydrolyzed into ethanol. The theoretical yield of ethanol is 0.511 and 0.489 for CO_2 on a basis of glucose metabolized. In the process of glycolysis two ATP molecules are produced in which the

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yeasts are to consume in order to biosynthesis. If no consumption of ATP takes place the metabolism of glucose will not take place, hence, no glycolysis (Bai et al., 2008).

Factors such as; temperature, sugar concentration, pH, fermentation time, agitation rate, and inoculum size influences the production of bioethanol. Temperature directly affects the growth rate of these microorganisms, high temperatures may be unfavourable for growth and may become a stress factor. The ideal temperature range for fermentation is between 20 and 35 °C. Free cells of *S. cerevisiae* have an optimum temperature near 30 °C whereas immobilized cells have slightly higher optimum temperature due to its ability to transfer heat from particle surface to inside the cell, (Azhar et al., 2017).

2.4.5 Common Yeast used in bioethanol production

2.4.5.1 *Pichia stipitis* sugar metabolism

Pichia stipitis originates from a group of yeasts isolated from rotting wood and the larvae of wood dwelling insects. As a results of its origins this yeast has the capability to use most sugars that are present in wood (Agbogbo and Kelly, 2008). The presence of numerous genes for endoglucanases and β-glucosidases, along with xylanase, mannanase and chitinase activities indicates that it could metabolize polysaccharides. Some researchers have also indicated that this yeast is able to utilize cellobiose and convert to bioethanol (Jeffries et al., 2007). *Pichia stipitis* is one of the limited number of naturally-occurring microorganisms that have the ability to ferment all of the glucose, xylose, mannose, galactose and cellobiose sugars, with high ethanol productivity. Its thick cell wall and high resistance to contamination makes it suitable for industrial usage. (Agbogbo and Kelly, 2008).

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2.4.5.2 *Kluyveromuyces marxianus* sugar metabolism

Kluyveromyces marxianus is described as a homothallic, hemiascomycetous yeast, is phylogenetically related to *S. cerevisiae*, and is a sister species to the better-known *Kluyveromyces lactis* (Lane and Morrissey, 2010). In contrast to other yeasts known for 2-phenylethanol production, *Kluyveromyces marxianus* has the GRAS status, which makes it especially suitable as industrial producer strain (Medeiros et al., 2001). Moreover, *Kluyveromyces marxianus* is Crabtree-negative, which is an advantage for future production processes, because the formation of ethanol as a toxic by-product under aerobic conditions can be avoided (Etschmann et al.,, 2002).

2.4.6 Fermentation of Starch

Starch is a polysaccharide made up of two polymers of D-glucose: amylose, an essentially unbranched α [1 → 4] linked glucan, and amylopectin, which has chains of α [1 → 4] linked glucoses arranged in a highly branched structure with α [1 → 6] branching links. Amylose and amylopectin make up 98–99% of the dry weight of native granules, while the remainder comprises small amounts of lipids, minerals, and phosphorus in the form of phosphates esterified to glucose hydroxyls (Copeland et al., 2009).

Cassava is a starch crop that can be used for bioethanol production. Although cassava is a good source of glucose with a high starch content (Sanchez and Cardona, 2008), they are mainly limited to a tropical climate. According to Jansson et al., 2009, cassava has a 150 conversion rate (L/tonne). Other starchy materials that may serve as a source for bioethanol production includes rye, barley, tricilate, sorghum, corn, and potato (Zhan et al., 2003).

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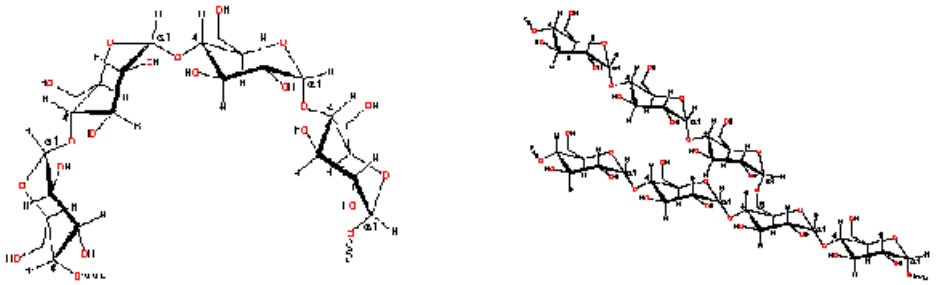

(a) (b)

(Chaplin, 2008)

Figure 3. Representative structure of amylose (a) and amylopectin (b)

2.4.7 Fermentation of raw ethanol with sugar raw materials

The conversion of sugars into bioethanol is a relatively simple process as compared to production from starch or lignocellulose material as no previous hydrolysis of the feedstock is required as the disaccharide can be broken down by the yeast cells (Cardona and Sanchez, 2007). In Brazil, the main feedstock is sugarcane while the United States of America (USA) produces ethanol from corn. Sugarcane is a C4 plant with high efficiency to convert atmospheric CO_2 and water into sugars through photosynthesis. Sugar Beet which is another sugar crop may also be used for bioethanol production. Ogbonna, 2001 presented that this crop is an alternative substrate for ethanol production since it does not require pH adjustment nor nitrogen supplements. Sweet sorghum may also be a suitable substrate for bioethanol production (Ratnavathi et al., 2011).

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2.4.8 Fermentation of raw ethanol with cellulose raw material

Cellulose also known as the sugar contained in the plant cell wall is the most plentiful polymer in the world The main component of lignocellulose is cellulose, a beta (1–4)-linked chain of glucose molecules (Wyman et al., 2005). It is as a result of the hydrogen bonds cellulose has resistance to degradation Hydrogen bonds between different layers of the polysaccharides contribute to the resistance. Hemicellulose, the second most abundant component of lignocellulose, is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose and xylose. Lignin is composed of three major phenolic components, namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Rubin, 2008). Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues and cell wall layers. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall (Chen et al., 2017). It is as a result of its glucose bonds that draws many interest in utilizing cellulose for bioethanol production.

2.4.9 Pretreatment of Lignocellulosic Material

Pretreatment of lignocellulose is mainly carried out as a result of the bonds between lignin, hemicellulose and cellulose being too strong. The conversion of lignocellulose to glucose and further production of ethanol has to undergo three main steps: delignification, depolymerization and enzymatic hydrolysis (or fermentation) (Saritha et al., 2014). Pretreatment process changes the microstructure, macrostructure, and chemical composition of lignocellulose. And it also alters the natural macromolecular structure of lignocellulose during decomposition to become susceptible to microbial degradation. In this process, lignin and hemicellulose which surround cellulose are broken down, lignin is removed, hemicellulose is degraded, and the crystalline structure of cellulose is changed to improve the availability and release of cellulose (Jönsson and Martin, 2016).

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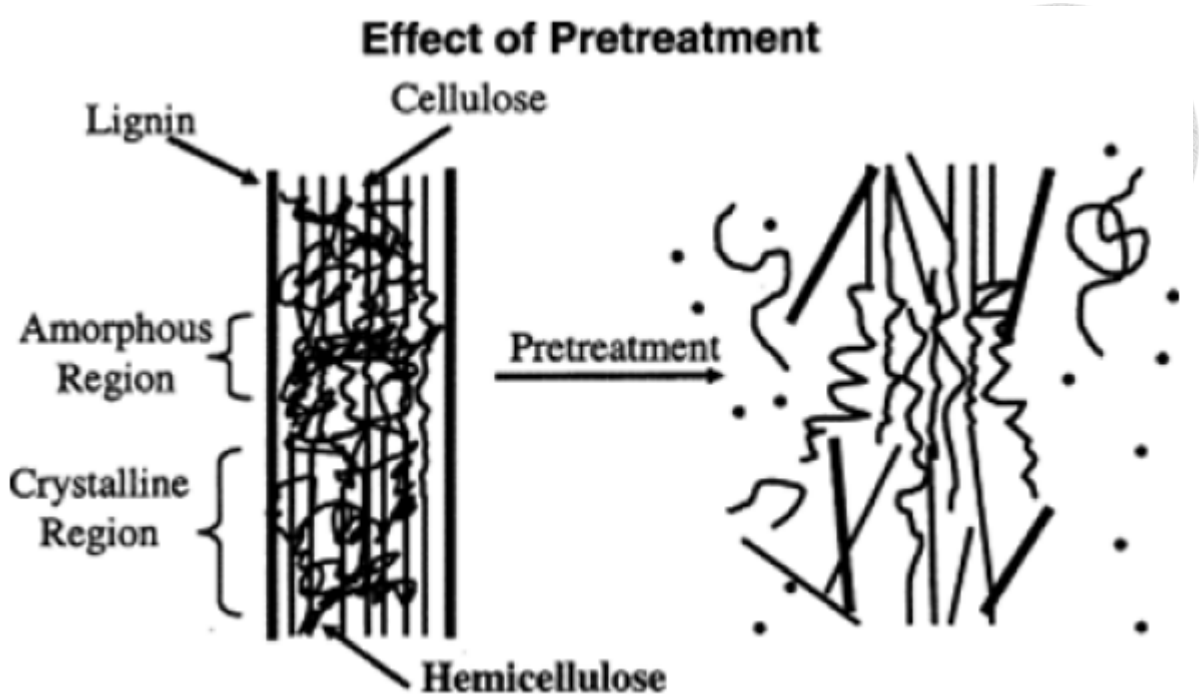

(Zhang and Shahbazi, 2011)

Figure 4. Shows the effects of pretreatment on lignocellulosic matter

2.4.9.1 Dilute acid hydrolysis

The purpose of dilute or concentrated acid hydrolysis is to break down the cellulose and hemicellulose polymers in lignocellulosic biomass to form individual sugar molecules which maybe be fermented into ethanol (Wymann, 1994). Advantages of acid hydrolysis are that the acid can penetrate lignin without pretreatment, the rate of acid hydrolysis is faster than enzyme hydrolysis, but glucose also degrades rapidly under acidic conditions. The acid hydrolysis process employs usually sulphuric acid and hydrochloric acid at concentrations of 1–10% using a moderate temperature (in the range of 100–150 °C) (Lenihan et al., 2010). Dilute acid and alkaline hydrolysis are the most common forms of hydrolysis, however, their uses vary for example alkaline pretreatment is most commonly used in the treatment of plants with higher lignin and acid hydrolysis is mainly used in plants with high cellulose (Carvalheiro, 2008; Meng et al., 2015; Loow et al.,2016).

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2.5 Fermentation

French chemist Louis Pasteur in the year 1854, determined that fermentation is caused by yeast. It was the earlier works of German scientist Theodor Schwann who helped develop the cell theory that acted as a stimulus in Pasteur's research. About 1840, Schwann determined that fermentation is the result of processes that occur in living things. In 1907, a German chemist named Eduard Buchner received the Nobel prize for showing that enzymes in yeast cells cause fermentation. Two years after, Arthur Harden and Hans Euler-Chelpin determined exactly how enzymes cause fermentation, and they won the Nobel prize for their work in 1929. By the 1940s, technology was developed to use fermentation to produce antibiotics.

Today fermentation is used to produce industrial chemicals, medicines such as antibiotics, and alcoholic beverages, as well as to make bread rise and to preserve many types of food. Fermentation may be defined as a metabolic process whereby microorganisms break down organic matter to produce energy. While some microorganisms derive energy, some by products are; lactic acid, butane, carbon dioxide, cellulose, nisin, ethanol. In ethanol fermentation, derivation of energy from sugars by either yeast or bacteria, produce carbon dioxide and ethanol are produced. As a result of yeast producing their energy without the need of oxygen, ethanol production is a facultative anaerobic process.

2.5.1 Separate Hydrolysis and Fermentation

Separate hydrolysis and fermentation is a fermentation strategy, it is however, different from simultaneous saccharification and fermentation being that the difference lies in two vessels are required; one for saccharification and one for fermentation. Unlike SSF the optimal conditions of enzymes and yeast will not be affected. The major drawback of SHF is that end products, i.e. glucose and cellobiose released in cellulose hydrolysis strongly inhibits the cellulase efficiency. Glucose inhibits β-glucosidase which results in an increase of cellobiose since β-glucosidase catalyse the hydrolysis of cellobiose to glucose. Cellobiose itself has an inhibiting effect of cellulases and thereby reduces the cellulase activity.

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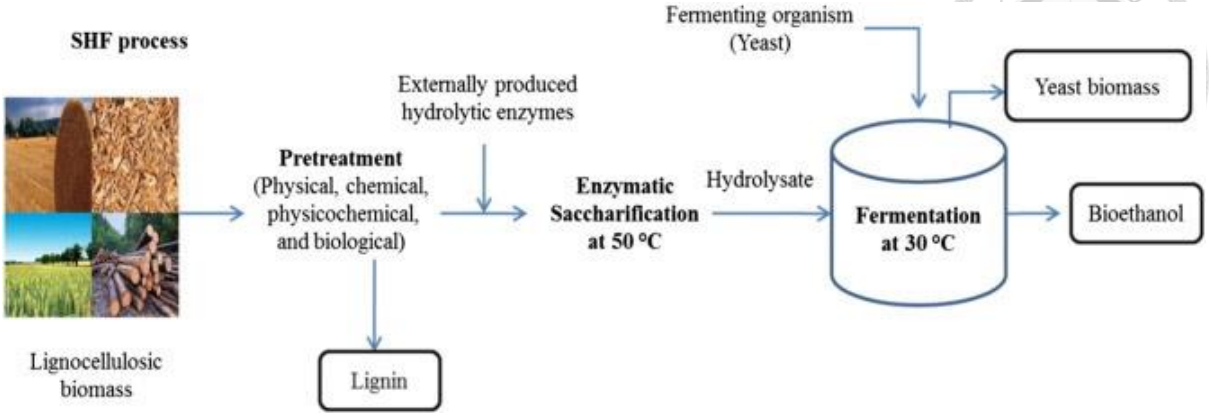

(Choudhary et al., 2016)

Figure 5. The process flow of separate hydrolysis and fermentation

2.5.2 Co-Fermentation

The ethanol yield can be increased by fermentation of hexoses and pentoses present in the hydrolysis broth (Erdei et al.,, 2013). Although wild-type *Saccharomyses cerevisiae*, commonly known as baker's yeast, is unable to ferment xylose into ethanol, it is the predominant microorganism used in large-scale processes due to the high ethanol yields and high productivities that can be achieved. Although solutions to the problem of pentose sugar fermentation have been proposed using different approaches (Demeke et al.,, 2013), there are still serious constraints regarding xylose fermentation. Co-cultivation of a pentose-fermenting microorganism with *S. cerevisiae*is an alternative approach to solving this issue (Chen, 2011).

