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Sustainable Production Of Biofuels And Biochemicals From Aquatic Biomass

Quazi Mahzabin Rahman
North Carolina Agricultural and Technical State University

Sustainable Production of Biofuels and Biochemicals from Aquatic Biomass

Quazi Mahzabin Rahman

North Carolina A&T State University

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Quazi Mahzabin Rahman

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Approved by:

Dr. Lijun Wang Major Professor	Dr. Abolghasem Shahbazi Committee Member
Dr. Shamsuddin Iias Committee Member	
Dr. Stephen Kinsley Department Chair	Dr. Sanjiv Sarin Dean, The Graduate School

Biographical Sketch

Quazi Mahzabin Rahman was born in 1988 and grew up in the city of Kolaroa, Bangladesh. She earned her B.Sc from the Department of Chemical Engineering at Bangladesh University of Engineering and Technology in 2011. During the period of her engineering education, she achieved brilliant academic performance while she was also involved in many campus activities. Quazi was a positive volunteer in serving any non-profit social activities. After her graduation she was awarded a research assistantship to pursue her M.S degree at North Carolina Agricultural and Technical State University. Quazi has been awarded for her excellent academic performance. Her GPA for the master degree program is 3.88.

Quazi has participated in 'National Conference on Advances in Environmental Science and Technology' at September 12, 2013, and made a poster presentation on 'Ethanol production from Fermentation of Green Microalgae by Escherichia coli'

After completing her M.S. degree, Quazi is going to serve as an engineer in Chemical and Bioengineering.

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Abstract

In recent years, growing attention has been devoted to the conversion of biomass into biofuels and biochemicals. Biomass has several reasons to be considered as an attractive energy source. The main reason is that energy produced from biomass can contribute to sustainable development. Aquatic biomasses—microalgae, duckweed, and cattail—are considered as promising biomass sources due to their favorable characteristics such as high growth rate and low lignin content. The objective of this study was to produce biofuel- ethanol through Simultaneous Saccharification and Fermentation (SSF) of fresh algae, duckweed and cattail biomass by *E. coli* and to produce acetic acid by *C. thermoaceticum* fermentation of cattail, using ultrasonic pretreatment and 4% NaOH pretreatment, respectively. Effect of change in process parameters (Enzyme concentration, temperature, *E. coli* concentration) on ethanol yield and protein content was investigated for microalgae. For microalgae, highest ethanol yield at 77.7% of theoretical value was obtained at 37°C with 0.5g/L *E. coli* concentration, with 15 FPU cellulase/g glucan and with 0.5% (w/v) biomass concentration. The protein content of fresh algae after SSF was increased and the highest protein of the algal residue was 10%. For fresh duckweed fermentation, combined heat treatment and ultrasonic treatment resulted in the highest ethanol yield at 96% of theoretical value obtained at 37°C, 15FPU enzyme concentration and 1% (w/v) biomass concentration. For cattail, the ethanol yield at 8% of theoretical value was obtained. Although the ethanol yield from SSF of fresh cattail was very low, cattail juice was used successfully to grow microalgae *Chlorella* sp. Acetic acid of 0.212g/g of biomass was obtained by *Clostridium* fermentation of pretreated cattail. Production of acetic acid was hindered by the initial acetate concentration.

CHAPTER 1

Introduction

The quick growth of the world population and rapid progress of a number of emerging economies have both directed to a sharp increase in global energy consumption. However, the increasing cost of fossil fuels as well as the escalating social and industrial awareness of the environmental impacts associated with the use of fossil fuels has created the need for more sustainable fuel options which are both more economic and environmentally friendly. Biomass is one of the most promising renewable resources used to generate different types of biofuels such as biodiesel and bioethanol [1, 2]. In recent years, growing attention has been devoted to the conversion of biomass into fuel ethanol, considered the cleanest liquid fuel alternative to fossil fuels. Significant advances have been made towards the technology of ethanol fermentation [3]. Biomass has several reasons to be considered as an attractive energy source. The main reason is that energy produced from biomass can contribute to sustainable development. Resources are often locally obtainable, conversion of biomass into secondary energy carriers such as biofuels is feasible without high capital investments and biomass energy can play an important role in reducing greenhouse gas emissions. Furthermore, since energy plantations may also generate new employment opportunities in rural areas, it also contributes to the social aspect of sustainability. In addition, application of agro-industrial residues in bioprocesses not only provides alternative substrates but also helps solve their disposal problem. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new opportunities have opened for the effective utilizations of biomass [3-5].

Corn grain is currently the dominant feedstock for bioethanol production in the United States [6, 7]. In 2009, the US ethanol industry produced a record of 40 billion liters of ethanol from corn starch, at an increase of 18% over the previous year [6, 8]. However, since corn is also an important food source, its conversion for energy purposes would put much stress on food supplies [9]. Moreover, intensive corn production has raised environmental concerns such as high requirements for agricultural inputs, which results in substantial environmental pollution, and soil erosion than that of any other crop [5, 6]. Therefore, it is necessary to investigate novel biomass sources to supplement corn starch to make ethanol industry more sustainable and environmentally friendly [6].

Currently, most of bioethanol research is focused on the exploitation of (ligno-) cellulosic sources either from non-food crops, or waste residues from agriculture (second generation biofuels). Lignocellulosic biomass generally contains 55–75% (of dry matter) carbohydrate [10]. Although lignocellulosic biomass are favorable because of its superiority in productivity, second generation biofuels are uneconomic, due to high cost involved in biomass handling and pretreatment process. Lignocellulosic biomass contains a large amount of lignin which reduces the accessibility of cellulose by cellulase enzyme. Indeed less than 20% of cellulose in native biomass can be enzymatically saccharified unless effective and energy-intensive pretreatments are carried out [11].

Aquatic biomass such as microalgae, duckweed and cattail are considered as promising biomass sources for the production of biofuels due to their favorable characteristics such as high growth rate and low lignin content. Microalgae are gaining interest in the current energy scenario due to their high photosynthetic rate, fast growth potential coupled with relatively high contents of lipid, carbohydrate and nutrients. All of these properties render them an excellent

source for biofuels; as well as a number of other valuable pharmaceutical products [12, 13]. The current interests in producing bioethanol are focused on microalgae as a feedstock for fermentation process [14]. Microalgae like *Chlorella, Chlamydomonas, Dunaliella, Scenedesmus,* and *Tetraselmis* possess high carbohydrate content. These carbohydrate-rich microalgae can be used for bioethanol production via various hydrolysis strategies and fermentation processes. To date, few studies have reported using microalgae for ethanol production [1]. Duckweed, from the family *Lemnaceae*, is the world's smallest angiosperm and a small, free-floating aquatic plant with the characteristics of fast multiplication, easy to grow, and resistance to bacteria [15]. Depending on the duckweed species and the growing conditions applied, starch content ranging from 3 to 75% and cellulose content of 10-25% have been reported. Duckweed has the potential to decontaminate effluent streams from swine wastewater and food processing. Duckweed also has low-lignin content. Hence it could provide a more suitable source of cellulose for the production to biofuels [6, 11]. *Typha* species, commonly known as Cattails have been identified as a particularly suitable biomass crop for wetlands mainly because of their superiority in productivity (40+ metric ton/ha standing crops). Furthermore, Cattails have better pest resistance, adaptability, and chemical composition than some of the available lignocellulosic biomass. It has been reported that cattails contain 47.6% cellulose and 21.9% lignin. Based on this composition, it is possible that, after appropriate fractionations, cattails could be a good source for the production of fuel ethanol [16, 17].

In this study three aquatic biomasses—algae, duckweed, and cattails were chosen as biomass sources to produce biofuel—ethanol through simultaneous saccharification and fermentation process by *E. coli*. Fresh biomass was used instead of dry biomass to reduce the cost involved drying during conventional biomass handling and to take advantage of high

moisture content of these three aquatic biomasses. Besides ethanol production, cattail juice was used as a growth medium of microalgae and recycled to the fermentation process to enhance the sustainability.

1.1 Thesis Objectives

The overall objective of this research was sustainable production of biofuels and biochemicals from three fresh aquatic biomasses—algae, duckweed, and cattail (Figure 1-3).

The specific objectives of this thesis research were to:

1. Investigate and compare the conversion of fresh biomass of cattail, duckweed and microalgae into biofuel-ethanol through Simultaneous Saccharification and Fermentation (SSF) process using various pretreatment methods;
2. Analyze the effect of fermentation on protein content of microalgal biomass;
3. Investigate the usefulness of liquid juice separated from biomass as a culture medium for microalgae; and
4. Investigate the conversion of lignocellulosic biomass cattail into acetic acid by the fermentation of *Clostridium thermoaceticum*.

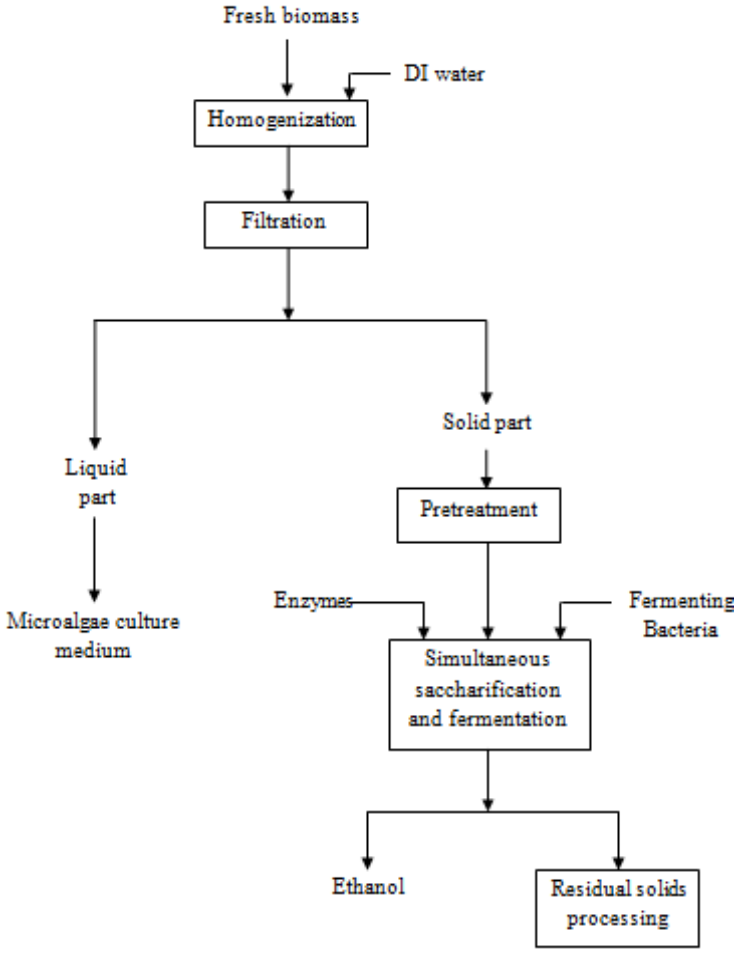

Figure 1. Block diagram of ethanol production from fresh cattail biomass.