Co-culture has already been invested in the production of ethanol based on a few researches and has led to an increase in ethanol. The production of ethanol by fermentation of starches and cellulosic materials is gaining increasing interest because of the increasing economy of bioethanol production caused by the high oil price. Abate et al., (1996) described

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ethanol production by a co-culture of *Zymomonas mobilis* and *Saccharomyces sp*. with higher yields and production rates than with either micro-organism in pure culture. The utilization of inulin from artischoke as a substrate for ethanol production by a co-culture of *Z. mobilis* and *Kluyveromyces fragilis* was described by Szambelan et al., (2004). They achieved a conversion of 94% of the theoretical maximum. In case of sorghum as a substrate for the ethanol production, Mamma et al., (1996) suggested a co-culture fermentation process with *Saccharomyces cerevisiae* and *Fusarium oxysporum.* Hydrolysis of cellulose and fermentation of the released sugars occurs simultaneously in this example. Another combination of a mould and a yeast for the production of ethanol was reported by Ward et al., *(1995). Kluyveromyces marxianus* and *Talaromyces emersonii* were cocultivated at a temperature of 45°C in this example.

2.6 Seaweed Overview

Macroalgae or better known as seaweeds may be characterized by three phyla based on their pigmentation: brown (including the pigments of fucoxanthin and chlorophyll a and c), red (possessing the pigments of phycoerythrin and phycocyanin), and green (with the pigments of chlorophyll a and b together with various characteristic xanthophylls) (Moroney, 2015). Plants specialist refers to these broad groups as *Phaeophyceae, Rhodophyceae* and *Chlorophyceae*, respectively. Brown seaweeds are largest of the macroalgae with size that could reach up to 30 meters in length, however the size may vary in this species since the smaller could reach lengths of 30-40 cm long. The red and green seaweeds are smaller and their size are almost similar in length. Seaweeds are termed macroalgae as a result of being larger in size as compared to microalgae (*Cyanophyceae*) which are microscopic in size, (FAO 2003).

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(Tabassum et al., 2017)

Figure 6. Shows some species of seaweed

The structure of the seaweed consists of the thallus i.e. the entire body of the seaweed, lamina i.e. a flattened structure with the resemblance of a leaf, sorus i.e. a cluster of spores spore, air bladders, a hollow, gas-filled structure organ which helps the seaweed float, found on the blade), stipe i.e. a stem-like structure, not all seaweeds have these, holdfast i.e. a specialized structure on the base of a seaweed which acts as an "anchor" allowing it to attach to a surface**,** haptera i.e. finger-like extensions of holdfast anchoring to benthic substrate (MESA, 2015).

Macroalgae play an important role in the aquatic environment; for instance, they are a food source for many maritime creatures such as sea urchins and fishes, and form the base of some food webs. They also provide shelter and a home for numerous fishes, invertebrates, birds, and mammals. As a result of their high nutritional qualities and pharmaceutical values, some macroalgae are consumed as food, or as herbal medicine for treating gall stones, stomach ailments, eczema, cancer, renal disorders, scabies, psoriasis, asthma, arteriosclerosis,

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heart disease, lung diseases, ulcers, and so on. They may also serve other purposes as fodder, fertilizer, fungicides, dietary supplements, and so on, (Peng et al., 2015). Among natural sources, seaweeds are known for their richness in fiber, minerals, and certain vitamins. Seaweeds also contain interesting bioactive substances, such as polysaccharides, proteins, lipids, and polyphenols, which are of interest since they possess many antibacterial, antifungal, and antiviral properties.

2.6.1 Seaweed Ecological Issues

Although seaweed may possess some favourable qualities, there are instances where they may pose a threat to the local environment. In the periods of 2011 to 2016 peculiar events of washing ashore of pelagic *Sargassum* occurred in the Lesser Antilles and Caribbean region. Irregularly huge amounts of algae of the genus *Sargassum* washed up on the beaches of islands of the Lesser Antilles from the Virgin Islands to Barbados and Trinidad. Every year, tons of algae are deposited on the coast and accumulate on the exposed beaches of windward coastlines in the Lesser Antilles. Consequently, the coastal environment is faced with significant damages and non-survival of many organisms, Marechal et al., 2017. As a consequence of these depositions habitats of organisms may be affected such as sea turtles. Sea turtles usually go on shore in order to lay eggs, however, as a result of the huge amount of seaweed there will be a struggle in movement. Another problem identified is that once the turtle has successfully lay its eggs, waves may carry more seaweed over the nesting area which may have an effect on the temperature of the eggs and this would live to variations in the sexes of the turtles, Maurer et al., 2015.

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(Tabassum et al., 2017)

Figure 7. Immense quantities of sargassum have been washing ashore on Long Island, around Antigua, and across the Caribbean in waves over the past 5 years.

(Tabassum et al., 2017)

Figure 8. Hatchlings, such as this neonatal hawksbill turtle that succumbed in the nearshore waters of eastern Antigua, may struggle through the dense mats of *Sargassum* as they attempt to begin offshore migrations.

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2.6.2 Use of Seaweed for the Production of Bioethanol

First generation biofuels are defined as the created of ethanol from edible crops, however, this sources of fuel are considered not feasible as it will result in competition between the future population for food. This led to the second generation biofuels which are fuels that may be generated from various types of biomass, conversely, these sources are yet again not reliable as they may have consumed arable land and additional cost of fertilizers. This situation has led to what is known as third generation biofuels, 'the use of algae', (Allen et al., 2015).

Seaweeds are promising candidates for bioethanol as they contain relatively low lignin or no lignin at all and these algae possess various types of glucan which are polysaccharides composed of glucose. The glucans found in green and red seaweeds are cellulose and starch, and brown seaweeds contain cellulose and β-1,3-glucan (Yanagisawa et al., 2011).

Table 6. Carbohydrate composition of macroalgae, microalgae, and lignocellulosic biomass

Macroalgae[a]			Microalgae[b]	Lignocellulosic biomass
Green algae polysaccharide	Red algae polysaccharide	Brown algae polysaccharide	Starch total carbohydrate	Cellulose Hemicellulose
Manan Ulvan Starch Cellulose monosaccharide Glucose Mannose Rhamnose Xylose uronic acid glucoronic acid	Carrageenan Agar Cellulose Lignin monosaccharide Glucose Galactose Agarose	Laminarin Mannitol Alginate Fucoidin Cellulose monosaccharide Glucose Galactose Fucose Xylose uronic acid mannuronic acid guluronic acid glucuronic acid	Arabinose Fucose Galactose Glucose Mannose Rhamnose Ribose Xylose	Lignin

(Jung et al., 2013)

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Yazdani et al.,, 2015 utilized the macroalgae *Nizimuddinia zanardini* for bioethanol utilizing treatments of sulphuric acid and hot water, resulting in a maximum yield of 34 g/kg of the dried biomass using hot water pretreatment. The carbohydrate contents of green, red, and brown algae are 25–50%, 30– 60%, and 30–50% dry wt., respectively. Many attempts have been taken to utilize various carbohydrates in macro algal biomass by using physicochemical hydrolysis, as in the saccharification of lignocellulosic biomass such as dilute acid pretreatment (Jung et al., 2013). Yanagisawa et al., suggested a combination of both dilute acid pretreatment and saccharification in order to obtain a higher ethanol product. In the experiment a 5.5 % ethanol concentration was produced which is higher than 4-5% concentration that was confirmed economically feasible. The combination of both chemical and enzymatic hydrolysis could yet again be identified in the work of Abd-Rahim et al., (2014). According to the results of their experiment they obtained a maximum reducing sugar yield of 62.35% using the macro-algae *Kappaphycus alvarezii*.

2.6.3 Overview *Ulva lactuca*

Ulva is commonly known as “sea lettuce” or “green laver”. In appearance this species of algae has colour variations ranging from green to dark green depending on its occurrence i.e. underwater or at beach. They form irregular, however, round ruffled edge shaped translucent soft sheet fronds (leaf blades) from slight yellowish to blackish green. Fronds are attached to rocks with the help of holdfast (a type of root like structure that enables anchoring). It gains 20-30 cm diameter that is quite smaller. *U. lactuca* is harvested from beaches in the world every year in huge quantity, (Qing et al., 2016).

(Source: http://www.seaweed.ie)

Figure 9. *Ulva* growing in ocean

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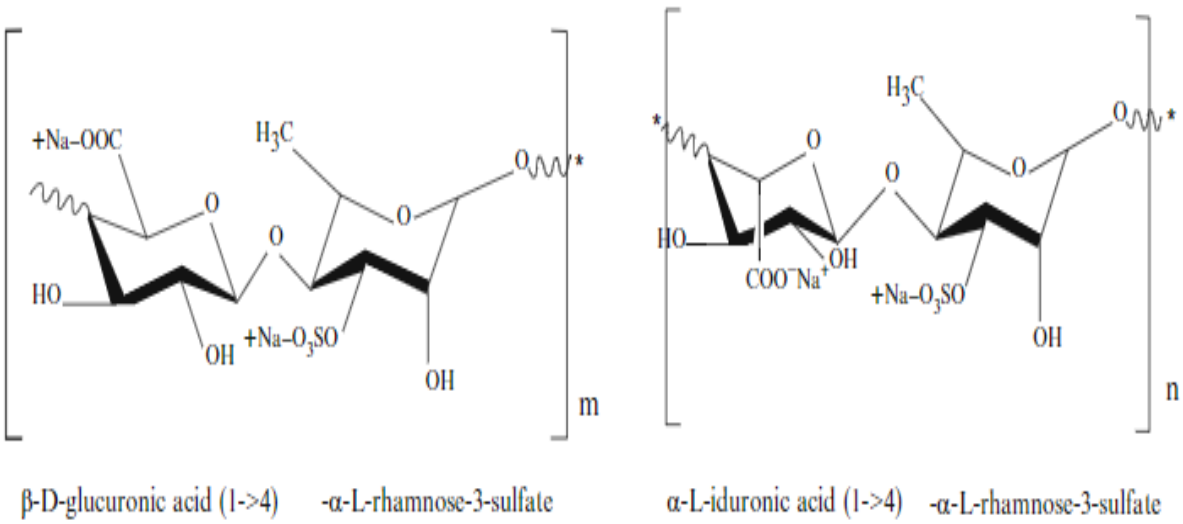
Among the polymers synthesized by these algae, cell wall polysaccharides represent around 38-54% of the dry algal matter. These include four polysaccharide families in *Ulva* sp.: two major ones, the water soluble ulvan and insoluble cellulose, and two minor ones, a peculiar alkali-soluble linear xyloglucan and a glucuronan. Their distribution and associations in *Ulva* cell wall have been summarized in a model which takes into account recent cytochemical and physicochemical data.



β-D-glucuronic acid (1->4) -α-L-rhamnose-3-sulfate α-L-iduronic acid (1->4) -α-L-rhamnose-3-sulfate

(Anbu et al., 2015)

Figure 10. Shows the structure of Ulvan

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The yield ranges from 8% to 29% of the algal dry weight, depending on the extraction and purification procedures.13-21 Recovery of ulvan is generally done by precipitation by adding an alcohol or a quaternary ammonium salt. Composition. The pioneering work of Brading et al and McKinnel and Percival, (1962) established that sulfate, rhamnose, xylose, and glucuronic acid are the main constituents of ulvan. They also identified that glucuronic acid and rhamnose occur mainly in the form of the aldobiouronic acid, 4-O-â-Dglucuronosyl-L-rhamnose. Rhamnose (16.8-45.0% dw), xylose (2.1-12.0%), glucose (0.5-6.4%), uronic acid (6.5-19.0%), and sulfate (16.0-23.2%) have since then been reported in ulvan from several Ulvales species,14,16-19,24-30 but it is only after the work of Quemener et al., (1997) that iduronic acid (1.1-9.1%) was recognized as a constituent carbohydrate unit in ulvan. Variable amounts of mannose and galactose have been reported, but their belonging to ulvan has been questioned since they form a distinct neutral fraction in *U. mutabilis* (Bryhni,1978). Arabinose was reported to be present in *U. lactuca* ulvan collected in Egypt (Fattah and Edrees, 1973) and 3-O-methyl L-rhamnose in *E. compressa* and *Enteromorpha* sp. (McKinnel and Percival,1962). The biochemical composition of *Ulva lactuca* may vary according to location and season (Bikker et al., 2016). Fattah and Edrees (1973) showed that for *U. lactuca* harvested at the Mediterranean coast, rhamnose varied from 1.5 % (November) to 28 % (April), while total proteins ranged from 8.7 % (April) to 33.8 % (August).

As previously mentioned the sugars present in *Ulva lactuca* are arabinose, mannose, and so on. Table 8. Shows the price and quantity for some sugars. Though the sugars present in *Ulva lactuca* may not be enough to compare with first and second generation biofuels. They do have a very fast growth rate and require fewer inputs as comparison (Trivedi et al., 2013).

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Table 7. Starch, total monomeric sugar and fibre content (% of DM) of *Ulva lactuca* and extracted fraction.

	Starch	**Rha**	**Gal**	**Glc**	**Xyl**	**NDF**	**ADF**	**ADL**
Ulva lactuca	4.2	9	0.7	11.3	2.9	25.9	13.5	6.9
Extraction fraction	0.3	1.7	0.2	3.4	0.5	20.3	17.9	10.6

Bikker et al., 2016

Table. 8. Commercial cost of the sugars present in *Ulva lactuca.*

Raw material	Unit	Cost	Source
Glucose	(1 kg)	NTD 2330	Sigma Aldrich
Xylose	(500 g)	NTD 6580	Sigma Aldrich
Rhamnose	(1 kg)	NTD 700	

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2.6.4 Bioethanol from *Ulva*

Ulva species has a fast growth rates and are known to possess various polysaccharides; those features can be comparable with terrestrial plants for bioethanol production. Trivedi et al., (2013), performed an experiment using a species of *Ulva* known as *Ulva fasciata* Delile and resulted in an ethanol yield of 0.45 g/g and an ethanol efficiency of 88.2%; the results of their experiment demonstrated the possibilities for the utilization of this alga for bioethanol production. In another experiment performed by Trivedi et al., (2015), an ethanol yield of 0.47 g/g reducing sugars was obtained which corresponded to a 93.81% conversion efficiency.