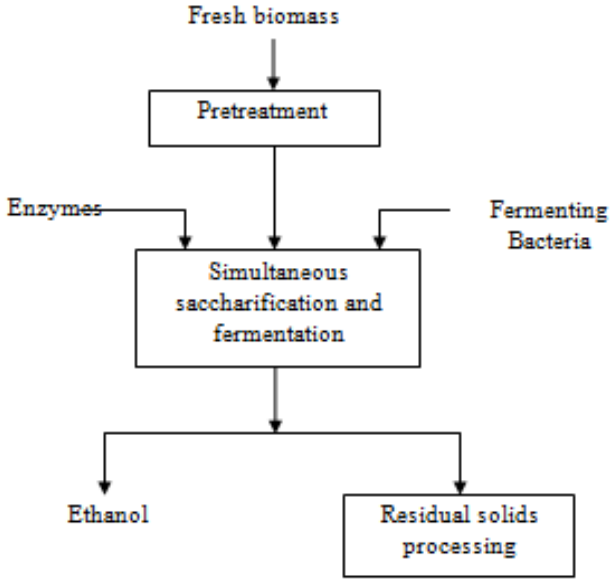

Figure 2. Block diagram of ethanol production from duckweed and algae biomass.

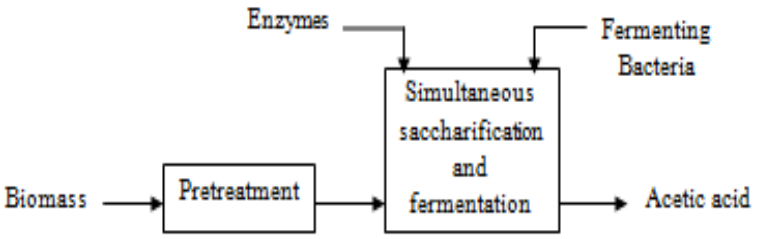

Figure 3. Block diagram of acetic acid production from biomass.

CHAPTER 2

Literature Review

2.1 Current Status of Production and Utilization of Bioethanol

During the last decade, the production of ethanol from biomass materials received more attention in the United States (U.S.) and worldwide. In the U.S., bioethanol is primarily produced from corn starch feedstocks while in Brazil biofuel is mainly produced from sugarcane juice and molasses. Together, these two countries account for 89% of the current global bioethanol production. Table 1 shows the world production of fuel ethanol in 2008. According to the statistics conducted by the renewable fuels association, the production of bioethanol in the U.S. by year 2009 was 10.9 billion gallons representing 55% of the worldwide production [18]. On average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol [19].

Table 1

World Production of Fuel Ethanol in 2008 [20] and [21]

Country	Millions of gallons	Country	Millions of gallons
USA	9000.0	Other	128.4
Brazil	6472.2	Thailand	89.8
European Union	733.6	Cambodia	79.29
China	501.9	India	66.0
Canada	237.7	Australia	26.4
Total	17,335.2		

European countries produce only 5% of the total amount of bioethanol worldwide while biodiesel produced in Europe primarily in France and Germany remains by far more substantial and accounts for approximately 56% of the global production. Although, most of the remaining

countries in the world collectively account for only 5% of the global bioethanol production, China, Thailand as well as India are continuing to invest substantially in agricultural biotechnology and emerge as potential biofuel producers [18].

2.2 Biomass for Bioethanol Production

Various biomass sources have been identified as alternate source of energy fuels. These biomass sources range from various kinds of bio-wastes such as food wastes, municipal wastes and agricultural wastes, energy crops such as switchgrass, edible and non-edible oilseeds and various aquatic plants [13]. Currently, bioethanol is mainly derived from sucrose (e.g., sygarcabe) and starch crops (e.g., corn) as well as lignocellulosic materials (e.g., rice straw and switchgrass) [22]. However, the use of agricultural crops or agricultural wastes as feedstock for bioethanol production still presents a number of problems such as high demand of arable lands and water supply, and high costs involved in converting lignocellulosic materials into ethanol [1].

Cellulosic ethanol is attractive because feedstocks that include the crop residues such as wheat straw, corn stover, grass, paper, cardboard, wood chips, and other fibrous plant material, are cheap and abundant. As the lignoellulosic feedstock is outside the human food chain, it does not raise moral or ethical issues like the use of corn. The conversion of cellulosic feedstocks into ethanol requires less fossil fuel compared to corn so the production of lignocellulosic ethanol can reduce more greenhouse-gas emissions than corn ethanol [20].

Cellulosic biomass contains three main components: cellulose, lignin, and hemicelluloses. On average, lignocellulosic biomass has around 26% lignin, 44% cellulose and 30% hemicellulose [20, 23]. Cellulosic biomass also contains sugars, but they are much harder to extract than those in corn, sugarcane, and other starchy biomass. Therefore, special

pretreatments are necessary to release the sugars. Three major steps are involved in production of cellulosic ethanol: pretreatment, hydrolysis, and fermentation. Several by-products formed during the pretreatment process can inhibit fermentation, and also some of the sugars from cellulosic biomass are difficult to be fermented by a microorganism. A process flow diagram showing the essential steps of production of ethanol from cellulosic biomass is given in Figure 4.

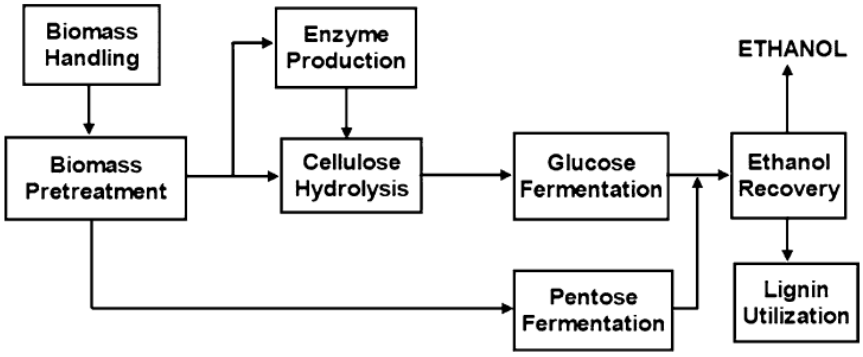

Figure 4. Processing of cellulosic biomass for ethanol production [20].

2.3 Aquatic Biomasses for the Treatment of Wastewater and Production of Biofuels

2.3.1 Cattail. The genus *Typha*, a member of the grass family group is extremely resilient in that it can grow in a wide variety of wet conditions. They can tolerate high quantities of salts and are useful in phytoremediation, not only cleaning waste from water supplies but taking up heavy metals, chemicals, and consume microbes. Cattails planted in shallow sewage wastewater plants can be used as effective secondary sewage treatment and achieve two to three times of their growth in a natural environment [16, 24].

The majority of the starch in cattails is in the rhizomes which constitute the majority of the dry weight of the plants. The rest of the plant is mostly cellulose with a small but significant quantity of fermentable sugars in the leaf and stock. Hence the entire plant can be processed to supply sugars for fermentation. It has been reported that cattails contain 47.6% cellulose and 21.9% lignin. Glucose from cattails cellulose can be efficiently fermented to ethanol with an

approximately 90% of the theoretical yield [25]. Different pretreatment methods such as dilute acid pretreatment, alkaline pretreatment and hot water pretreatment have been used to treat dry cattail for the production of bioethanol [16, 25]. In this study fresh green cattail pretreated with an ultrasonic homogenizer was used for ethanol production to save the energy for drying cattail.

2.3.2 Duckweed. Duckweed is a small, green floating aquatic plant belonging to the *Lemnaceae* family that can be easily found in quiescent or slowly flowing waters and also in relatively polluted waters worldwide [26]. It has a longer production period than most other plants, even growing year-round in some areas with a warm climate [27]. It accumulates its biomass at more rapid rates than other higher plants, including agricultural crops. Duckweed has a doubling time of 2–7 days [15]. Besides, duckweed shows a high ability to remove nutrients from wastewater, and it has been widely applied for the treatment of municipal and industrial wastewaters in many countries, including Bangladesh, Israel and the USA [28-30].

Currently, duckweed is used as animal fodder due to its high protein content. One way to produce large amounts of duckweed with low cost may be to use nutrients in animal waste lagoons [31, 32]. *Lemna minor* 8627 that was selected from 41 geographic isolates was found to remove 83% TKN, 100% NH_3-N, 49% P, and 68% TOC within 12 days when grown on 50% swine lagoon effluent [33]. The biomass production of duckweed was higher when grown in a municipal settling pond with a yield ranging from 10-14 g/m^2/day or 40-56 dry tons/hectare/year [26]. Thus, the co-production of protein and bio-fuels from duckweed grown in swine lagoons has the strong potential to be economically feasible.

Duckweed has a dry weight protein content around 35%, cellulose content around 12%, hemicellulose around 14%, lignin around 3%, starch around 3.4%, and crude fat around 3.5% [34]. The annual yields of the duckweed *Spirodela polyrrhiza* and *Lemna gibba* were 20.4 and

54.8 t/ha in dry matter (DM) [35, 36], compared to the annual yields of 5.22 and 7.66 t/ha for corn and cornstover respectively [37]. With duckweed's high biomass production rate, most methods to upgrade duckweed into more valuable bioethanol render yields on a per area/per year basis competitive with any other feedstock available. As duckweed has a very little amount of lignin compared to other lignocellulosic biomass, enzymatic hydrolysis or dilute acid hydrolysis is the most feasible approach to release the sugars from the duckweed for the conversion of duckweed to bioethanol. Additionally, duckweed contains several co-products that could reduce the overall cost of ethanol production. There are several previously unaccounted benefits to duckweed. First, duckweed has a high protein content of approximately 35% of its dry mass, which could be extracted and used as animal feed. Secondly, when duckweed grows, it absorbs nutrients from the water; thus, duckweed acts as a bioremediator. This feature would be beneficial to farmers with swine lagoons or potentially integrated into a waste water treatment facility. Thus, when viewed as part of a bigger, integrated system designed to treat wastewater, to generate protein rich animal feed, and to produce cellulosic ethanol, duckweed becomes a very interesting plant.

Research has also shown that duckweed is a potential starch source for ethanol production. Depending on the duckweed species and the growing conditions, starch contents ranging from 3 to 75% have been reported. Using enzymatic hydrolysis and yeast fermentation of high-starch duckweed biomass, 94.7% of the theoretical starch conversion was achieved in pilot scale study [6]. However, there are only a few published reports on ethanol fermentation from duckweed [22], [24]. This study was to improve the ethanol yield from duckweed by increasing sugar release through enzymatic treatment and acid hydrolysis. Finally, ethanol production from pretreated duckweed by *E. coli* KO11 was also investigated.

2.3.3 Microalgae. Microalgae have recently been considered as a third generation feedstock for biofuel production [22], with the focus on the production of biodiesel from microalgae [38, 39]. However, since some microalgae species have high carbohydrate content in the forms of starch and cellulose, they are also excellent substrates for bioethanol production. The use of carbohydrate-rich microalgal biomass for bioethanol production is advantageous, since microalgae grow faster and fix CO_2 at a higher rate than terrestrial plants. In addition, microalgae based carbohydrates are mainly in the form of starch and cellulose with the absence of lignin, are thus much easier to be converted to monosaccharides compared with lignocellulosic materials [12, 40, 41].

Like other plants, many algal species have rigid cellulose-based cell walls and accumulate starch as their main carbohydrate storage compounds and cell wall structure, which contains an astonishingly diverse range of simple and complex carbohydrates. Some of marine algal species contain up to 70% of polysaccharides, i.e., cell wall polysaccharides (cellulose, hemicelluloses, xylan, and mannan), intercellular polysaccharides (sulfated glucuronoxylorhamnan, algine, agar, and carrageenin), and storage polysaccharides (amino pectin, laminaran and floridean starch). Both intercellular and cell wall polysaccharides can be converted into fermentable sugars. The majorities of algal polysaccharides are potential biochemical feedstock and can be fermented into ethanol [42]. Table 2 shows the amount of carbohydrates and protein measured from different algal species.

Table 2

Amount of Protein and Carbohydrates from Various Species of Microalgae on a Dry Matter Basis (%)[40, 43].

Algae strains	Proteins	Carbohydrates
Scenedesmus obliquus	50–56	10–17
Scenedesmus quadricauda	47	–
Scenedesmus dimorphus	8–18	21–52
Chlamydomonas rheinhardii	48	17
Chlorella vulgaris	51–58	12–17
Chlorella pyrenoidosa	57	26
Dunaliella bioculata	49	4
Dunaliella salina	57	32
Euglena gracilis	39–61	14–18
Prymnesium parvum	28–45	25–33
Tetraselmis maculate	52	15
Porphyridium cruentum	28–39	40–57
Spirulina platensis	46–63	8–14
Spirulina maxima	60–71	13–16
Synechoccus sp.	63	15
Anabaena cylindrical	43–56	25–30

The microorganisms of bacteria, yeast or fungi are used to ferment sugars hydrolyzed from carbohydrates into ethanol under anaerobic conditions. Besides the main product of ethanol, carbon dioxide and water are also formed as by-products. In general, according to simplified reaction equation below, theoretical maximum yields are 0.51 kg ethanol and 0.49 kg CO_2 per kg of carbon sugar, glucose [40].

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

The gross chemical composition of microalgae is highly dependent on environmental factors such as light intensity, temperature and availability of nutrients. Generally, microalgae contain varying proportions of proteins, lipids, carbohydrates, nucleic acids, pigments and vitamins. Carbohydrates in microalgae in the form of starch, glucose, sugars and other polysaccharides are present in concentrations ranging from 5% to 50% dry mass weight [39, 44]. Microalgae like *Chlorella, Chlamydomonas, Dunaliella, Scenedesmus*, and *Tetraselmis* have been shown to accumulate a large amount of carbohydrates [45]. Microalgae are a potential source of fermentable substrate as they may have high levels of carbon compounds in their composition, directly available for fermentation or after pre-treatment depending on their growth conditions. Several microalgae can be used to produce ethanol via fermentation, such as *Chlamydomonas sp., Chlorella sp., Oscillatoria sp., Cyanothece sp.*, and *S. platensis* [46]. The *Chlorella vulgaris* microalgae can be used as a source for the production of ethanol due to its high carbohydrate content, with conversion efficiency above 65.0%. Ueno et al. [46] obtained the maximum formation of ethanol from *Chlorella* sp. cultivated at 30.0°C of 448.0 μmol/g in dry weight [47].

Figure 5 shows the summary of the upstream and downstream of microalgal processing steps to produce ethanol. The carbohydrates from the cell wall must be hydrolyzed before they can be used as a feedstock for fermentation, which can be accomplished by pretreatment followed by enzymatic hydrolysis of the biomass into suitable fermentable sugars. The most effective enzyme concentration for a high ethanol yield should be 0.001–0.05%, based on the volume unit of the enzyme for every weight unit of the feedstock [48]. Compared to untreated microalgae, pretreatment increased the efficiency of the fermentation process by more than 33% [49] and the ethanol production by more than 60%. However, pretreatment increases the energy

consumption by up to 30% of the overall energy requirement for the fermentation process. Ethanol yields from microalgae can be further improved by combining them with a hydrolysis separation process. The production of ethanol from microalgae can be improved by using yeast in an immobilized fermenter. The immobilized reactor is capable of increasing ethanol yields by approximately five-fold relative to the 50% increases of glucose in feedstock [50]. The capital cost of ethanol production from algae was estimated to be approximately $1.75 per gallon reported by the Solution Recovery Service Company [50]. Thus, the estimated sale price of ethanol should be higher than this. The net life cycle energy consumption for the production of ethanol from microalgae was estimated to be 0.2–0.55 MJ for every 1 MJ of ethanol produced [13].

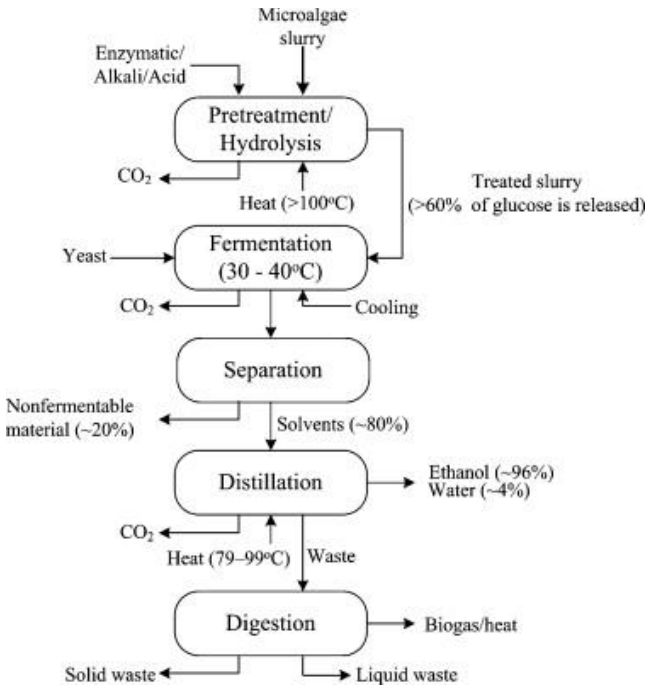

Figure 5. An overall summary for ethanol production from microalgae.

Very less research work has been reported on the fermentation of algae for ethanol production. Moen [51] showed that brown seaweed produced higher bioethanol than other algae species. Ueda et al. [52] patented a detailed system for microalgae fermentation; where,

microalgae were fermented in anaerobic and dark environment to produce ethanol and remaining algae biomass after fermentation were used in anaerobic digestion process. The ethanol produced from fermentation can be purified to be used as fuel and produced CO_2 was recycled to algae cultivation ponds as a nutrient to grow microalgae [40].

Even though limited reports on algae fermentation were found, a number of advantages were observed in order to produce bioethanol from algae. A fermentation process requires less energy and is simpler than a biodiesel production process. Besides, CO_2 produced as by-product from fermentation process can be recycled as a carbon source to cultivate microalgae to reduce the overall greenhouse gases emissions of the system. However, the production of bioethanol from microalgae is still under investigation and this technology has not yet been commercialized [40]. In this study fresh green microalgae pretreated with ultrasonic homogenizer were used for ethanol production and the effect of fermentation on protein content was studied.

2.4 Process Engineering of Ethanol Production from Biomass

The overall process of biological conversion of biomass into ethanol through fermentation consists of four major unit operations: pretreatment, hydrolysis, fermentation, and ethanol separation or purification [53].

2.4.1 Pretreatment. Biomass pre-treatment is one of the most crucial and expensive process steps that has been widely studied [54]. Hemicellulose and lignin content, cellulose crystallinity and available surface area of biomass are some of the major factors that affect the hydrolysis of cellulose and xylose for fermentation. The pretreatment step is necessary to reduce the crystallinity of the biomass and increase the surface area to enhance substrate digestibility. Pretreatment is used to improve the efficiency of hydrolysis by disrupting the cell wall [55]. Through the pretreatment process, carbohydrates entrapped in the cell wall become free and it

also helps to breakdown complex carbohydrates into fermentable sugars for bioethanol production [56]. After the disruption, the carbohydrates are released from the intracellular medium requiring further processing to obtain monosaccharides [45].

Pretreatment must meet the following criteria: (a) Improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (b) Avoid the degradation or loss of carbohydrate; (c) Avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (d) Be cost-effective. In order to disrupt the cell wall, various methods have been tested-Physical, physic-chemical, chemical, and biological processes as a pretreatment. The physical methods could be high-pressure homogenizers, bead beating, freezing, sonication, and autoclaving [9, 45, 56].

2.4.1.1 Physical pretreatment. Physical pretreatment involves the reduction of particle size and cellulose crystallinity. The reduction in particle size leads to an increase in surface area. Physical pretreatment are normally done by the comminution of the biomass materials via chipping, grinding, milling, bead-beating, freeze fracturing, ultrasonic cell disruptions and other mechanical methods.