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Table 9. Ethanol production from various marine algae feedstocks (a modified version).

Seaweed	Conditions	Sugars released (g/g)	Ethanol Yield (g/g)	Ethanol Efficiency	References
Ulva fasciata	Hot buffer + enzyme	0.205	0.45	88.2	Trivedi et al., (2013)
Gracilaria verrucosa	Enzyme	0.87	0.43	84.31	Kumar et al., (2013)
Kappaphycus alverzii	Acidic	NA	0.369	72.35	Meinita et al., (2012)
Gelidium amansii	Dilute Acid	0.422	0.38	74.50	Park et al., (2012)
Kappaphycus alverzii	Acidic	0.306	0.40	80.39	Khambhaty et al., (2012)
Saccharina japonica	Thermal acid	0.456	0.169	33.13	Jang et al., (2012)
Gelidium amansii	Acid + enzyme	0.566	Na	-	Kim et al., (2011)
Laminaria japonica	Acid + enzyme	0.376	0.41	80.39	Kim et al., (2011)
Sargassum fulvellum	Acid + enzyme	0.096	NA	-	Kim et al., (2011)
Ulva lactuca	Acid + enzyme	0.194	NA	-	Kim et al., (2011)
Gracilaria Salicornia	Acid + enzyme	16.6	0.079	15.49	Wang et al., (2011)
Sargassum sagamianum	200^{o}C and 15 MPa for 15 min	NA	0.386	75.68	Hyeon et al., (2011)
Sargassum sagamianum	_	NA	0.133-0.233	26.07-45.68	Yeon et al., (2011)

(Trivedi et al., 2013)

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Table 10. Shows a summary of *Ulva* used as feedstock for bioethanol production.

Seaweed	Conditions	Reducing sugars	Ethanol yield	Ethanol efficiency (%)	References
Ulva fasciata	Hot buffer + enzyme	0.205	0.45	88.2	Trivedi et al., (2013)
Ulva lactuca	Acid + enzyme	0.194	NA	-	Kim et al., (2011)
Ulva rigida	Sonication	0.196	0.333	64.7	Korzen et al., (2015)
Ulva lactuca	Enzyme	-	7.2 g/L	-	Yanagisawa et al., (2011)
Ulva lactuca	Hot water + enzymatic	-	0.35 g/g	-	H. van der Wal et al., (2013)
Ulva lactuca	Acid + enzyme		13.3 g/g of sugar/l	52%	Sayed et al., (2016)

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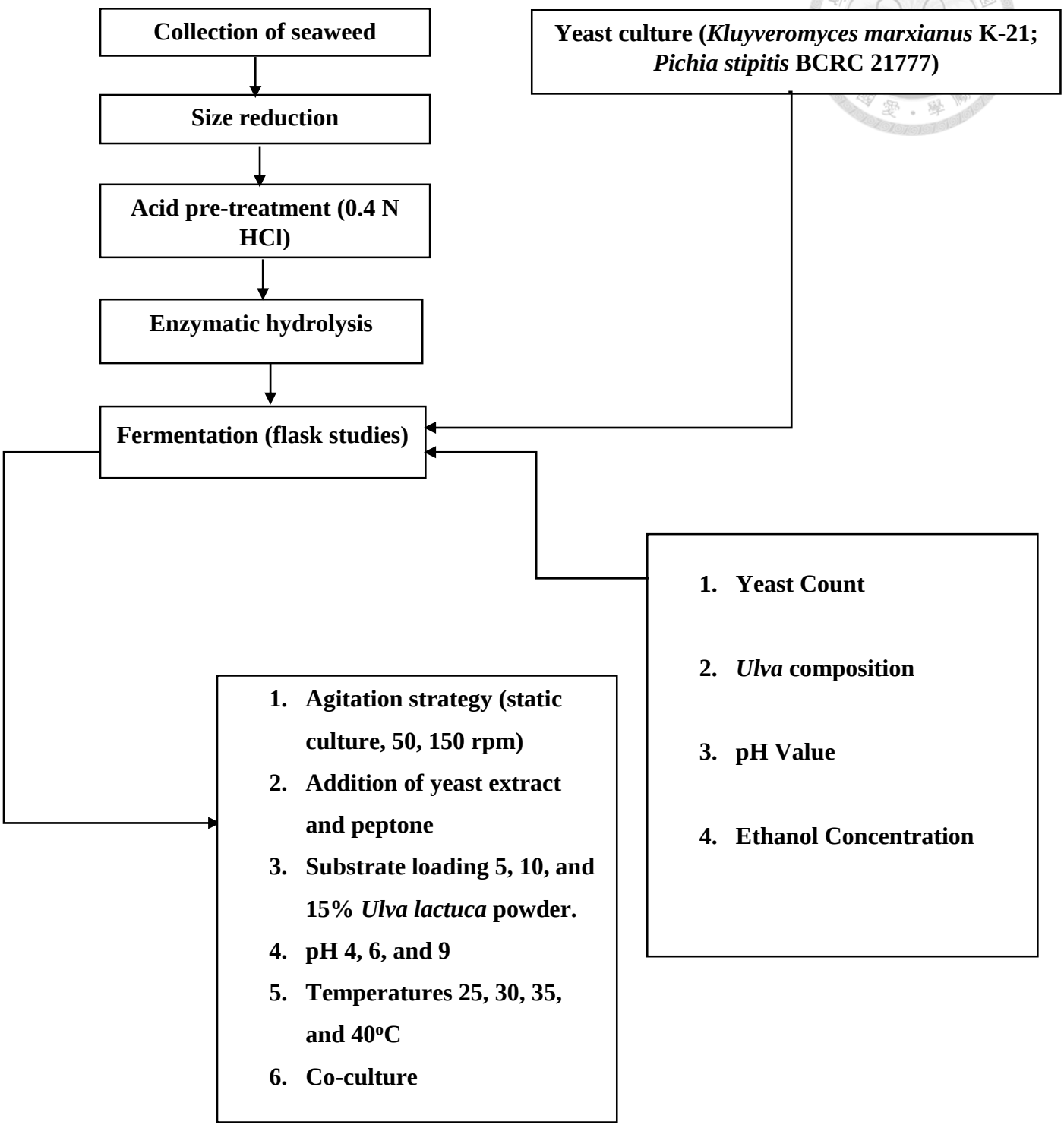
3. Experimental Design



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3.1 Materials and Methods

3.1.1 Chemical reagents

- Agar (Sigma Aldrich, Canada Inc., Burlington, ON, L7L6A4)
- D-glucose (Merck, 64271, Darmstadt, Germany)
- D-xylose
- L-Rhamnose monohydrate (Sigma Aldrich., 3050 Spruce Street, St.Louis, MO 63103)
- 3,5-Dinitrosalicyclic acid (DNS) (Acros Organics, New Jersy, USA)
- Ethanol absolute (Sigma alrich., 3050 Spruce Street, St.Louis, MO 63103)
- Glycerol (Union Chemical Works LTD, Taiwan)
- Hydrochloric acid (Showa, Japan)
- Peptone (Sigma Aldrich, Canada Inc., Burlington, ON, L7L6A4)
- Potassium sodium tartrate, K-Na (Showa, Japan)
- Yeast extract (Lab M Limited l, Quest Park, Moss Hall Road, Heywood, Lancashire BL97JJ, United Kingdom)
- Sulfuric acid 98%, H_2SO_4 (AENCORE, 景明化工股份有限公司, Miaoli, Taiwan)
- Sodium Hydroxide, NaOH (Showa, Japan)
- Malt extract (Lab M Limited l, Quest Park, Moss Hall Road, Heywood, Lancashire BL97JJ, United Kingdom)

3.1.2 Equipment

- Laminar flow: Lian Shen Enterprise Company LTD
- Autoclave: TM-328 (Yihder Technology Co., Ltd, New Taipei city, Taiwan)
- Centrifuge (Model EBA 12R, Hittich-Zentrifugen, Tuttlingen, Germany), (Universal 320R, Hittich-Zentrifugen, Tuttlingen, Germany)
- Oven (Type FD 115, WTC Binder, Tuttlingen, Germany)
- Distilled water maker (Model 315, Buchi, Flawil, Swizerland)
- 96 well plate (Micro well plate, NUNC Co., Rochester, NY, USA)
- ELISA microplate reader (VersaMax TM tunable microplate reader, Molecular Devices Co., CA, USA)
- High Performance Liquid Chromatography Analyzer (HPLC)

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- Degassing system (Model DG-2410, Sanwa Tsusho Co., Kyoto, Japan)
- HPLC pump (880-PU, Jasco Co., Kyoto, Japan)
-Column oven (800-LC, Jasco Co., Kyoto, Japan)
-Detector refractive index detector (2414; Waters, Milford, MA, USA)

- Incubator (JSL ORBITAL SHAKER INCUBATOR, Taiwan)
- -80^{o}C Freezer
- Electronic analytical balance
- Hot plate magnetic stirrer
- Microcentrifuge
- pH meter (HANNA Instruments Woonsocket RI USA)
- 4^{o}C refrigerator
- 0.22μm syringe filters
- Pulverizer (RT-08 350G, Rong Tsong Precision Technology Co. Taichung Taiwan)
- Sieve

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3.1.3 Materials

3.1.3.1 Samples

Seaweed powder (1 kg) was purchased from Taiwan Fertilizer Co. LTD.

3.1.3. 2 Microorganism

The yeasts used in this experiment were *Kluyveromyces marxianus* K21 and *Pichia stipitis* BCRC 21777 purchased from the Bio-Resource Conservation and Research Center of the Food Industry Development Institute. K21 was activated in YM medium with a composition of 3 g / L yeast extract, 3 g / L malt extract, 5 g / L peptone and 10 g / L glucose in 1 liter culture medium. While BCRC 21777 was activated on YPD medium consisting of 20 g glucose, 20 g peptone, and 10 g yeast extract. Add 10 g of agar into the medium. Commercial cellulase used for this experiment was purchased from Bio dragon (Cellulase AP3: 1490 U/g).

3.2 Method

3.2.1 Preparation of seaweed powder

The seaweed powder was rinsed using ddH_2O for 30 minutes in-order to get rid of possible contaminants. Following this samples was dried in the oven for 5 days at 45^oC, then pulverized to powder using a pulverizer. After this process the powder was placed in a container and stored at 4^oC until use.

3.2.2 Hydrolysis of Samples

3.2.2.1 Hot Acid Hydrolysis

5, 10, and 15 g (dry weight) *Ulva lactuca* powder was added to 100 mL 0.4 N HCl and treated with 121^oC autoclave for 20 minutes.

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3.2.2.2 Enzymatic Hydrolysis

After pretreatment, addition of 4560 U Cellulase (Cellulase AP3 1490 U/g; 1 unit (U) = 1 mole glucose/min) was added to the hydrolysate and reacted at 45ºC for 24 hours (initial pH 4.5).

3.2.3 Fermentation

3.2.3.1 Agitation Strategies of static, 50 rpm and 150 rpm on bioethanol production.

Fermentation was conducted by adding 47.5 mL of the hydrolysate and adding 5 % (v/v) yeast into separate flasks, then placing the flasks at 30ºC at the above mentioned conditions in order to determine the optimum fermentation conditions. Fermentation was conducted for a total of 72 hours. Sampling intervals were 0, 2, 6, 12, 24, 36, 48, and 72 hours (3 mL per sample). No supplemental nutrients were added such as peptone or yeast extract.

3.2.3.2 Addition of Nitrogen and Protein sources to the hydrolysate

As a result of the higher ethanol production from the static culture fermentation, it was then utilized further in these experiments. 1% and 1 % yeast extract and peptone was added to *Ulva* hydrolysate. Fermentation was conducted by adding 47.5 mL of the hydrolysate and adding 5 % (v/v) yeast into separate flasks, then placing the flasks at 30ºC (static culture) 72 hours. Sampling intervals were 0, 2, 6, 12, 24, 36, 48, and 72 (3 mL per sample). No supplemental nutrients were added such as peptone or yeast extract.

3.2.3.3 Influences of substrate loading on bioethanol production (10 and 15 %)

Fermentation was conducted by adding 47.5 mL of the hydrolysate and adding 5 % (v/v) yeast into separate flasks, then placing the flasks at 30ºC static culture for 72 hours. Sampling intervals were 0, 2, 6, 12, 24, 36, 48, and 72. No supplemental nutrients were added such as peptone or yeast extract. 3 mL samples were taken after each interval for analysis of sugar composition and ethanol content.

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3.2.3.4 Influence of temperature and pH on bioethanol production

Since 10% loading resulted in a higher bioethanol production it was utilized further in the bioethanol studies. A total of 47.5 mL *Ulva* hydrolysate was added to a 250 mL Erlenmeyer flask using temperatures 25, 35, 40^{o}C and at pHs' of 4, 6 and 9 at static culture for 72 hours. A sample of 3 mL was withdrawn from the fermentation medium at the following time intervals 0,2, 6, 12, 24, 36, 48, and 72 hours.

3.2.3.5 Influence of co-culture on bioethanol production

For ethanol production by co-culture of *Kluyveromyces marxianus* and *Pichia stipitis*, yeast cells were placed into 47.5 mL hydrolysate into 250 mL Erlenmeyer flask at 35^{o}C for 72 hours under static conditions. A sample of 3 mL was withdrawn from the fermentation medium at the following time intervals 0,2, 6, 12, 24, 36, 48, and 72 hours.

3.2.3.6 Preservation and activation of fermenting yeast

For the preservation of the yeast, the yeast was inoculated into 10 ml YM medium (*Kluyveromyces marxianus* K-21) for *Pichia stipitis* BCRC 21777 it was placed into YPD medium. The optical density value was determined by using a spectrophotometer (OD_{600nm}>1.0). The yeast was added with 30% glycerol and stored in -80^{o}C. For the activation of the yeast, the yeast was retrieved from the -80^{o}C refrigerator and using a sterilized wire loop a streak was made on the agar plates (YM agar for K-21; YPD agar for BCRC 21777) and was then placed into the incubator at 30^{o}C for 24 hours.