Bead-beating is a method in which the mechanical damage is caused directly by the collision of minute glass or ceramic beads spinning on high speed with the microalgal biomass. The small particles are vigorously agitated by shaking or stirring. This method has been used both in the laboratory and at an industrial scale [57]. Freeze-fracturing, has also been used for the disruption of cellular walls and membranes in microorganism concentrates and plants and in animal tissues. This method consists in freezing the cells at very low temperature using liquid nitrogen and grounding them in a mortar. The cells are then disrupted due to their brittle nature and the abrasive action of ice crystals. The final product is a powder like material that can be

further processed by other methods [58]. Ultrasonic disintegrators are also widely used to disrupt cells. These devices generate intense sonic pressure waves in liquid media. Under the right conditions, the pressure waves cause the formation of micro bubbles which grow and then collapse violently in a process called cavitation. The resulting implosion generates a shock wave with enough energy to break cell membranes and walls and even covalent bonds. Ultrasonic disintegrators also generate a considerable amount of heat during processing, thus the sample should be cooled if needed [59, 60]. It was found that using sonication before enzymatic pretreatment increased the oil yield up to 97% for Jatropha [61].

2.4.1.2 Chemical pretreatment. The chemical lysis is a different approach to the biomass processing, where chemical agents of an acid or alkaline must be added (e.g., hydrochloric or sulfuric acid, sodium hydroxide) in order to hydrolyze the biomass into its constituent molecules [62]. Acids are used to solubilize hemicellulose, degrade the lignin and make cellulose accessible to enzymatic hydrolysis. Acid pretreatments are done with concentrated, dilute and weak organic acids. Strong acids such as sulfuric acid and hydrochloric acid in their concentrated and dilute forms have been used in the fractionation of lignocellulosic biomass. Acid hydrolysis of biomass releases oligomers and monosaccharides in a homogenous reaction where the acid catalyzes the breakdown of cellulose to glucose [10].

Alkaline pre-treatment has been reported to be promising in treating various biomass feedstocks. This method of pre-treatment is preferable due to the lower temperature and pressure involved. In addition, alkaline pre-treatment reduces the degree of inhibition during fermentation and provides a lower production cost compared with other pre-treatment methods. Dilute sodium hydroxide (NaOH) pretreatment of lignocellulosic biomass has been reported to cause swelling leading to a decrease in cellulosic crystallinity and degree of polymerization. An

increase in biomass surface area, separation of structural linkages between lignin and carbohydrates and disruption of lignin structure has also been reported by dilute sodium hydroxide pretreatment [54, 63, 64]. The alkaline pretreatment process can be improved further by the application of ultrasound [65]. The acid pre-treatment is more preferable as it provides higher efficiency in converting cellulosic materials. During an acid pre-treatment process, various parameters significantly influence the total amount of fermentable sugars released. These include process time, temperature, amount of substrate loading and acid concentration [56, 58].

2.4.1.3 Biological pretreatment. The biological pre-treatment involves the utilization of microbes and enzymes to degrade the biomass in order to release the fermentable sugars [56]. Some fungi and bacteria have been identified to have the ability to degrade lignin and some hemicellulose off the lignocellulosic materials. These microorganisms have very little effect on cellulose since the cellulose has more resistance than the other parts of lignocelluloses to be biologically attacked. Several fungal species (e. g. brown, white and soft rot fungi) have been used in biomass pretreatment [66]. For example, virus infection has been utilized to disrupt microalgal cell wall. This mechanism has been reported to have the potential to be applied in the pretreatment of biomass [67]. However, biological pretreatment is associated with a low hydrolysis rate which prolongs the completion time of the process step [56, 68]. In spite of the many cell disruption methods tested, the most efficient pretreatment method for microalgae has not yet been unequivocally confirmed by the scientific world [58].

2.4.2 Enzymatic hydrolysis. Enzymatic hydrolysis follows the pretreatment to break down the cellulose component of the lignocellulose into reducing sugars that can be further fermented to ethanol using a microorganism. Hydrolysis is the process of converting

carbohydrate polymers into monomeric sugars including hexoses and pentoses, which can be done chemically by acids or enzymatically by cellulases. Cellulases are mixtures of several enzymes that act as a group to reduce cellulose to glucose for fermentation. Cellulase enzymes are produced by both bacteria and fungi. Three main types of enzymes can be found in cellulases- endocellulases, exoglucanase or cellobiohydrolase and β-glucosidase [69]. Since the first application of microbial enzyme in the food industry in the early 1960s, many efforts have been made to replace traditional acid hydrolysis with enzymatic hydrolysis in almost all glucose production due to higher yields under mild conditions, less by-products, and no corrosion issues. Thus currently enzymatic hydrolysis is preferred in both researches and industries [68].

Mechanisms of cellulose hydrolysis have been reviewed on numerous occasions for second generation bioethanol production [14]. Three steps were considered in enzymatic hydrolysis of cellulose: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase. Cellulase activity decreases during the hydrolysis, which is partially due to the irreversible adsorption of cellulase on cellulose. Thus some studies introduced surfactants during hydrolysis to modify the cellulose surface property for minimizing the irreversible binding of cellulase on cellulose [9].

Many factors influence the enzymatic hydrolysis of cellulose including substrate concentration, accessible surface area of substrate, cellulase activity, enzyme loading, presence of inhibitors and reaction conditions such as temperature, pH, and other parameters [68]. Substrate concentration is one of the major factors that affects the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally consequences in an increase of the yield and reaction rate of the hydrolysis. However, high substrate concentration can lead to substrate inhibition, which considerably lowers the rate

of hydrolysis. The degree of substrate inhibition depends on the fraction of total substrate to total enzyme. The optimum substrate concentration during enzymatic hydrolysis is 10% (w/v) if rheological problems are to be avoided. Hydrolysis is carried out at specific pH and temperature which are normally 4.5-6.5 and 45-50°C. The susceptibility of cellulosic substrates to cellulases depends on the structural features of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin [9, 68]. Increasing the dosage of cellulase can enhance the yield and rate of the hydrolysis to some extent, but would significantly increase the cost of the process.

A cellulase dose of 10 FPU/g-cellulose is often used in the laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48-72 h) at a reasonable enzyme cost. Cellulase enzyme loadings in hydrolysis vary from 7 to 33 FPU/g substrate, depending on the type and concentration of substrates [9]. The formed product ethanol is also an inhibitor to the yeasts/bacteria that perform the fermentation. This puts a limit to the concentration of fermentable sugars. In addition, furfural, soluble lignin compounds in the liquid can inhibit, or even stop the fermentation [55].

Effectiveness of enzymatic hydrolysis, however, is still not cheap enough to make the hydrolysis of cellulose economically feasible, thus it is important to identify methods to overcome above barriers [68].

2.4.3 Microorganisms. Several reports and reviews have been published on production of ethanol through the fermentation by microorganisms, and several bacteria, yeasts, and fungi have been reportedly used for the production of ethanol [3]. Table 3 shows bacterial species mostly used by various researchers for production of ethanol as main fermentation product.

Table 3

Bacterial Species Used by Various Researchers for Production of Ethanol as Main Fermentation Product [20, 70].

Mesophilic organisms	mmol ethanol produced per mmol glucose metabolized
Clostridium sporogenes	Up to 4.15a
Clostridium Indoli (pathogenic)	1.96a
Clostridium sphenoides	1.8a (1.8)b
Clostridium sordelli (pathogenic)	1.7
Zymomonas mobilis (syn. Anaerobica)	1.9
Zymomonas mobilis subsp. Pomaceus	1.7
Spirochaeta aurantia	1.5 (0.8)
Spirochaeta stenostrepta	0.84 (1.46)
Spirochaeta litoralis	1.1 (1.4)
Erwinia amylovora	1.2
Escherichia coli KO11	0.7–0.1
Escherichia coli LY01	40–50 g ethanol produced/I
Leuconostoc mesenteroides	1.1
Streptococcus lactis	1.0
Klebsiella oxytoca	0.94–0.98
Kelbsiella aerogenes	24 g ethanol produced/I
Mucor sp. M105	–

Saccharomyces Cerevisiae is the primary species that has been exploited for ethanol production. Other microorganisms such as *Zymomonas mobilis*, and *Escherichia coli* have also been intensively studied over the past three decades [70]. *S. cerevisiae* can produce ethanol at a concentration of as high as 18% of the fermentation broth, and thus is the preferred one for most

ethanol fermentation processes. This yeast can grow on both simple sugars such as glucose and the disaccharide sucrose [3].

Zymomonas mobilis is an unusual gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homoethanol fermentation pathway and tolerates up to 120 g/l ethanol. It has a higher ethanol yield (5–10% more ethanol per fermented glucose) and has a much higher specific ethanol productivity than *Saccharomyces sp*. Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for the conversion of all the biomass resources because it ferments only glucose, fructose, and sucrose [3].

Recently, advancements in metabolic engineering and synthetic biology have led to the ability to use *Escherichia coli* as a biocatalyst for the production of a wide variety of potential biofuels from several biomass constituents. High productivities and yields of biofuels can be attained with *E. coli* due to its high growth and metabolic rates and tolerance to high concentrations of substrate and products. *E. coli* is capable of utilizing a variety of carbon sources such as glucose, xylose, galactose and arabinose, as well as noncarbohydrate carbon sources such as glycerol and fatty acids, which together encompass the main constituents of biomass [71].

The native pathway for producing ethanol in *E. coli* starts with the intermediate metabolite pyruvate. The two step reduction of acetyl-CoA to ethanol results in the consumption of two reducing equivalents and proceeds with acetaldehyde as the intermediate. Therefore, on a 3-carbon basis, the glycolytic pathway to pyruvate generates one reducing equivalent, while the synthesis of ethanol from pyruvate consumes two reducing equivalents (Figure 6).

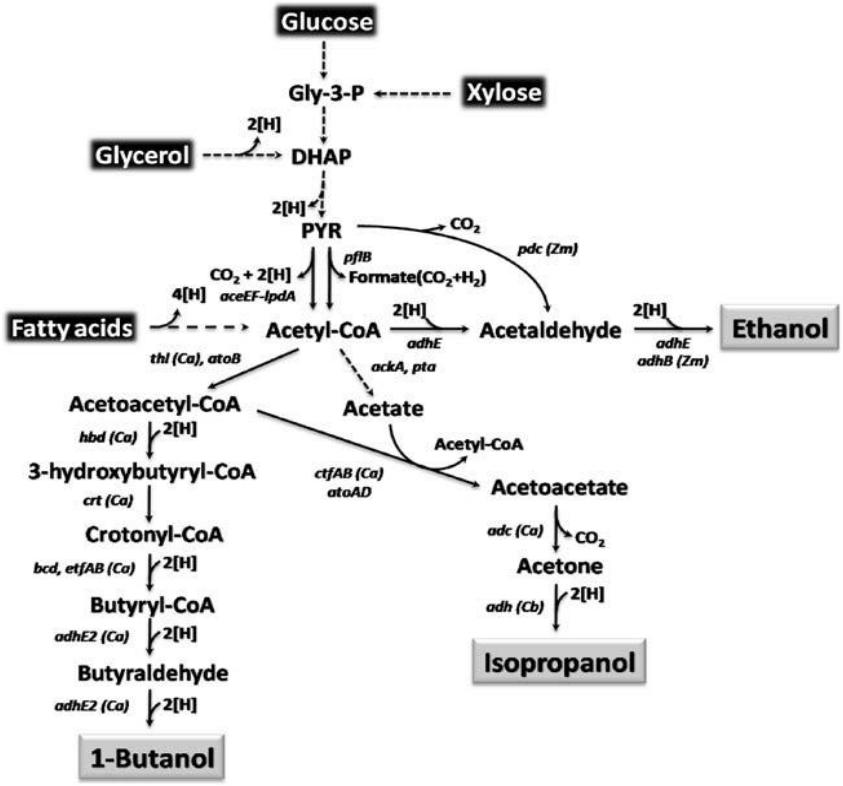

Figure 6. Fermentative pathway for the production of biofuels in *E. coli* [71].

This hinders homoethanol fermentation, as more oxidized products such as acetic acid must be produced to maintain an overall redox balance. In order to alleviate this drawback, metabolic engineering strategies have been implemented to enable higher yields of ethanol production from sugars. In contrast to the native *E. coli* pathway, the pathway for ethanol production in *Zymomonas mobilis* is a two-step process in which pyruvate decarboxylase (PDC) converts pyruvate directly to acetaldehyde and CO_2, and alcohol dehydrogense (ADH) then converts acetaldehyde into ethanol. Further improvement of ethanol yield was accomplished by generating a strain (KO11) which reduced succinic acid production by 95% and produced ethanol at 100% theoretical yield when grown on glucose or xylose rich medium. In order to utilize *E. coli* for the viable commercial production of ethanol, improvements in ethanol tolerance are required, as significant growth inhibition results from ethanol concentrations

upwards of 35 g/L while yeasts that are used to commercially produce ethanol are capable of exceeding 90 g/L ethanol [72]. In the past few years, a growing number of literatures have centered on *E. coli* as a model organism for optimizing metabolic pathways [73]. Bioethanol fermentation from wheat straw hydrolysate by the recombinant *E. coli* strain resulted in a maximum ethanol yield of 0.24 g ethanol/g biomass [74] where *E. coli* KO11 resulted in an ethanol yield of 0.4 g ethanol/g of sugars from marine algal biomass [75].

2.4.4 Fermentation. Fermentation is a biological process where microorganisms such as bacteria and yeast convert reducing sugars such as glucose, xylose, fructose, sucrose etc into ethanol and carbon-dioxide while obtaining energy for growth and maintenance. It can be carried out in both anaerobic and aerobic process. Approximately 80% of all ethanol generated in the world is obtained by biological fermentation and 20% by conversion of petroleum. Theoretically the maximum yields that can be obtained from the conversion of pentose and hexose sugars to ethanol are 0.51kg/kg C6 sugar and 0.49 kg/kg C5 sugar [76]. Stoichiometrically, pentose and hexose fermentation can be represented as:

Pentose fermentation: $3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$

Hexose fermentation: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

Two major strategies have been developed for the enzymatic and microbial conversion of polysaccharides to ethanol. The process economies and optimization determine the best strategy for optimum yield of end products. The two common fermentation approaches are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) [77].

2.4.4.1 Separate hydrolysis and fermentation (SHF). In the SHF process, the cellulosic material is hydrolyzed to reducing sugars by cellulase and hemicellulase enzymes operating at

their optimum condition first. In the second stage, these sugars in the hydrolyzates after enzymatic hydrolysis are then subjected to fermentation by yeast or other fermenting bacteria in another chamber under different operating conditions. This fermentation strategy has some disadvantages like product inhibition during the hydrolysis stage. The activities of the enzymes are lessened due to the presence of hydrolysis products such as cellobiose and glucose. As a result of severe product inhibition of enzyme activity, a batch hydrolysis process of 10% substrate requires an enzyme loading approximately 33 FPU/g substrate if a 73-75% glucose yield is to be obtained. A fivefold reduction in enzyme loading can be achieved if the product sugars generated can be removed gradually from the hydrolysis reactor [78]. Product removals or simultaneous fermentation of produced sugars are feasible remedies to alleviate the high enzyme loading and eliminate product inhibition.

2.4.4.2 Simultaneous saccharification and fermentation (SSF). Simultaneous saccharification and fermentation is a single stage process in which both enzymatic hydrolysis and alcoholic fermentation are carried out within the same reactor. The optimum temperature for SSF is a compromise between that of hydrolysis (45-50°C) and fermentation (20-40°C depending on the microbes used for fermentation). As a result of low temperature range under which SSF is conducted, hydrolytic enzymes operate below their optimum and require a longer period of time to fully convert cellulose to glucose for fermentation. Depending on the type of substrate, SSF can be run from 3 to 7 days.

In the SSF process**,** hydrolysis and fermentation are carried out simultaneously in the same reactor vessel. It greatly reduces the product inhibition to the hydrolysis as the sugars that are produced from the hydrolysis or saccharification of cellulose are simultaneously fermented to ethanol. Hydrolysis is usually the rate-limiting process in SSF. Thermo tolerant yeasts and

bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature [9].

SSF has the following advantages: (a) Simultaneous conversion of sugars into ethanol increases hydrolysis rate, (b) Lower enzyme requirement, (c) Higher product yields; (d) Shorter process time; and (e) less reactor volume. However, ethanol may also exhibit inhibition to the cellulase activity in the SSF process. Wu and Lee [79] found that cellulase lost 9%, 36% and 64% of its original activity at ethanol concentrations of 9, 35 and 60 g/l, respectively, at 38°C during a SSF process. The disadvantages of a SSF process include: (a) Incompatible temperature of hydrolysis and fermentation, (b) Ethanol tolerance of microbes, and (c) Inhibition of cellulase enzymes by ethanol [9].

2.5 Acetic Acid Utilization and Production

With a US production of 2.2 billion kg/yr, acetic acid is a widely used commodity chemical [80]. It has wide applications in food industry (as acidulant and preservatives), plastic industry, textile industry and pharmaceuticals [81]. It is an important feedstock for many chemicals including vinyl acetate polymer, cellulose acetate, terephthalic acid/dimethyl terephthalate, acetic acid esters, acetic anhydride, and calcium magnesium acetate.

2.5.1 Biological production of acetic acid. Since the late 1970s, production of acetic acid via fermentation using renewable biomass feedstock has been studied by numerous researchers as an alternative to acetic acid production from petroleum or natural gas feedstocks. In conventional vinegar production, glucose is first fermented to ethanol by yeast and then the ethanol is oxidized by a bacterium to acetic acid. The theoretical maximum yield of this conventional route is 2 moles of acetic acid from 1 mole of glucose or 0.67 g/g glucose. In commercial practice, actual acetic acid yield is 0.50-0.55 g/g glucose or roughly 75-80% of

theoretical. But this conversion route cannot utilize five carbon sugars because of the inability of commercially available yeast to ferment them to ethanol [82].

Although a variety of bacteria can produce acetic acid, mostly members of acetobacter, gluconacetobacter, and gluconobacter are used commercially in vinegar manufacturing industries. They are widespread in nature and most of them are capable of oxidizing ethanol to acetic acid. This characteristic makes acetic acid bacteria to be often involved in foods and beverages, either in a beneficial, neutral or detrimental way [83]. Acetic acid bacteria produce acetic acid by oxidative fermentation. Acetic acid bacteria (AAB) are strictly aerobic, Gram-negative, catalase positive and rod shaped microorganisms that are characterized by a unique resistance to ethanol and acetic acid. These microorganisms belong to the Acetobacteraceae family and are classified into 12 genera and 59 species. Among AAB, several members of the genera Acetobacter and Gluconacetobacter are involved in the production of vinegar. These bacteria oxidise ethanol to acetic acid by two sequential reactions equation 1 & 2. Equation 1 & 2 represents the conventional vinegar production.

$$C_6H_{12}O_6 \xrightarrow{S.\ cerevisiae} 2C_2H_5OH + 2CO_2 \qquad \text{(Eq. 1)}$$

$$2C_2H_5OH + 2O_2 \xrightarrow{A.\ aceti} 2CH_3COOH + 2H_2O \qquad \text{(Eq. 2)}$$

Species of anaerobic bacteria, including members of the genus *Clostridium* or *Acetobacterium* can convert sugars to acetic acid directly, without using ethanol as an intermediate. Strains of *Clostridium sp.* mostly used are- *Clostridium thermoaceticum* (ATCC 27407); *Clostridium thermoaceticum* (ATCC39073); *Clostridium thermocellum* (ATCC27405) and *Clostridium lentocellum* SG6.

The overall chemical reaction conducted by these bacteria may be represented as: $C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$ [84]. This ability of *Clostridium* to utilize sugars directly, or to

produce acetic acid from less costly inputs, means that these bacteria could potentially produce acetic acid more efficiently than ethanol-oxidizers like *Acetobacter*. The acetate yield in batch fermentation by *C. formicaceticum* is about 0.91 g/g fructose but the volumetric productivity is low (0.19 g/1 h) [85]. However, the inability of this acetogen to ferment glucose limits its utility as a biocatalyst. Co-cultures of two or more organisms have been constructed to increase the substrate range of this acetogen [86].

However, *Clostridium* bacteria are less acid-tolerant than *Acetobacter*. Even the most acid-tolerant *Clostridium* strains can produce vinegar of only a few per cent acetic acid, compared to *Acetobacter* strains that can produce vinegar of up to 20% acetic acid. At present, it remains more cost-effective to produce vinegar using *Acetobacter* than to produce it using *Clostridium* and then concentrate it. As a result, although acetogenic bacteria have been known since 1940, their industrial use remains confined to a few niche applications [87].