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3.3 Analytical Methods

3.3.1 Yeast cell count

Fermentation liquid was diluted with 0.9% Na Cl solution. Pipette 0.1 ml and spread plate onto agar plate and incubate for 24 hours for K-21. Count the yeast colony and expressed as CFU/ml.

3.3.2 **Sugar and ethanol analysis by high-performance liquid chromatography (HPLC)**

Sample was filtered with 0.22µm filter. HPLC was used to determine the sugar composition of *Ulva* hydrolysate after hydrolysis and fermentation. 5 mM H_2SO_4 was used as the mobile phase with 0.4 ml/min flow rate. The temperature of the column oven was 80^oC. The amount of sample injected was 20 µL. Columns are design to separate sugars and alcohols according to their molecular weight and then will be detected with the refractive index detector. Results of the experiment will be compared with a standard.

4. Results and Discussion

4.1 Composition of *Ulva lactuca* using HPLC

HPLC analysis was conducted to confirm the simple sugars present in the *Ulva lactuca* hydrolysate after the series of hydrolysis and fermentation process. The results shown in Table 11 the monosaccharide composition of the hydrolysate after pretreatment and enzymatic hydrolysis. According to this table the dominant sugars present in the hydrolysate were glucose, xylose and rhamnose. According to the results of Othman, (2014) the presence of other monosaccharides could be noticed such as: glucose, galactose, rhamnose, xylose, maltose, mannose, and arabinose. In the current experiment, there were other peaks noticed which may have belonged to the previously mentioned monosaccharides (glucose, galactose, rhamnose, xylose, maltose, mannose, and arabinose). The most abundant sugar was glucose. Korzen *et al.,* (2015) explained in his results that as a result of its cellulose and starch content glucose is most likely the most abundant monosaccharide. Based on the results of this experiment the loading may not have resulted in a doubling of the glucose, xylose or rhamnose content could be related to the enzyme loading as it was kept to the same amount and also related to viscosity (Rosgaard et al., 2007).

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Table 12. HPLC analysis of the monosaccharide composition of 5, 10, and 15 g *Ulva lactuca* powder.

5 g	Glucose (g/L)	Xylose (g/L)	Rhamnose (g/L)
Pretreatment	9.18±0.1	2.09±0.7	5.27±0.2
Enzyme	32.92±0.3	2.10±0.6	6.05±0.4
10 g	Glucose (g/L)	Xylose (g/L)	Rhamnose (g/L)
Pretreatment	12.4±0.35	2.5±0.19	8±0.80
Enzyme	33.4±0.13	3±0.23	13.8±0.13
15 g	Glucose (g/L)	Xylose (g/L)	Rhamnose (g/L)
Pretreatment	14.8±0.29	3.1±0.34	9.3±0.12
Enzyme	36.1±0.22	4.2±0.14	16.3±0.57

Data were expressed as mean ± SD (n=3). Values significantly different by ANOVA analysis ($p<0.05$). Add 4560 U cellulase (1490 U/g).

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4.2 Fermentation study

4.2.1 Yeast fermentation and reducing sugar utilization by yeast

4.2.1.1 Agitation Study

Agitation could be beneficial to the growth and performance of the microorganism cells by improving the mass transfer characteristics with respect to substrates, products/byproducts and oxygen. Thus, agitation results in a better mixing of the fermentation broth, helping to maintain a concentration gradient between the interior and the exterior of the cells (Zhou et al., 2018). Such a concentration gradient works in both directions; through better diffusion it helps to maintain a satisfactory supply of sugars and other nutrients to the cells, while it facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells. Agitation also favors oxygen supply to the cells that is important for high biomass concentration (Khongsay et al., 2012).

Bioethanol was produced by using the yeast *Kluyveromyces marxianus* K-21 and *Pichia stipitis* BCRC 21777. The agitation used in this experiment were static culture, 50 rpm and 150 rpm. According to the results of this experiment static culture resulted in a higher ethanol production 7.8 g/L (0.78 %) (Figure 16). Ethanol production decreased for the agitations 50 rpm and 150 rpm for both yeast; respectively *Kluyveromyces marxianus* K-21 (6.1, 5.9 g/L) (Figure 18 and 20), *Pichia stipitis* BCRC 21777 (4.9, 4.8 g/L). It was also noticed that fermentation stopped after 24 hours for both yeast at 150 rpm. A relatively high agitation speed was also favorable for sugar consumption. The specific sugar consumption rate shows a similar tendency to specific growth rate, and a relatively high specific sugar consumption rate was achieved at high agitation speed.

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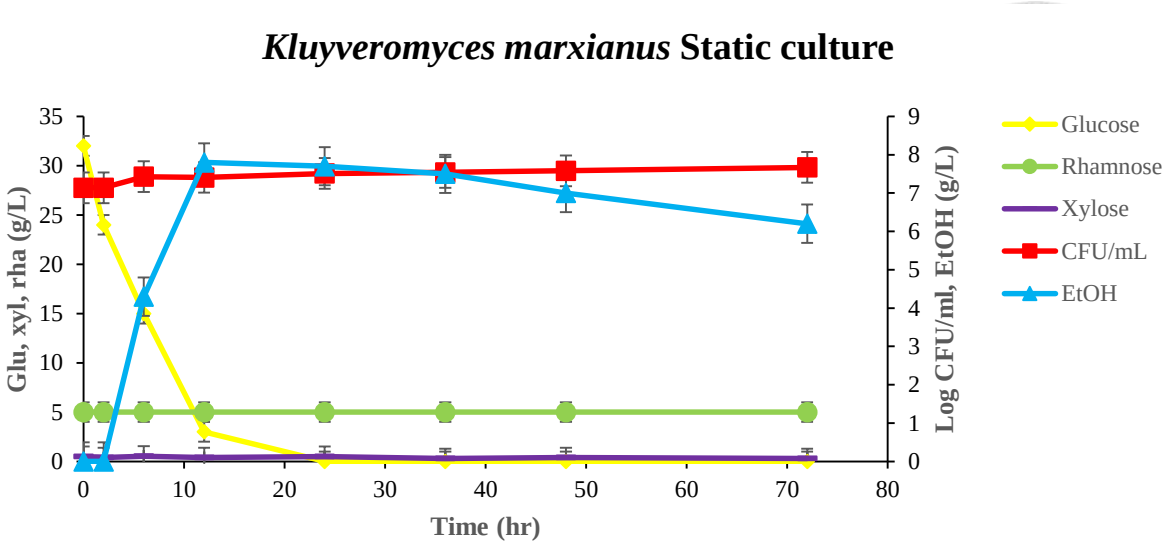

Figure 11. The production of ethanol from 5% *Ulva lactuca* inoculated with 5% *Kluyveromyces marxianus* K-21 and fermented for a 72- hours period. The condition was at static conditions.

Ethanol production was the highest in the 12th hour for *Kluyveromyces marxianus* (Figure 11) at static culture. Glucose was completely consumed within 24 hours and some amounts of xylose was still remaining after the 72-hour period, however, there was completely no influence of this fermentation strategy on rhamnose utilization. Figure 12 shows the bioethanol production using *Pichia stipitis* according to the diagram peak ethanol was noticed within the 72-hour period at static culture, however, ethanol concentration cannot be as compared to *Kluyveromyces marxianus* which according to this experiment is a better ethanol producer.

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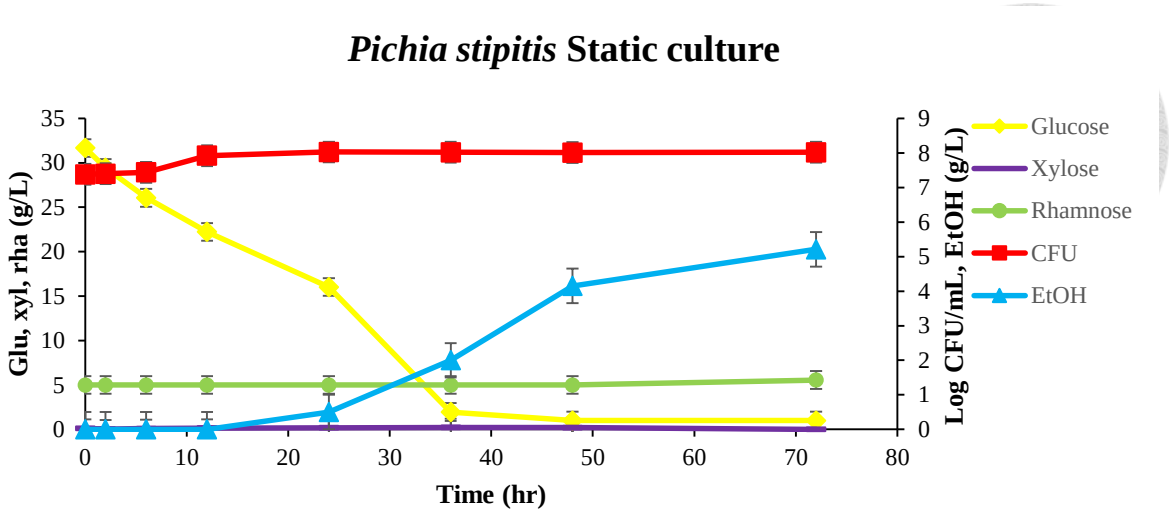

Figure 12. Ethanol production in static culture using *Pichia stipitis* BCRC 21777.

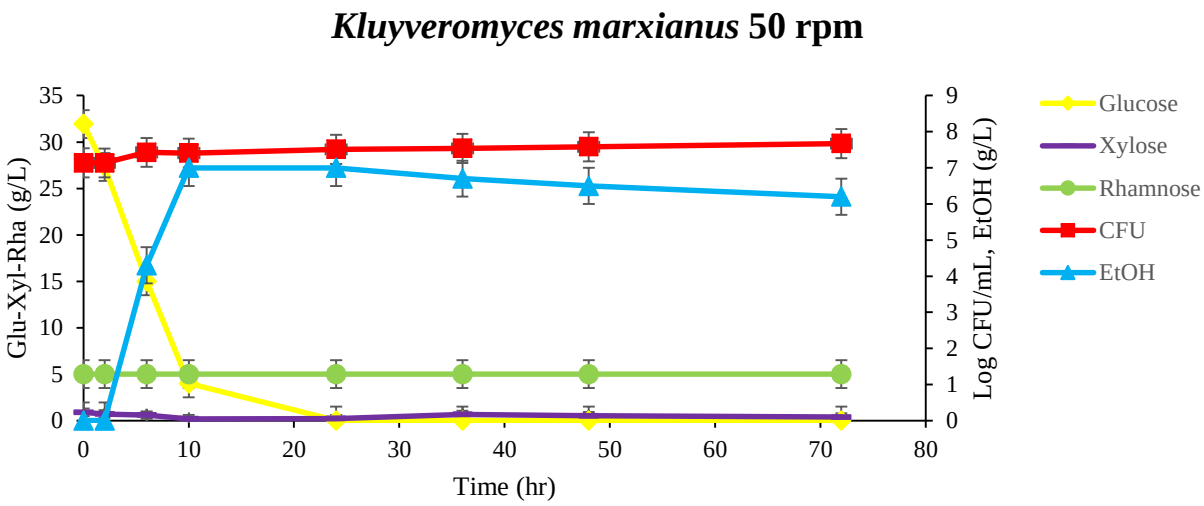

Figure 13. Ethanol production at 50 rpm using the yeast *Kluyveromyces marxianus* K-21

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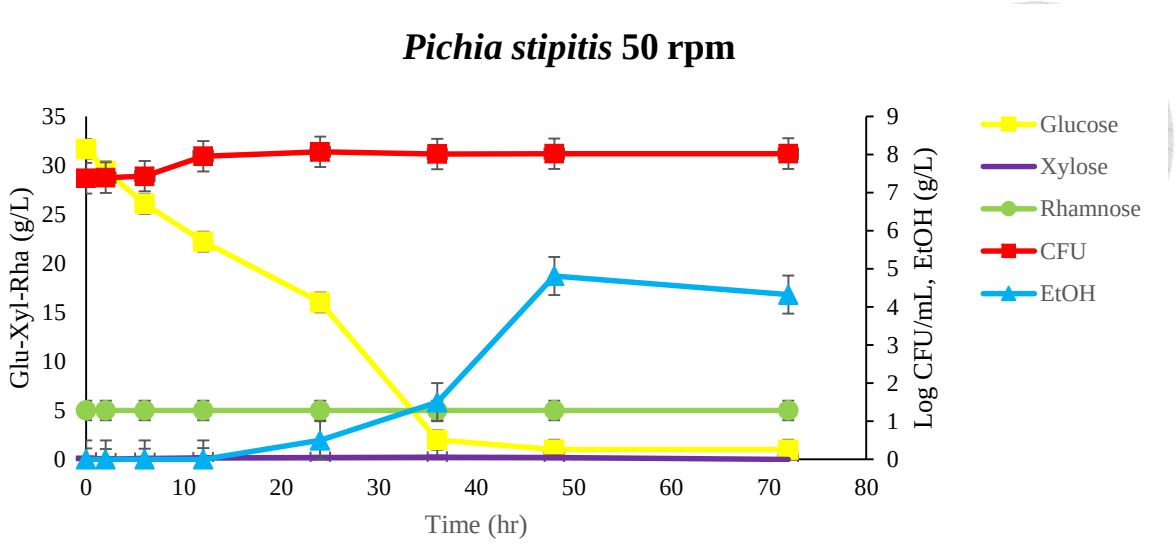