2.5.1.1 Acetic acid production by Clostridium thermoaceticum. In 1942, Fontaine and co-workers discovered a thermophilic bacterium, *Clostridium thermoaceticum* that catalyzed the near stoichiometric conversion of glucose to acetate via a metabolic process (Figure 7) that is now referred to as the acetyle-CoA pathway or Wood-Ljungdahl pathway. *C. thermoaceticum* was reclassified as *Moorella thermoacetica* in the mid-1990s. Although numerous acetogens have been isolated, *M. thermoacetica* has served and continues to serve as the primary model acetogen in the laboratory [84].

Fermentation with C. thermoaceticum offers a significant advantage in terms of acetate yield comparing to the conventional vinegar fermentation, because *C. thermoaceticum* can theoretically produce 3 moles of acetic acid from 1 mole of glucose (equation 3). In practice,

85% of the sugar may be converted to acetic acid. *C. thermoaceticum* can also ferment fructose and xylose (Equation 4).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H^+ + 8e^-$$

$$2CO_2 + 8H^+ + 8e^- \rightarrow CH_3COOH + 2H_2O$$

Net reaction: $C_6H_{12}O_6 \rightarrow 3CH_3COOH$ (Eq. 3)

For C5 sugar the net reaction: $C_5H_{10}O_5 \rightarrow 5CH_3COOH$ (Eq. 4) [84]

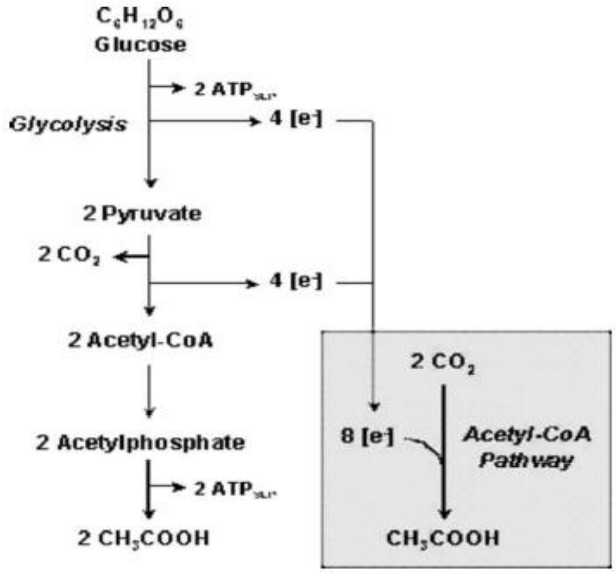

Figure 7. Homoacetogenic conversion of glucose to acetate [84].

The production of acetic acid by *C. thermoaceticum* has several important advantages comparing to the vinegar process. It is an anaerobic process, and thus should be cheaper that the aerobic process traditionally used for vinegar production. The process can make use of at least three sugars (xylose, fructose, and glucose) that can be derived from biomass, and it can produce high yields of acetic acid. The theoretical yield is 1 g acetic acid per gram glucose, although actual yields reported in the literature are 0.8-0.93. *C. thermoacetica* is a homoacetogen that is capable of producing acetic acid as its sole product. Its major limitation was relatively low concentration produced by the wild strains (20 g/l) and low productivity (0.15-0.25 g $l^{-1}h^{-1}$) [88].

Numerous studies have evaluated the potential use of *M. thermoacetica* to commercially produce both acetic acid and calcium-magnesiumacetate (an environmentally safe road de-icer). Advantages of *M. thermoacetica* are: 1) it is the most metabolically diverse acetogen thus far characterized. This bacterium utilizes very diverse substrates, grows both autotrophically and heterotrophically and has only one organic nutritional requirement, nicotinic acid; 2) *M. thermoacetica* is a robust and hearty thermophile, and its metabolic diversity is likely the primary basis for the organism's ability to compete with other microbes for substrates. However, other factors also contribute to the survival strategies of this acetogen. For example, the spores of *M. thermoacetica* are among the most heat-resistant spores characterized. The ability of *M. thermoacetica* spores to survive high temperatures demonstrates that this anaerobic bacterium can also survive standard commercial canning procedures. There are several disadvantages: 1) *M. thermoacetica* can tolerate small amounts of O_2; 2) *M. thermoacetica* is inhibited by high concentrations of acetate and does not grow under acidic conditions. These limitations have made it impossible to commercialize the acetogenic abilities of both wild-type and mutant strains of *M. thermoacetica* [84].

M. thermoacetica or *C. thermoaceticum* needs to be acclimatized to a xylose environment to obtain high yields of acetic acid. It preferentially consumes xylose over glucose when grown in a medium containing a mixture of glucose and xylose. To maintain viability for xylose fermentation, it is necessary to grow the organism in xylose and glucose medium alternately. In batch fermentation at pH 6.9 and temperature 59°C, a concentration of initial xylose concentration of 15 g/L resulted in a maximum yield of acetic acid at 0.84 (g acetic acid / g xylose consumed). The maximum concentration of product achieved was 15.2 g/L which occurred with a 20 g/L xylose concentration with a yield of 76%. With increases in xylose

concentration, the amount of unconsumed xylose in the medium increased which decreased the yield [89]. The organism consumes arabinose, mannose, and galactose only when each of these is present with xylose in the medium. In a batch fermentation of a mixture of sugars, the extent of consumption of mannose, arabinose, and galactose is <20% in 130 h. Fed-batch operation did not result in increased yield of acetic acid, because the organism lost viability after a certain period and was not revived by adding extra nutrients or trace elements. This proves to be a major drawback for acetate production from this strain using xylose as the carbon source [90].

2.5.2 Biological production of acetic acid from biomass. At present all the acetate products are made from petroleum-derived acetic acid [90]. Fermentation is potentially a cost-effective alternative for acetic acid production. The advantage of the fermentation route is that it relies on renewable resources rather than nonrenewable (petroleum) resources. Production of acetic acid via fermentation using renewable biomass feedstock has been studied extensively since the late 1970s. Traditionally, hydrolyzates of corn starch and corn-steep liquor have been used for glucose/nitrogen sources for this process. Cellulosic biomass (α-cellulose, pulp mill sludge etc), mixture of cellulose, hemicellulose and lignin-derived components (Japanese beech wood in hot compressed water) were also used as alternative feedstock [91, 92].

A strain of *Clostridium thermoaceticum* (ATCC 49707) best produces acetate from glucose at pH 6.0 and 59°C with a yield of 83% of theoretical. In a fed-batch operation of SSF with a cellulosic biomass α-cellulose, an overall acetic acid yield of 60 wt% was obtained [93]. Co-culture with *C. thermoaceticum* (ATCC 39073) and *C. thermocellum* (ATCC 27405) increases the fermentability for a wide range of biomass components. Almost all compounds produced from beech wood in hot-compressed water were found to be converted to acetic acid when using these microorganisms in combination [94].

A cellulolytic, acetic acid producing anaerobic bacterial isolate identified as *Clostridium lentocellum* SG6 can become a potential strain for acetic acid production because of its high fermentation yield and sporulating character. The strain SG6 can ferment cellulose at high substrate concentrations [81]. It produced acetic acid as a major end product from cellulose fermentation at 37°C and pH 7.2. Acetic acid production was 0.67 g/g cellulose substrate utilized in cellulose mineral salt (CMS) medium. This is the highest acetic acid fermentation yield in monoculture fermentation for direct conversion of cellulose to acetic acid [95].

2.6 Enzyme Assisted Extraction of Lipid and Protein from Biomass

The common methods of oil/lipid extraction include physical or mechanical processes, chemical procedures or a combination of these. During the conventional oil extraction processes, some of the oil not extracted remains in the solid residue. In order to effectively recover oil enclosed in the cell, the cell walls must be destroyed. Several methods including enzymatic pretreatment have been proposed to improve oil extraction procedures [96]. Hot acid hydrolysis, microwave irradiation, sonication, high-pressure homogenization, bead beating, and swelling by osmotic pressure have been used for cell disruption [62, 97]. These methods have some restrictions. During hot acid hydrolysis, cells are disrupted concurrently with the degradation of other cellular components, leading to excess acid loading and reduction of co-products. Physical or mechanical approaches for cell disruption are not effective for large-scale operations.

Enzymatic treatment of oilseeds is an established technology in the vegetable oil industry [98], where enzymes are used to hydrolyze structural polysaccharides of the cell wall of oilseeds as well as proteins associated with lipid bodies [99]. Enzymatic processes are potentially useful to the edible oil industries due to their high specificity and low operating temperatures. Enzyme

applications in edible oil processing include: facilitating pressing, increasing the oil yield of solvent extraction, and facilitating the aqueous extraction [100]. It has been demonstrated that pre-extraction enzyme digestion increases cellular degradation and significantly increases oil recovery upon extraction. The enzyme treatment, besides giving higher oil yields, significantly increased the qualitative standard of the oil [101].

Enzymatic treatment is also used to extract lipids produced by oleaginous microorganisms as a potential feedstock for biodiesel production and chemical synthesis. Microorganisms that can accumulate lipids to more than 20% of their biomass are defined as oleaginous species [102]. Some yeast strains, such as *Cryptococcus* sp., *Lipomyces* sp., *Rhodosporidium* sp. and *Rhodotorula* sp. can accumulate intracellular lipids as high as 60% of its cell dry weight when using glucose as the carbon source [97]. Constitutive fatty acids of those lipids are mainly long chain ones that are quite similar to those of conventional vegetable oil. However, the carbon sources for oleaginous microbes need extend to lignocellulosic biomass and related raw materials so that large volume of microbial lipids can be secured [103]. With a heat pre-treatment with microwave, enzymatic treatment with the recombinant β-1,3-glucomannanase, plMAN5C, and extraction with ethyl acetate, 96.6% of the total lipids were extracted from *R. toruloides* cells at room temperature and atmospheric pressure directly from the culture without dewatering. Therefore, this process could significantly reduce energy consumption and costs for lipids extraction from the yeast [99].

Similar to the lipid/oil extraction, protein extraction can also be enhanced by enzymatic treatment. There is a demand for the formulation of innovative and alternative proteinaceous food sources due to an insufficient supply from traditional protein sources. Much interest has been focused on the potential of converting abundantly available waste to single-cell protein

(SCP) [104]. Algae, fungi and bacteria are the major sources of the microbial protein that can be utilized as SCP [105]. The production of the microbial biomass is achieved either by a submerged or solid state fermentation process. After fermentation, biomass is harvested and may be used as a protein source or subjected to processing steps like washing, cell disruption, protein extraction and purification [104].