Figure 14. Ethanol production at 50 rpm using *Pichia stipitis* BCRC 21777

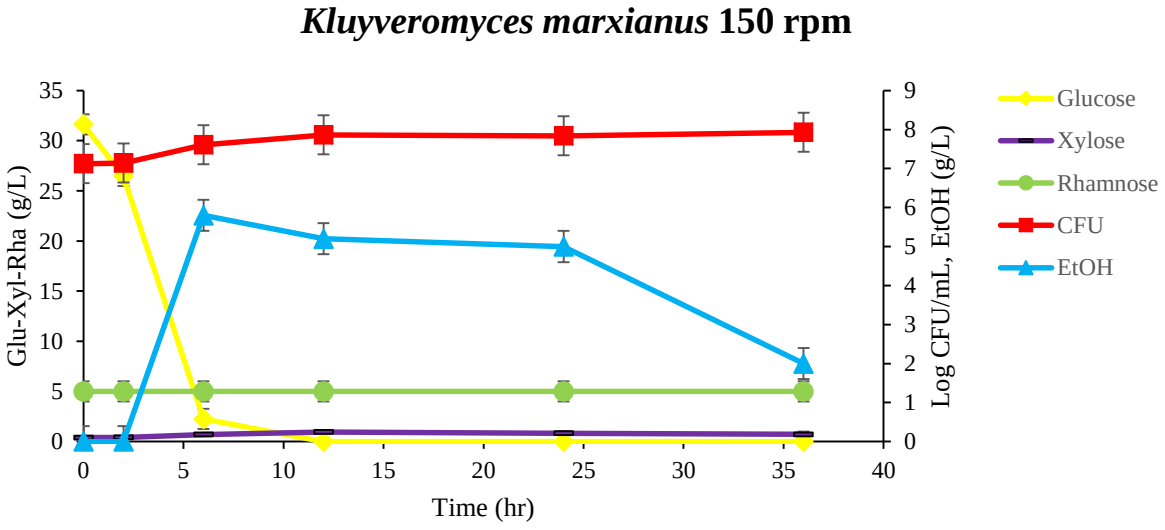

Figure 15. Ethanol production at 150 rpm using *Kluyveromyces marxianus* on 5% *Ulva lactuca* loading

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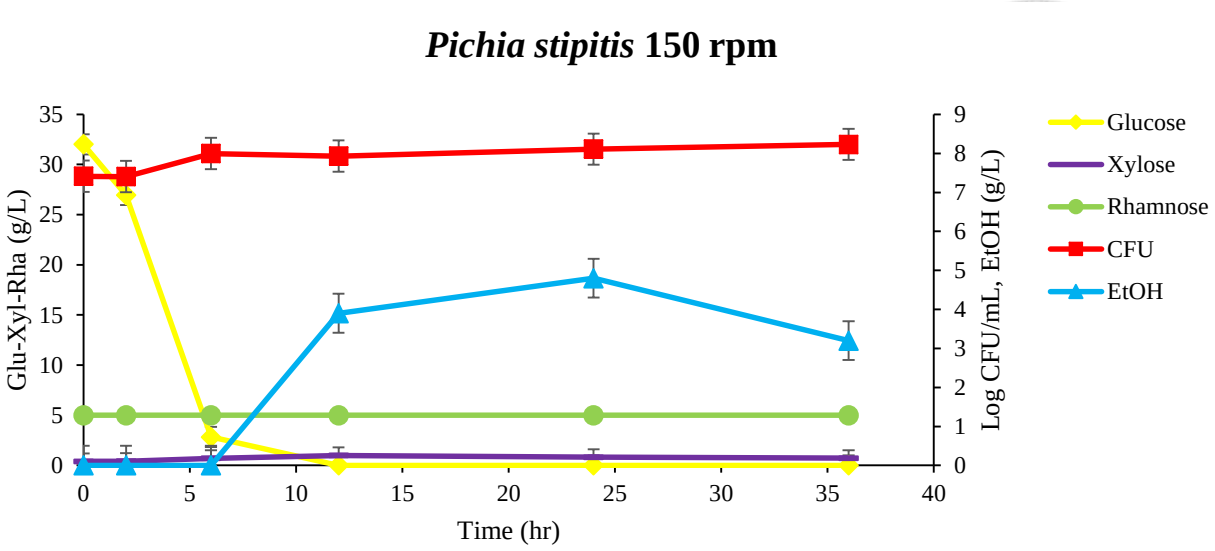

Figure 16. Ethanol production at 150 rpm using *Pichia stipitis* on 5% *Ulva lactuca* loading

To determine the relative differences in ethanol production of the different fermentation conditions ANOVA one-way analysis was carried out on the data and as a result there was no significant differences in ethanol production between the agitation, peak ethanol products of the different agitation conditions seemed only to differ on timing; so as a result agitation of static culture was chosen for the rest of the experiment because it was numerically higher than the rest of the agitations.

4.2.1.2 Influence of nitrogen and protein sources

An additional experiment was conducted using peptone and yeast extract (Table 13, Figure 17, and Figure 18). They were added to monitor whether they might influence ethanol production by increasing concentration. However, according to the analysis the ethanol production from the hydrolysate with nitrogen and protein sources and the hydrolysate without was not significant. This means that no additional nutrients could be added to the hydrolysate as it is already rich in nitrogen and protein sources. The results of this experiment can be compared with that of Trivedi *et al.*, (2013), Van der Waal *et al.*, (2013), and Yanagisawa *et al.*, (2011), who all performed researches on the nitrogen and protein availability of *Ulva lactuca* and *Ulva* species.

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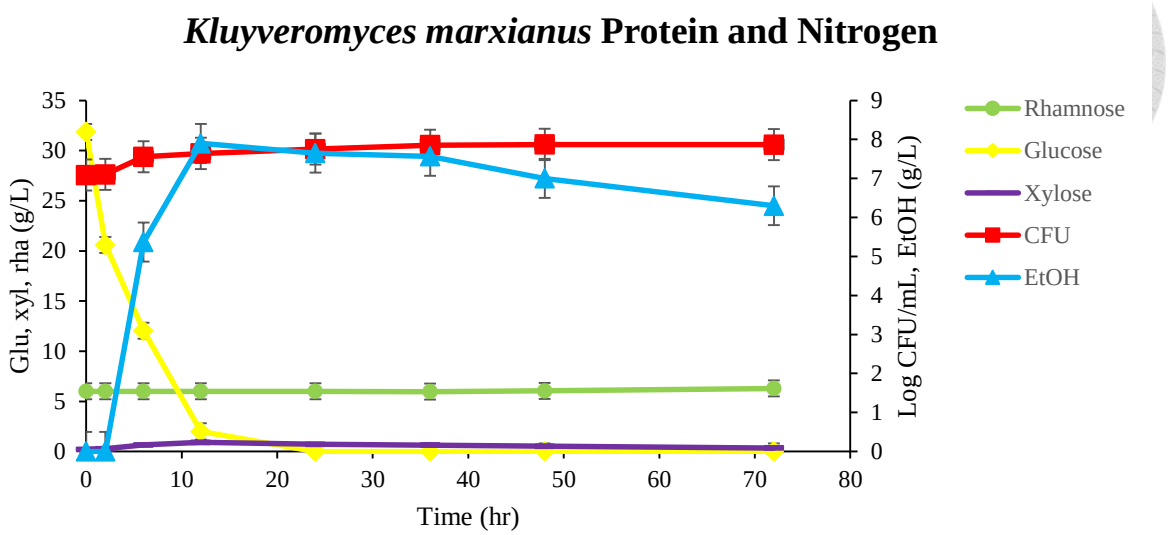

Figure 17. Ethanol production using *Kluyveromyces marxianus* on hydrolysate with protein and nitrogen sources

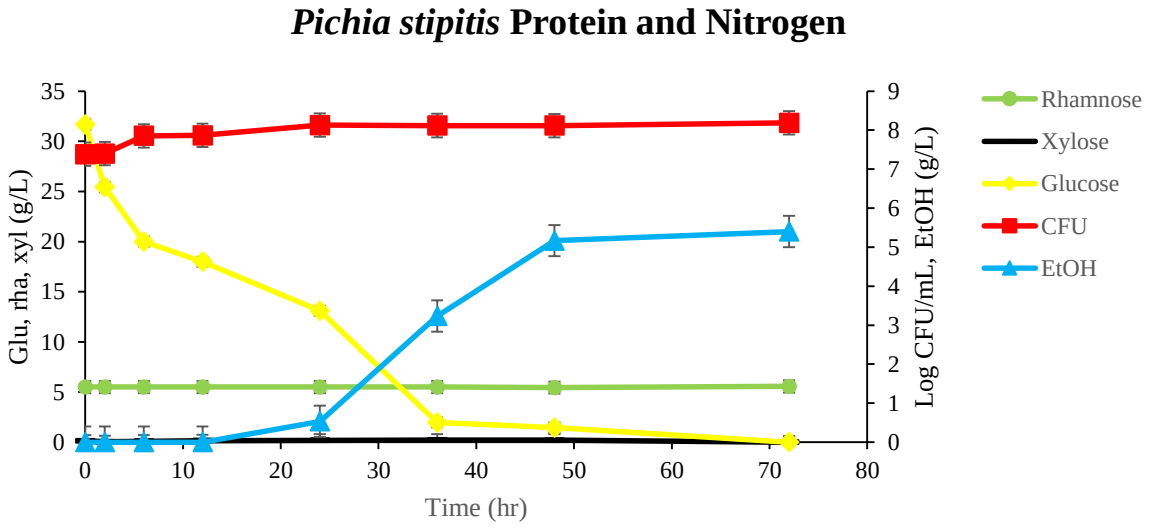

Figure 18. Ethanol production using *Pichia stiptis* on hydrolysate with protein and nitrogen sources

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Table 13. Agitation of static culture, 50, 150, and N+P (static conditions) on yeast strains.

Yeast strains	Static culture	50 rpm	150 rpm	N+P static
Kluyveromyces marxianus K-21	7.80±0.4	6.10±0.7	5.90±0.24	7.90±0.23
Pichia stipitis BCRC 21777	5.21±0.37	4.90±0.19	4.80±0.35	5.40±0.6

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4.2.1.3 Loading Study

According to literature, the threshold of economic profitability corresponds to bioethanol concentrations in the fermentation broth in the range 4–5 volume percent. Achieving this threshold entails the utilization of media containing higher amount of solids. Therefore, increasing substrate loading required to produce high-concentration ethanol so that the process is more profitable (Triwahyuni et al., 2015).

An attempt to increase ethanol concentration at high substrate loading was used in this study, *Ulva* hydrolysate was subjected to fermentation by *Kluyveromyces marxianus* K-21 and *Pichia stipitis* BCRC 21777 at a temperature of 30^{o} C. The fermentation process was conducted at loadings of 5%, 10% and 15% (g/L) substrate loading to evaluate ethanol production. The yeast was simultaneously inserted at the beginning of fermentation process. The sugar from the hydrolysate is immediately consumed by yeast for ethanol production in the fermentation process. Fermentation production rates may increase by reducing product inhibition (Obata et al., 2016). Since the static treatment resulted in a higher ethanol production it was then used in substrate loading testing.

In the experiments involving the 5% substrate loading a total amount of ethanol 7.80 g/L was produced (Figure 11) when using the *Kluyveromyces marxianus* K-21, for the *Pichia stipitis* a total amount of 5.21 g/L (Figure 12) of ethanol was produced. 10% substrate loading *Kluyveromyces marxianus* K-21 reached a total of 10.2±0.5 (g/L) (Figure 19) ethanol while the amount of *Pichia stipitis* also increased to 6.25±0.1 (g/L) (Figure 20). It was then noticed that after loading of 15% the ethanol production decreased slightly to 9.4±0.2 g/L (Figure 21) for *Kluyveromyces marxianus* K-21 and for *Pichia stipitis* decreased to 3.8±0.4 g/L (Figure 22). Reasons for this could have been attributed to viscosity of a higher substrate loading (Wu et al., 2015). The results from this experiment can be compared to that of Othman, 2014 who obtained similar results after a substrate loading of 10%, however, results of 15% loading differed as the substrate loading resulted in a slightly higher ethanol production for Othman, 2014. Reasons for this could be attributed to a high viscosity and slightly different conditions. Triwahyuni et al., 2015 explained that high substrate loading can also result in limited fermentation and enzymatic digestibility can be significantly reduced.

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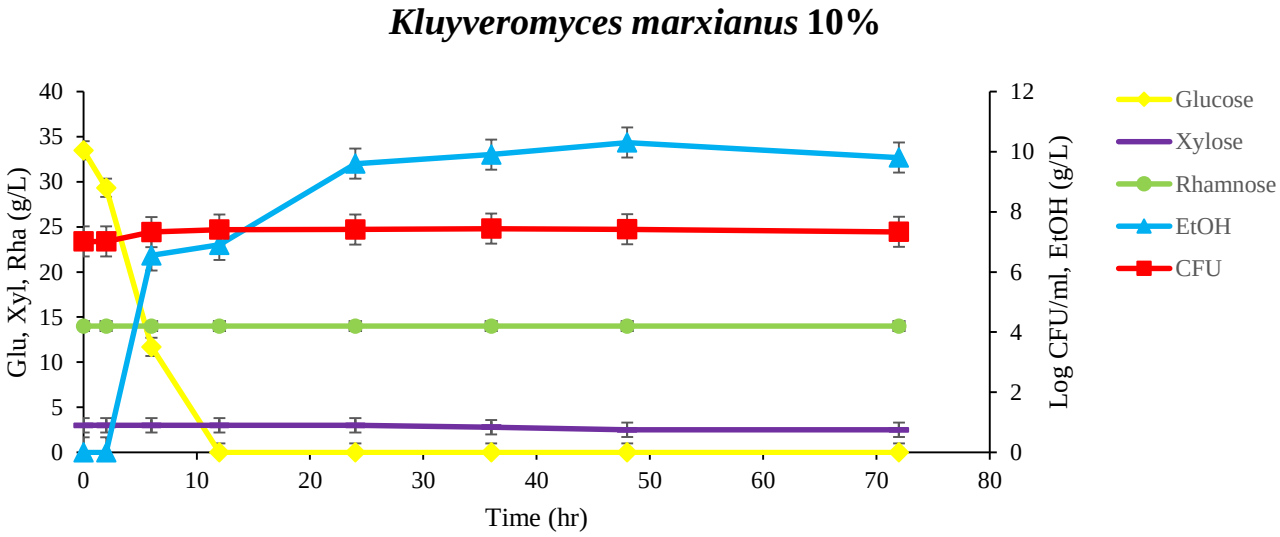