Aqueous extraction process (AEP) has been considered as a good option for simultaneous extraction of oil and protein. AEP avoids serious damage to the oil and proteins of the seed and allows production of food-grade proteins instead of feed-grade products. It also eliminates chemical refining steps and the oil produced through this process is more suitable for human consumption due to its better nutritive quality. Nevertheless, low oil recovery is one of the major challenges for this process which may be overcome by utilizing selected enzymes. Enzyme-assisted aqueous extraction (EAAE) is gaining importance as an alternative for simultaneous extraction of oil and protein. It is thought to be environmentally-friendly, safe and cheap [98, 106].

Increasing in the protein content of cellulosic residues to improve their nutritional value has been proposed by several investigators. This process is potentially useful in reducing the environmental impact of these residues and in enhancing animal feed and human food supplies. Protein enrichment of cellulosic biomass such as sugar beet pulps, wheat bran, citrus waste etc has been investigated by various researchers. Based on the original crude protein content of the substrates, 2-3 folds of protein increments of the cellulosic pulp were achieved by solids state fermentation using microorganism *Neurospora sitophila* [107]. Although many cellulose-degrading microorganisms, mostly fungi, are known, few would qualify as food or feed grade which can be used for solid state fermentation for protein enrichment of cellulosic biomass.

2.7 Biorefinery of Green Biomass and Microalgae

Green biorefineries are multiproduct systems, which utilize green biomass as an abundant and versatile raw material for the manufacture of industrial products. The basic idea of this concept is to utilize the whole green biomass like grass, alfalfa and various other sources and generate a variety of products. Besides biobased materials, fuels and energy may be supplied by this technology [108].

In green biorefinery, the first step is to use wet or green fractionation technology to isolate the green biomass substances in their natural form. Thus, green biomasses are separated into a fiber-rich press cake and a nutrient-rich press juice. Both fractions can produce valuable bioproducts through different processing operations. Figure 8 illustrates the array of potential products of a green biorefinery that can be generated by the downstream processing of press juice and press cake.

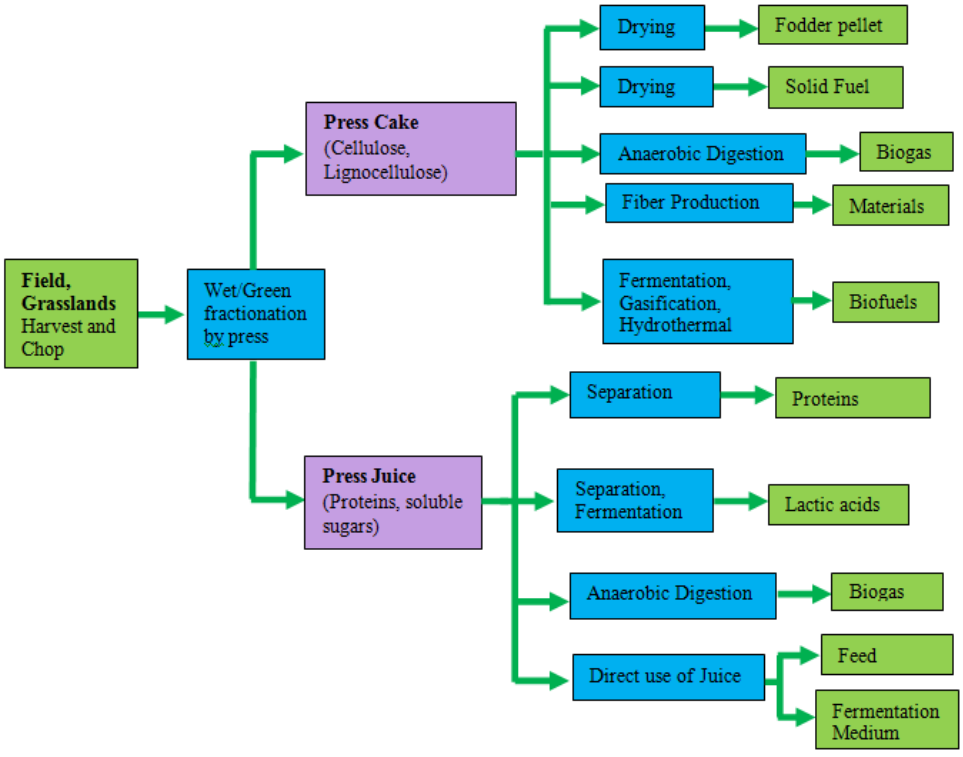

Figure 8. A green biorefinery system for green biomass utilization [108].

2.7.1 Feedstocks and Current Processing Technologies for Green Biorefinery.

Currently, green grasses are the main feedstocks for green biorefinery. The valuable components of fresh biomass are proteins, soluble sugars, and the fiber fraction (cellulose, hemicelluloses, and lignin part). Table 4 shows the chemical composition of representative types of green biomass. In general, biomass is typically composed of 75-90% of sugar polymers, with the other 10-25% of biomass principally being lignin [109].

Table 4

Composition of Representative Biomass Resources [108]

Biomass type	Aquatic plant		Herbaceous		Woody	
Name	Spirulina algae	Duckweed	Bermuda grass	Switch grass	Poplar	Pine
			Component (dry wt. %)			
Celluloses	<1	11.9	31.7	37	41.3	40.4
Hemicellulose	1	13.8	40.2	29	32.9	24.9
Lignin	<1	3.2	4.1	19	25.5	34.5
Crude protein	64	35.1	12.3	3	1.7	0.7
Crude lipid	5	5	11.9	–	–	–
Ash	11	16.5	5	6	0.8	0.5
Total	100	92.4	93.3	94	102.9	101.0

Most of attention has focused on the cellulose, hemicelluloses, and lignin presented in biomass materials so far. However, there are also considerable amounts of protein available in these biomass materials as Table 4 indicated. For the biomass species listed, aquatic organisms has higher protein content than other biomass resources. Since a kilogram of protein is generally much more valuable than an equal weight of carbohydrate, aquatic organisms can be used as a good candidate for green biorefinery in respect of high value protein recovery. Microalgae are usually composed of lipids, proteins, nucleic acids and no-cellulosic carbohydrates. In the green

refinery, the wet fraction of algae can be used as nutrient-rich green juice for value-added co-product developments like proteins [108].

Mechanical fractionation is usually the first unit operation in green biorefinery plant. Machinery, screw express has been primarily used to press the green juice out of the green biomass. For vegetative biomass like alfalfa, clover and grass, screw presses remove approximately 55-60% of the inherent liquid [110]. The green juice contains proteins, free amino acids, organic acids, dyes, enzymes, hormones, other organic substances and minerals. The green juice is a raw material for high quality fodder proteins, cosmetic proteins, human nutrition or platform chemicals like lactic acid and corresponding derivatives, amino acids, ethanol, and proteins [108]. Press juice also can be directly used as fermentation media for organic acids production. It has been found that juices from grass, clover and alfalfa can easily be converted to a stable universal fermentation media by adding more carbohydrates or for production of other organic acids or amino acids in the second stage fermentation [108, 111]. The press cake can be used for the production of green feed pellets/fodder pellet, as a raw material for the production of chemicals [108].

CHAPTER 3

Methodology

3.1 Biomass Preparation for Ethanol Production

Three aquatic biomasses including microalgae, duckweed and cattail which can be used to treat wastewater were used as the biomass sources in this research. Microalgae was cultured, harvested and used for pretreatment and fermentation. Duckweed was collected from NC A&T State University farm and homogenized without the addition of water and used directly for pretreatment and fermentation. Cattail was collected and homogenized with the addition of water. Then the slurry was filtered. Filtered solid part was used for pretreatment and fermentation and liquid part was used a nutrient medium to grow microalgae.

3.1.1 Microalgae culture and preparation. The strain of mixed *Chlorella* sp. was used in experiments. Figure 9 shows microalgae culture in lightbox and microalgae culture in large scale.

Figure 9. Microalgae culture in lightbox and microalgae culture in large scale.

Pre-cultured algal cells were inoculated using a proteose medium which is the original growth medium of this species in 1 L glass bottles. The 1 L glass bottles were kept in a light box at a room temperature for two weeks. The light intensity of the light box was 600 μmol $m^{-2}s^{-1}$.

The medium contains proteose peptone at 1 g/L and each of the following components at 10 ml/L: $NaNO_3$ (2.94 mM), $CaCl_2 \cdot 2H_2O$ (0.17 mM), $MgSO_4 \cdot 7H_2O$ (0.3mM), K_2HPO_4 (0.43 mM), KH_2PO_4 (1.29 mM) and NaCl (0.43 mM).

After inoculation, 40 mg/L chloramphenicol was used as an antibiotic to disinfect the growth medium for the growth of the algae. Bottles caps were kept loose and bottles were shaken manually every day to provide carbon-dioxide and release oxygen generated. Later, microalgae were grown in a large scale in the greenhouse using the same growth medium and antibiotic.

Grown microalgae were centrifugated by a centra-GP8R Centrifuge (Model 120, Thermo IEC) at 3400 rpm for 15 minutes (Figure 10). The liquid stream was discarded and solid streams of biomasses were collected to use for fermentation experiments.