Figure 19. Effects of loading of 10% substrate on bioethanol production using *Kluyveromyces marxianus* K-21

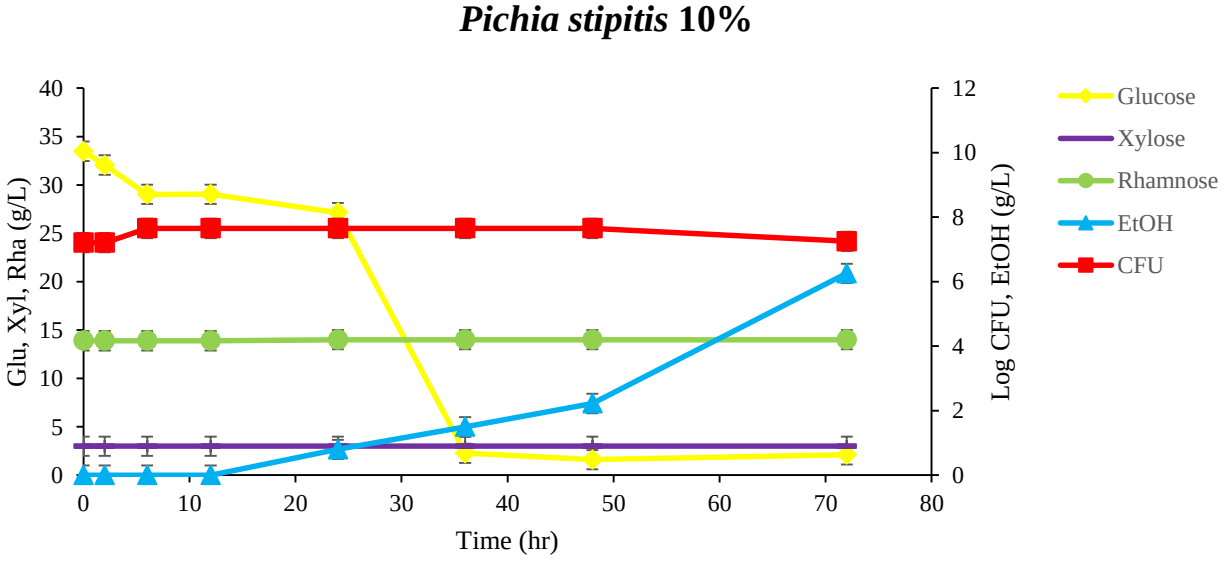

Figure 20. Effects of loading of 10% substrate on bioethanol production using *Pichia stipitis* BCRC 21777

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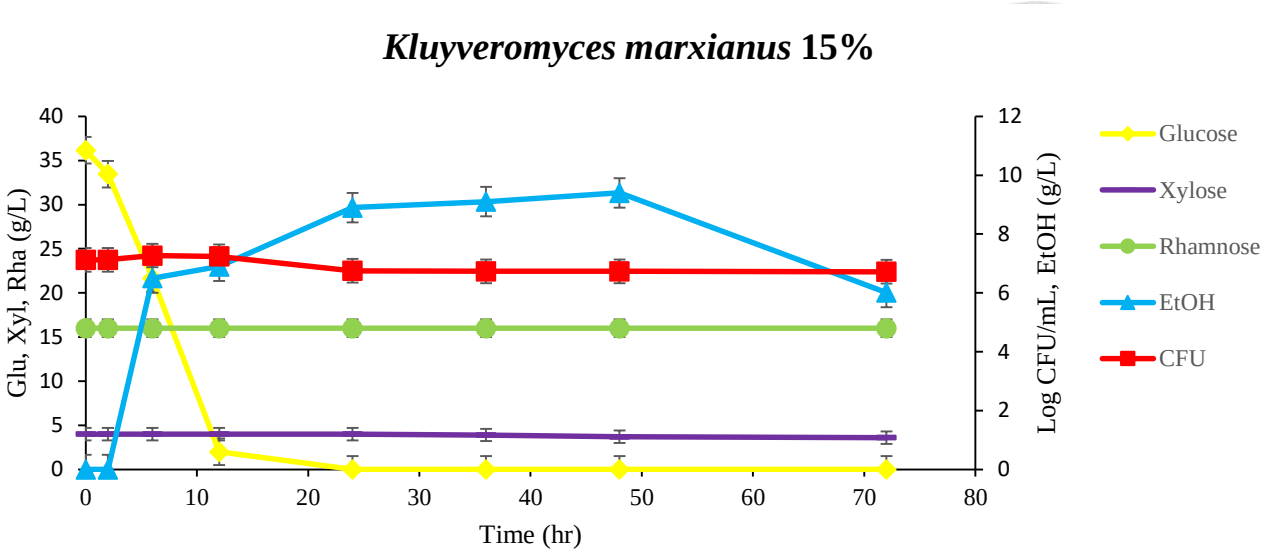

Figure 21. Effects of loading of 15% substrate on bioethanol production using *Kluyveromyces marxianus* K-21

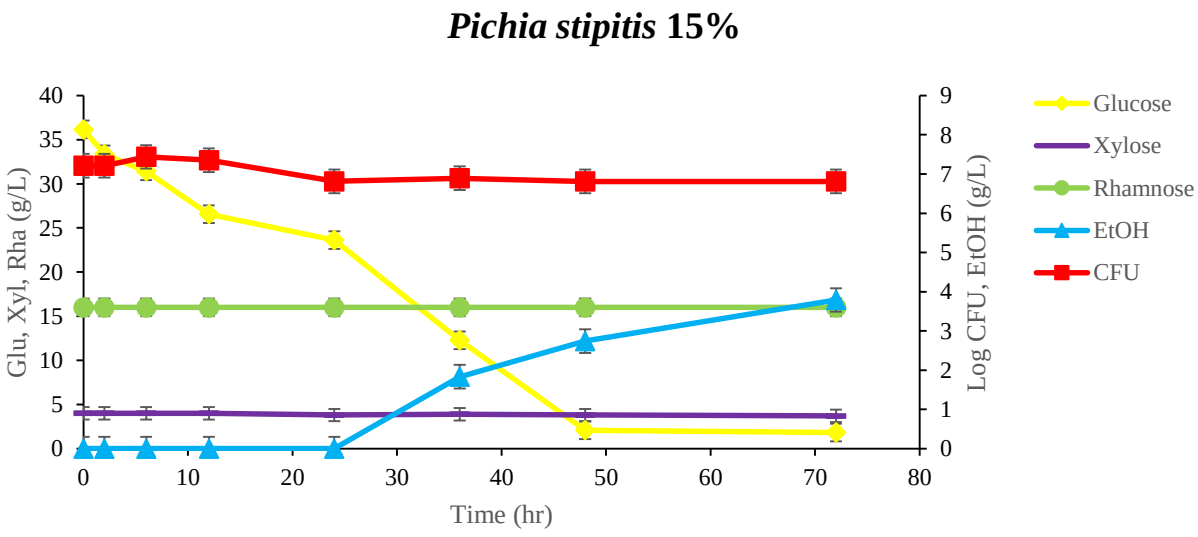

Figure 22. Effects of loading of 15% substrate on bioethanol production using *Pichia stipitis*

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Table 14. Bioethanol production of loading 5, 10, and 15%

Yeast strains	Loading	Ethanol (g/L)
Kluyveromyces marxianus	5%	7.8±0.4
	10%	10.2±0.5
	15%	9.4±0.2
Pichia stipitis	5%	5.21±0.37
	10%	6.25±0.1
	15%	3.8±0.4

Data expressed as mean ±SD (n=3). Values were significantly different at $p<0.05$ as determined by one-way ANOVA analysis.

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4.2.1.4 Temperature Study

Temperature is one of the major constraints that determine the ethanol production because temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. To know the optimum temperature for ethanol production, the fermentation media (hydrolysate) were kept at 25,30, 35, and 40ºC. The maximum amount of ethanol was achieved 10.6 g/L of ethanol at 35ºC from the *Kluyveromyces marxianus* K-21 according to the results of this experiment the amount began to decrease once the temperature went above 35ºC to 8 g/L ethanol at 40ºC. The ethanol concentration from 30 and 35ºC increased only slightly from 10.3 g/L to 10.6 g/L. Obata et al., 2016 achieved maximum ethanol concentration of 6 g/L using *Kluyveromyces marxianus* K-21 fermenting *L. digitata* and 0.7 g/L using *A. nodosum*.

For the *Pichia stipitis* BCRC 21777 what was noticed is that it is a poor ethanol producer at 35ºC a total of 6.8 g/L was achieved, like 30ºC ethanol was slightly increased from 4.5 g/L to 6.8 g/L. The results from this experiment may also be compared to that of Obata et al., 2016; comparing both *Kluyveromyces marxianus* and *Pichia stiptis* also found that *Kluyveromyces marxianus* always produced a higher amount of ethanol as compared to *Pichia stipitis* and at a faster time. Further, the increasing temperature reduced the percentage of ethanol production and it is mainly due to denaturation of the yeast cells (Periyasamy et al., 2009) as what was noticed in this experiment since no ethanol was produced by *Pichia stipitis* at 40ºC and a reduction of ethanol was noticed by *Kluyveromyces marxianus* at that same temperature. Khan et al., (2012) studied the effects of temperature on bioethanol production and observed that maximum bioethanol was produced at 35°C as compared to bioethanol produced at 23 and 28°C, respectively. Based on the temperature study there was little consumption of xylose and no consumption of rhamnose by the two yeast.

Figure 23-27 shows the Log CFU/mL for yeast at the varying temperatures. As can be seen, the maximum biomass concentrations for the temperature range between 25 and 35°C were of the same order of magnitude. At temperatures above 35ºC, the increase in temperature from 35 to 40°C was found to decrease the corresponding biomass concentration. For the *Pichia stipitis* as a result of its non-tolerant nature was unable to grow. This observation suggests that at a temperature of 35ºC, the increase in metabolic activity due to an increase in temperature is outweighed by the denaturation of the enzyme system within the cell; at temperatures above 35ºC, the effect of denaturation will become more and more pronounced over the increase in metabolic activities due to an increase in temperature

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(Kusmiyati and Shitophyta , 2015). Figure 23-27 also details the effect of temperature on total sugar utilization. The rate of total sugar utilization increased with temperatures up to 35°C but was found to decrease as the temperature was increased beyond 35°C.

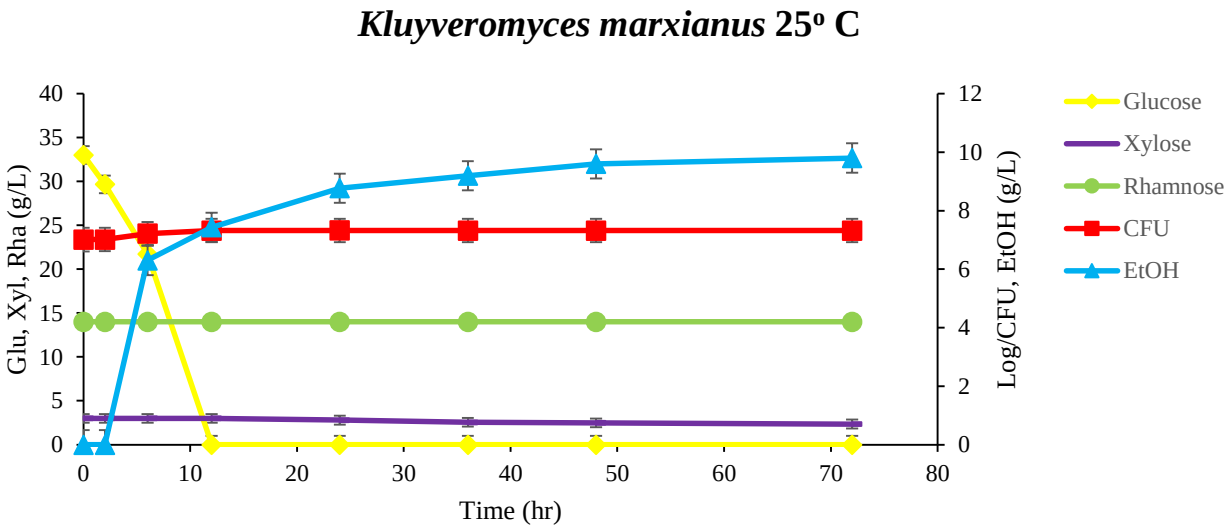

Figure 23. Substrate loading of 10% and fermentation at 25°C using *Kluyveromyces marxianus* K-21.

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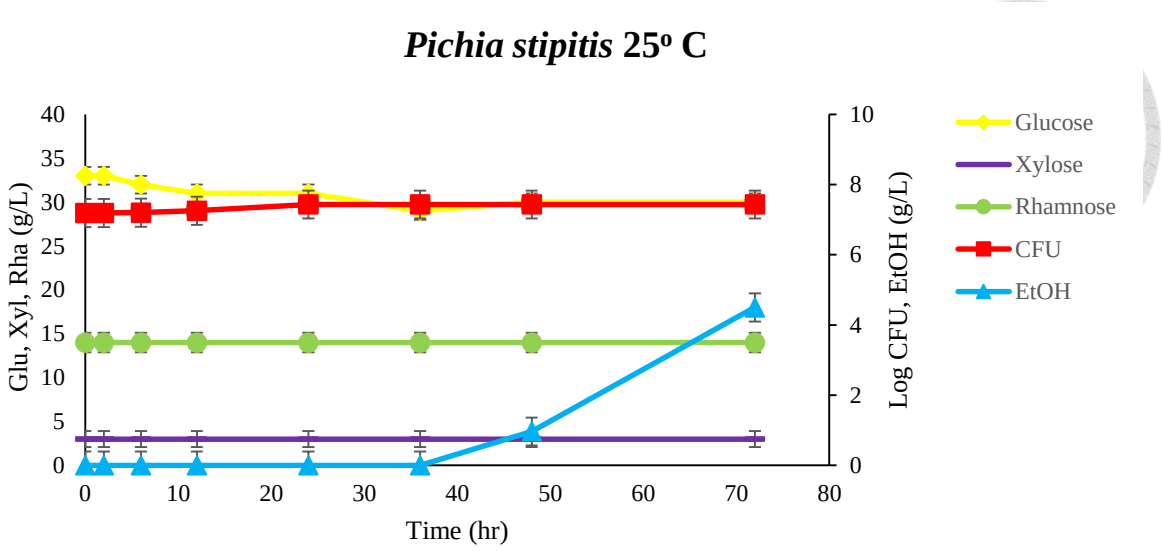

Figure 24. Substrate loading of 10% and fermentation at 25°C using *Pichia stipitis* BCRC 21777.

In this experiment as the temperature decreased to 25°C the glucose consumption for the *Pichia stipitis* BCRC 21777 was slower as compared to that of *Kluyveromyces marxianus* K-21, however, with the increase of temperature glucose consumption also increased. At this temperature xylose and rhamnose concentrations remained unused. Total amount of ethanol produced from *Pichia stipitis* BCRC 21777 was 4.5±0.35 g/L, while that of *Kluyveromyces marxianus* K-21 was 9.8±0.21 g/L.

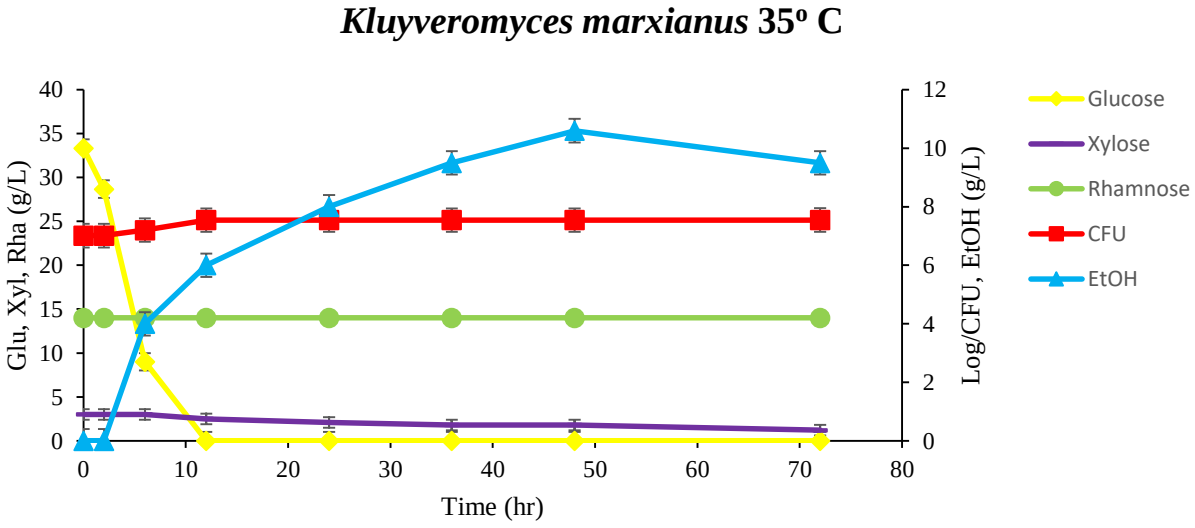

Figure 25. Substrate loading of 10% and fermentation at 35°C using *Kluyveromyces marxianus* K-21.

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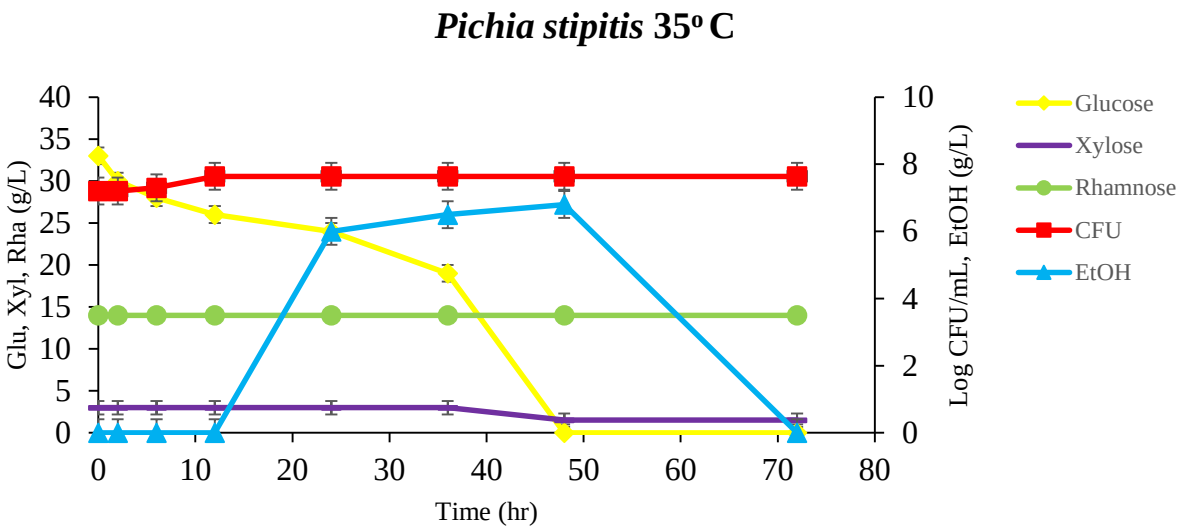

Figure 26. Substrate loading of 10% and fermentation at 35°C using *Pichia stipitis* BCRC 21777.

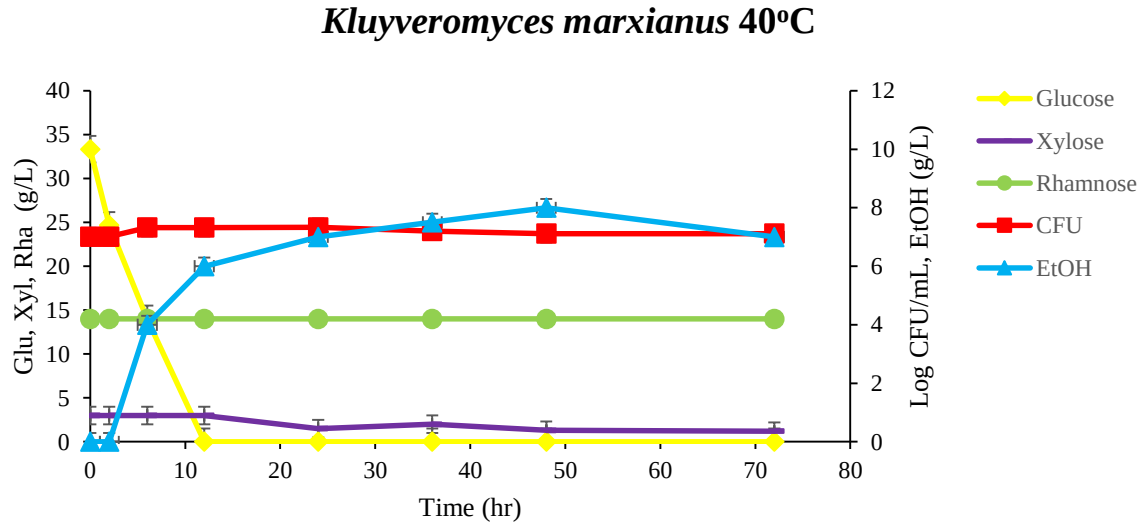

Figure 27. Ethanol production of *Kluyveromyces marxianus* (thermotolerant yeast) at 40°C at a substrate loading of 10%

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Table 15. Temperature influence on the bioethanol production of the two yeast strains.

Yeast strains	Temperature	Ethanol (g/L)
Kluyveromyces marxianus K-21	25	9.80±0.21
	30	10.2±0.50
	35	10.6±0.31
	40	8.00±0.26
Pichia stipitis BCRC 21777	25	4.50±0.35
	30	6.25±0.1
	35	6.80±0.2
	40	0.00

Data were expressed as mean ± SD (n=3). Values were significantly different at $p<0.05$ as determined by one-way ANOVA analysis.

4.2.1.5 pH Study

It was reported that high ethanol production was obtained by using initial pH 5.0 to 6.0 (Fadel, 2000), which was in agreement with the results of this study. The results for pH were much higher from a pH of 4 to pH 6. According to the results of this study *Kluyveromyces marxianus* K-21 at pHs' 4,6, and 9 produced ethanol with concentrations 9.50±0.25, 10.60±0.31, and 8.21±0.40. *Pichia stipitis* BCRC 21777 on the other hand produced ethanol concentrations of 6.20±0.40, 6.80±0.20, and 5.90±0.10 at those pH conditions. The ethanol concentration was increased from pH 4.0 to 6.0 and then decreased marginally above this value. Fadel (2000) reported that high ethanol production was obtained by using initial pH 5.0 to 6.0. Turhan et al. (2008) reported that maximum ethanol yield, maximum growth rate and biomass concentration were obtained at pH 5.5 on carob as a medium for ethanol production. Osman et al. (2011) tested wide initial pH range and found that at pH 3.0 no growth was observed and no ethanol was produced, while pH 6.0 was the optimum for both biomass and ethanol production. Similar results were obtained by Kadambini (2005). Mohanty et al. (2009) reported that pH 6.0 was optimum for bioethanol production from mahula (*Madhuca latifolia L.*) flowers by production from mahula (*Madhuca latifolia L.*) flowers by solid-state fermentation. Similar results were obtained by Togarepi et al. (2012) when *Ziziphus mauritiana* fruit pulp was used as a substrate (Akponah and Akpomie, 2012).

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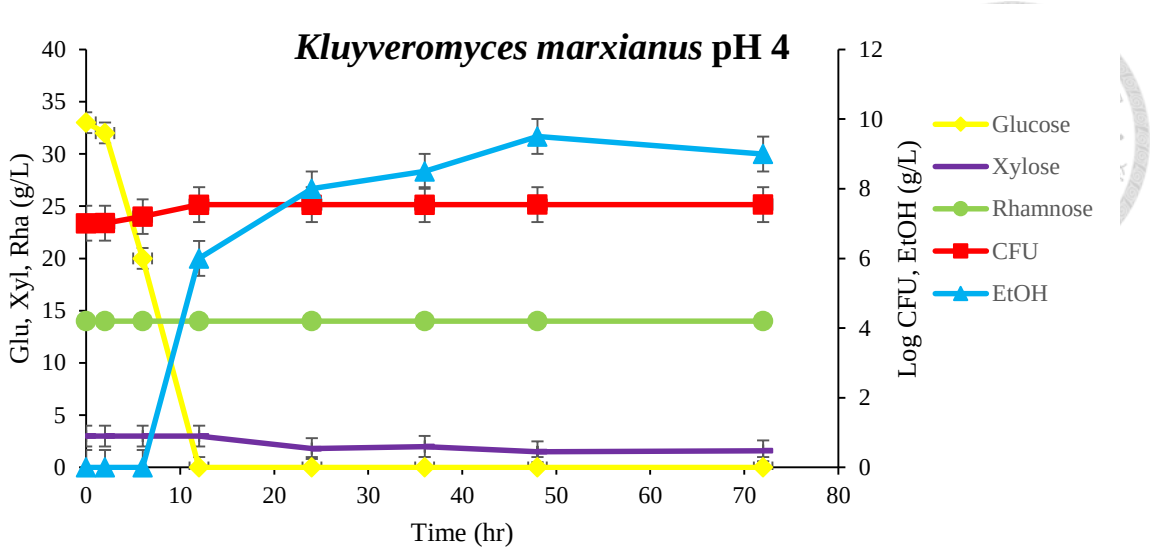

Figure 28. Fermentation of 10% hydrolysate using *Kluyveromyces marxianus* K-21 at pH 4.

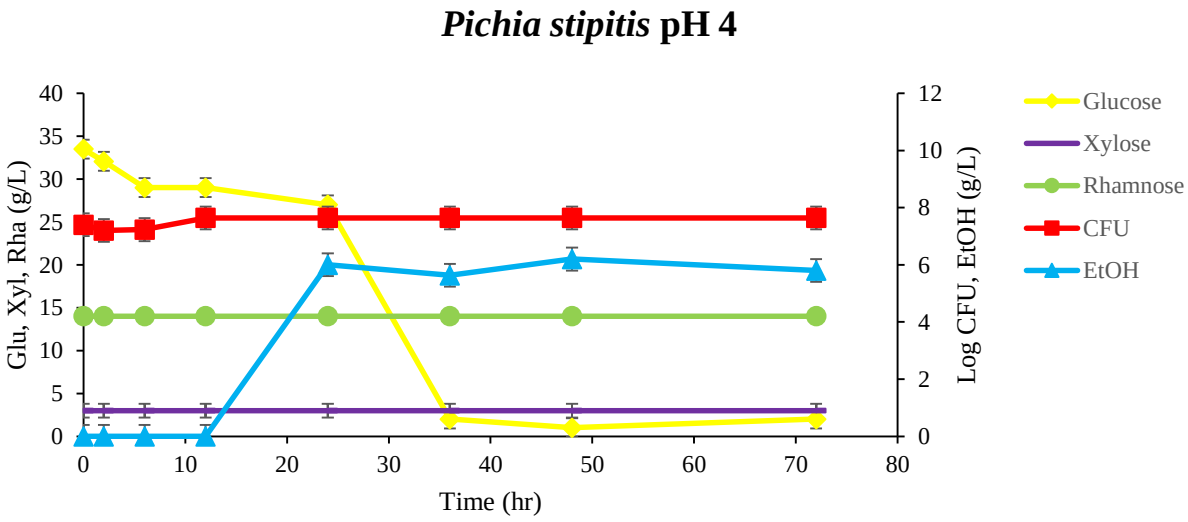

Figure 29. Fermentation of 10% hydrolysate using *Pichia stipitis* BCRC 21777 at pH 4.

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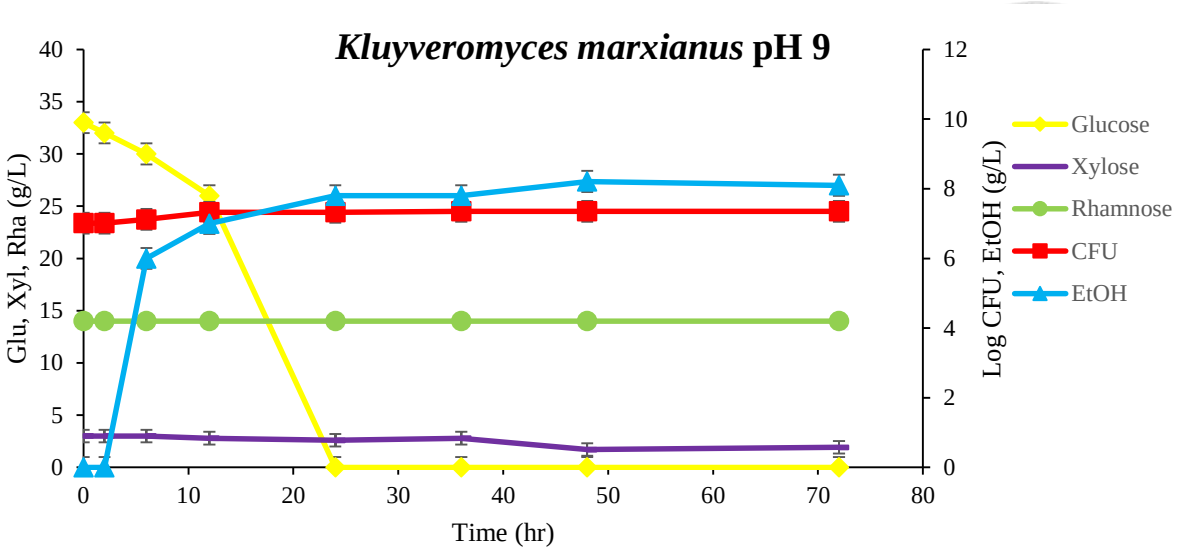

Figure 30. Fermentation of 10% hydrolysate using *Kluyveromyces marxianus* at pH 9

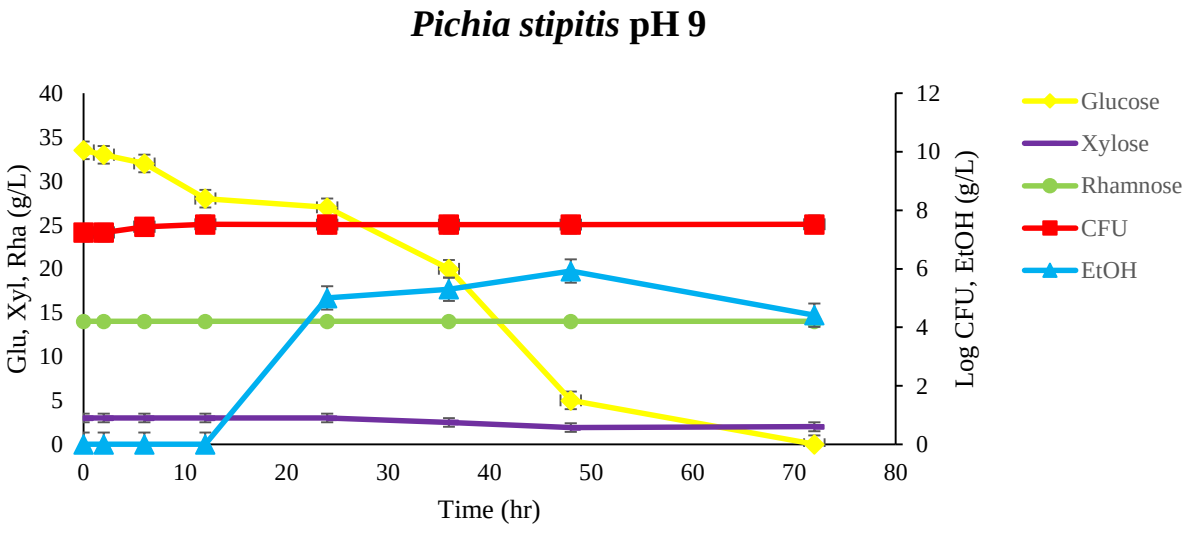

Figure 31. Fermentation of 10% hydrolysate using *Pichia stiptis* BCRC 21777 at higher pH 9.

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Table 16

The pH conditions that were tested against the optimum temperature of 35°C

pH	K-21 EtOH (g/L)	BCRC 21777 EtOH (g/L)
4	9.50±0.25	6.2±0.4
6	10.6±0.31	6.8±0.2
9	8.21±0.4	5.9±0.1

Data were expressed as mean ± SD (n=3). Values were significantly different at $p<0.05$ as determined by one-way ANOVA analysis.

4.2.1.6 Co-culture Study

Co-fermentation of biomass derived sugars with glucose and xylose consuming yeast has been proposed as a promising strategy for maximizing ethanol production from lignocellulose biomass hydrolysate. In this study co-culture has been optimized to maximize the amount of ethanol produced from *Ulva* hydrolysate. In the experiment both yeast was able to increase ethanol concentration significantly according to the ANOVA analysis to 11.5 g/L; it didn't increase by much this result has been evident in previous works of Rouhollah et al., 2007, Suriyachai et al., 2013 and Karagoz and Ozkan, (2014). In all the researches what is evident is the poor ethanol production from *Pichia stipitis* and also evident was how slow it is at producing ethanol peak ethanol as compared to *Kluyveromyces marxianus.*

Figure 32 shows glucose consumption was improved and as can be seen xylose consumption was also increased. In an experiment conducted by Rouhollah et al., (2007) utilizing monocultures or *Saccharomyces cerevisiae, Kluyveromyces marxianus* and *Pichia stiptis* and also co-cultures of these individual yeast, based on their results the best combinations were that of *Pichia stipitis* and *Kluyveromyces marxianus* and again based on their results the ethanol production only increased by little. Also, another experiment by Adivikatla et al., (2011) study of the comparism between monocultures of thermotolerant *Saccharomyces cerevisiae* and *Pichia stiptis* and then co-culture of the two yeast resulted in more ethanol production in the co-culture and still abiding to the results of the current experiment ethanol production only increased by a numbers. Another possible explanation for the lower ethanol production from *Pichia stiptis* could be explained as a result of its lower

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ethanol tolerance as compared to *Kluyveromyces marxianus* (Azhar et al., 2017 and Preez et al., 1989).

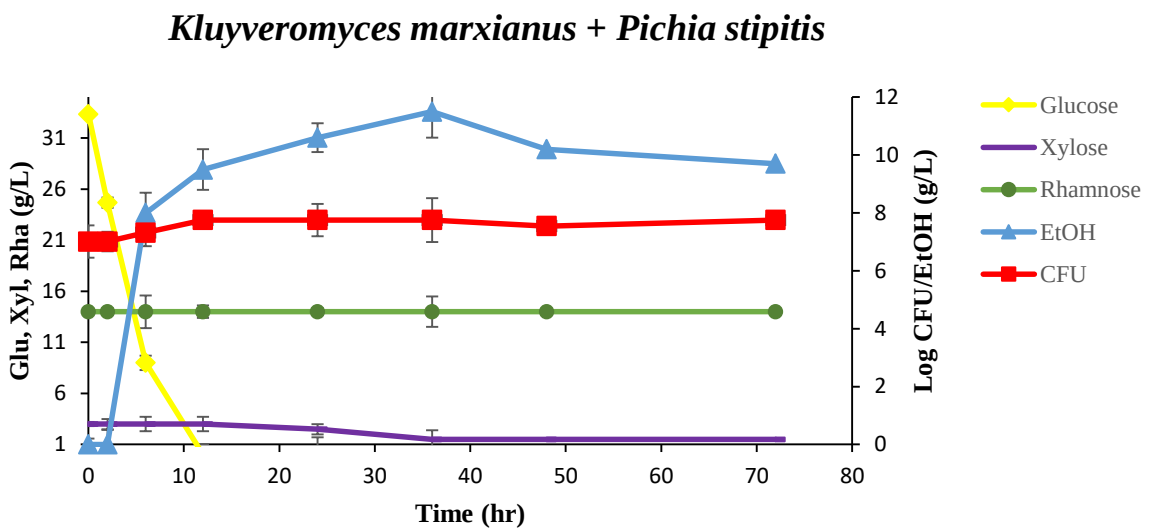

Figure 32. Co-culture of *Kluyveromyces marxianus* and *Pichia stipitis* on the hydrolysate

In co-culture experiment (Figure 32*), Kluyveromyces marxianus*, first, tend to increase in rate of hexoses fermentation and as a result xylose consumption will then started by *Pichia stiptis.* In this experiment peak ethanol was obtained at 36 hours and after that time period ethanol production gradually decreased. The ethanol concentration was only slightly higher as compared to an experiment performed by Othman, using different cocktails of yeast to study the effects on bioethanol production. In another experiment performed by Kim et al., 2011 ethanol production was increased using the co-culture of *Pichia stipitis* and *Saccharomyces cerevisiae*. In their experiment they also studied the effects of the co-culture on rhamnose degradation and their experiment observed the conversion of rhamnose to pyruvate by *Pichia stipitis* and then utilization of pyruvate into ethanol by *Saccharomyces cerevisiae*, however, the is phenomenon was not evident in the current research. One possible reason could be as a result of the fermentation conditions that the yeast was subjected to and as a result further information will be carried out in future experiments to determine better fermentative conditions for bioethanol production.

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5. Conclusion

In this experiment a total ethanol concentration of 10.60 ± 0.31 g/L using *Kluyveromyces marxianus* K-21 and 6.80±0.2 of ethanol was produced from 10% of *Ulva lactuca* powder at optimized conditions of 0 rpm, 35ºC and a pH of 6 using separate hydrolysis and fermentation strategy. Co-culture was proven to significantly improve the ethanol thought not by much to 11.5±0.7 g/L. Further experimental studies should be conducted to obtain a better understanding of co-culture on bioethanol production. In this experiment it also demonstrated that season and location plays a huge role in the carbohydrate composition of *Ulva lactuca*. Future work will be conducted to monitor the influences of co-culture on bioethanol production.

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7. Appendix

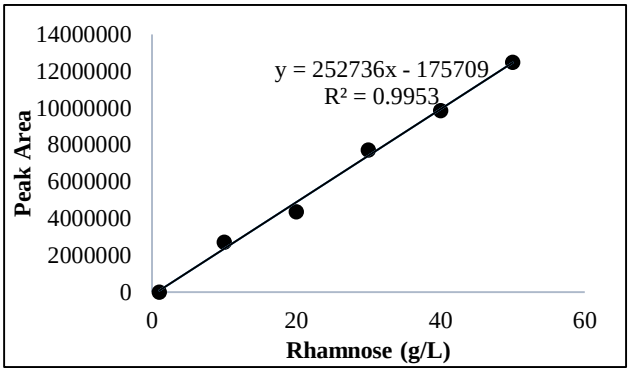

Figure S1. Rhamnose standard curve

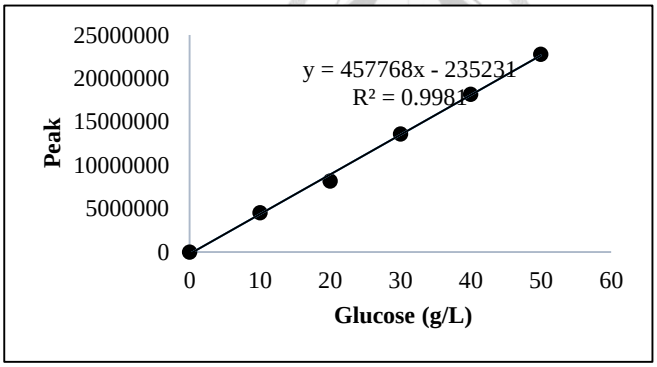

Figure S2. Glucose standard curve

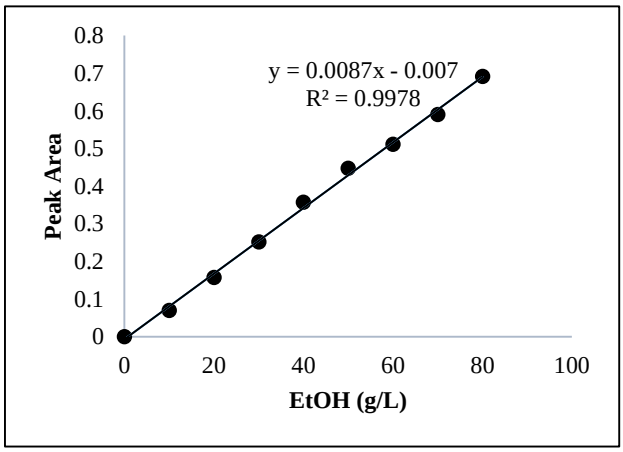

Figure S3. Ethanol standard curve

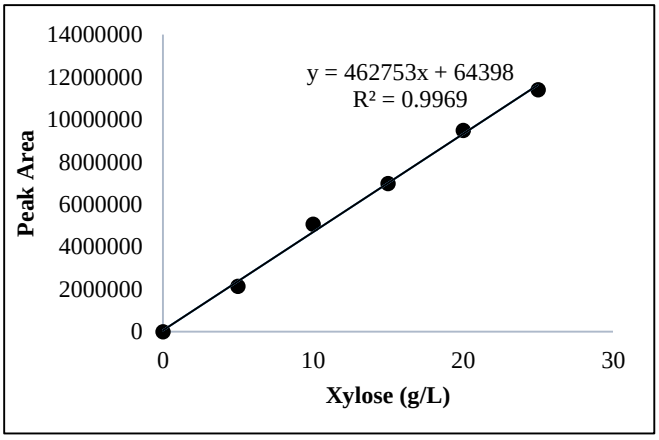

Figure S4. Xylose standard curve

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Time table

Ethanol Production from *Ulva lactuca*						
Platform	Feb	Mar	Apr	May	June	July
Hydrolysis	←	—	→			
Fermentation Flask study	←	—	→			
Fermentation Bioreactor study		←	—	—	→	
Writing thesis				←	—	→
Oral defense						↔

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Career Objective

To excel among the Professionals. Where I can utilize my skills with strong commitment and dedication, for the progressive growth.

Skills

- Computer literate; proficiency with MS word, Power Point
- Excellent language skills
- Stress and time management ability

Education

School/University Name	Year	Major	Degree
National Taiwan University	2016-2018 (Expected)	Food Microbiology	Master of Science (Pending)
National Pingtung University of Science and Technology	2011-2015	Animal Production	Bachelor of Science
Sir Arthur Lewis Community College	2009-2011	General Agriculture	Associate Degree

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