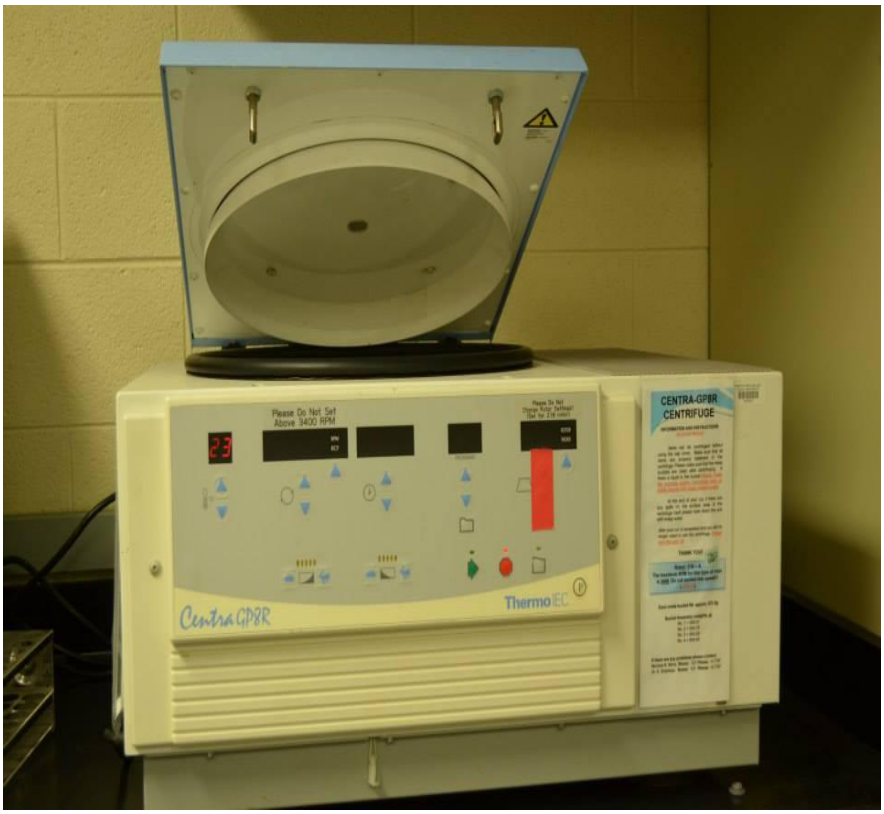

Figure 10. Centra-GP8R Centrifuge, model 120, by Thermo IEC.

3.1.2 Duckweed Collection and preparation. Duckweed, likely *Lemna minora,* was collected from a constructed wetland at NC A&T's farm. The constructed wetland contained some wastewater from a nearby swine unit. Figures 11a and 11b show duckweed floating in swine waste water and collected & homogenized duckweed respectively. The duckweed was collected between August 2013 and September 2013. Collected duckweed was stored at -20°C for further usage.

(a) (b)

Figure 11. (a) Duckweed in swinewaste water and (b) Collected and homogenized duckweed.

Some portion of the duckweed was spread and air-dried at the room temperature to a final moisture content of around 10%. Then the air-dried duckweed was kept at 50°C for 24 hours and then ground in a mortar and pestle to get fine powder of dried duckweed. Both green and dried ground duckweed was stored for following ethanol fermentation studies.

A Knife Mill Grindomix GM 200 (Figure 12) was used to homogenize fresh duckweed without any addition of water. The treatment time was 5 min and rotation speed was set to 4000 rpm.

Figure 12. Homogenizer Grindomix (GM 200).

3.1.3 Cattails collection and preparation. The cattails, *Typha latifolia*, were collected from a constructed wetland as shown in Figure 13a at NC A&T's farm chopped with pruning shears, cut into small pieces using scissors and stored at -20°C for further usage. Laboratory blender (Figure 13b) was used to homogenize the cattail with addition of water. Cattail to added DI water ratio was 1:5. The slurry was filtered using the whatman grade 595 filter paper. Separated liquid and solid biomass as shown in 13c was stored at -20°C for future use. Solid part was used for fermentation and liquid part was used for microalgae medium preparation.

(a) (b) (c)

Figure 13. (a) Cattail plants in the farm; (b) Laboratory blender; (c) Homogenized and filtered cattail.

3.2 Pretreatment of Green Biomass

An ultrasonic processor (Branson Sonifier S-250D Digital Ultrasonic Cell Disruptor/Homogenizer) as shown in Figure 14 was used for pretreatment of biomasses. Prepared biomasses which were described in previous section were diluted to a certain biomass concentration and were used for this pretreatment. The ultrasonic process was operated at a frequency of 20 kHz, supplied power of 200 W, using platinum probe with tip diameter 13 mm. Around 30 mL of diluted biomass sample at ambient temperature was placed in a 50 ml centrifuge tube. The tube was placed in a beaker. The ultrasonic probe was dipped at 2 cm into the slurry. Pretreatment time and amplitude were adjusted for each kind of biomass.

Figure 14. Branson Sonifier S-250D, Digital Ultrasonic Cell Disruptor/Homogenizer.

3.3 Microorganisms

3.3.1 *E. coli* KO11 for ethanol fermentation. The strain of recombinant *E. coli* KO11 (ATCC® 55124) was obtained from American Type Culture Collection and stored using LB medium (Sigma, L-3152) in the laboratory. This medium contained 1% tryptone, 0.5% yeast extract and 1% NaCl. 40 mg/L of antibiotic chloramphenicol was used for the growth of KO11.

In order to prepare enough *E. coli* cells for fermentation, seeds culture was scaled up in this way: 1 ml of seed *E. coli* was added to 4 ml of LB broth. The 5 ml of *E. coli* medium was

cultured in an incubator as shown in Figure 15 (incubator shaker series 126, New Brunswick Scientific Co., INC., Edison, New Jersey, USA) for 24 h at a shaking speed of 150 rpm and temperature of 37°C. In the same way, the 5 ml of *E. coli* was scaled up to 50 ml. All the transferring and operation were done on a sterilized clean bench to avoid contamination. The *E. coli* culture was centrifuged at 3400 rpm for 10 minutes. The supernatant was discarded. The precipitated *E. coli* was washed three times with a peptone solution. The washed *E. coli* cells were used for the ethanol fermentation.

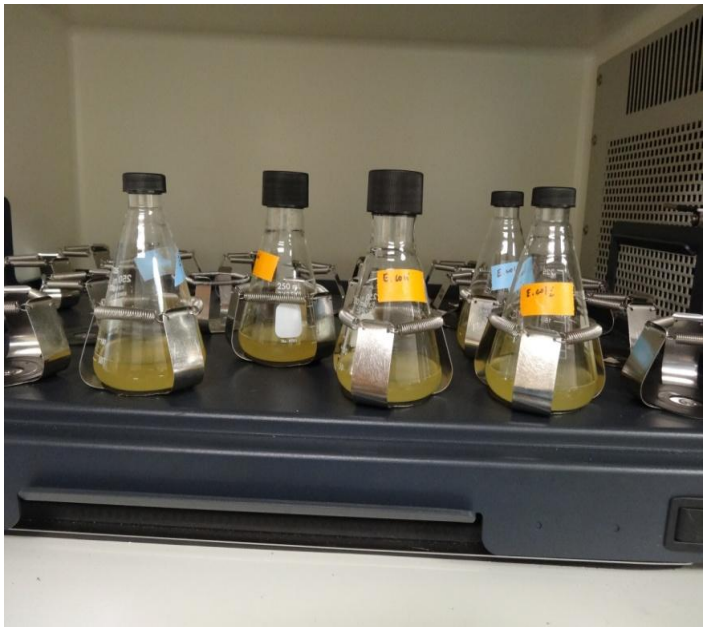

Figure 15. E. coli culture in environmental shaker.

3.3.2 *Clostridium thermoaceticum* (ATCC 49707) for acetic acid fermentation. The strain of *Clostridium thermoaceticum* (ATCC 49707) was obtained from American Type Culture Collection. The freeze-dried culture of *C. thermoaceticum* was rehydrated by Reinforced Clostridial Medium (BD 218081, Becton, Dickinson and company) according to the instructions of ATCC. Anaerobic chamber as shown in Figure 16 was used for all culture work for *Clostridium*. Nitrogen or carbon dioxide was used to achieve anaerobic environment.

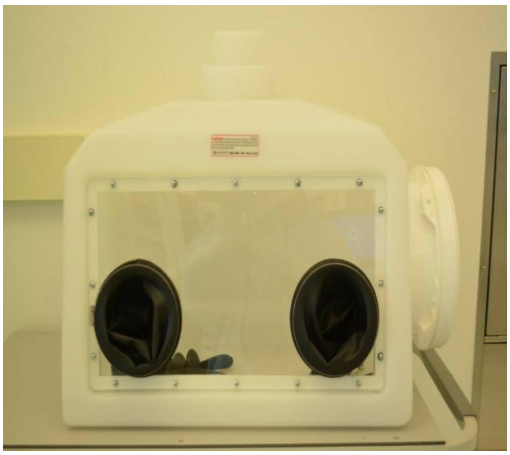

Figure 16. Anaerobic chamber used during *C. thermoaceticum* inoculation.

The activated *C. thermoaceticum* was stored in a 15% glycerol solution and then kept at -86°C refrigerator for future usage. The activated culture was scaled up to 50 mL volume using the Reinforced Clostridial Medium, which contained 10.0 g/L peptone, 10 g/L of beef extract, 3.0 g/L of yeast extract, 5.0 g/L of dextrose, 5.0 g/L of sodium chloride, 1.0 g/L of soluble starch, 0.5 g/L of cystein HCl, 3.0 g/L of sodium acetate and 0.5 g/L of agar, with a final pH of 6.8 ± 0.2.

3.4 Analysis of Biomass Chemical Composition

Compositional analysis of sugars in original air-dried biomasses was carried out using the Laboratory Analytical Procedures (LAP-002 and LAP-005) developed by the National Renewable Energy Laboratory. In those procedures, 0.3 ± 0.01 g dry samples were weighed. Each sample was run in duplicate. The first hydrolysis step was carried out by adding 3.00 ± 0.01 ml (4.92 ± 0.01 g) of 72% H_2SO_4 to each sample in the test tube which was incubated in the water bath at 30°C for 2 h by stirring the sample with a glass rod every 15 min to assure complete mixing and wetting. The second hydrolysis step was conducted by transferring each hydrolyzate to its own serum bottle and diluting to a 4% acid concentration by bringing the total weight up to 89.22 g (0.3 g sample, 4.92 g 72% H_2SO_4, and 84.00 g deionized water). The

samples in their sealed bottles were autoclaved at 121 ± 3°C for 1 h, to make sure that all the sugars were released to the liquid. After the completion of the autoclave cycle and cooling to a room temperature, 5 ml of each sample was obtained and neutralized with calcium carbonate to a pH between 5 and 6.

Neutralized hydrolyzate was filtered using a 3 ml syringe with a 0.45 µm filter to HPLC vials. The vials were stored in a 4°C refrigerator for the analysis of sugars using a HPLC (Waters, Milford, MA) as shown in Figure 17. It was equipped with a KC811 ion-exchange column and a waters 410 refractive index detector (RID). The mobile phase was a 0.1% H_3PO_4 solution at a flow rate of 1 ml/min. the temperatures of the detector and column were maintained at 35 and 50°C. The solid residues after the vacuum filtration were assumed to be lignin and ash, and were dried in an oven at 105°C for 24 h. After drying, the weight of residues was recorded.

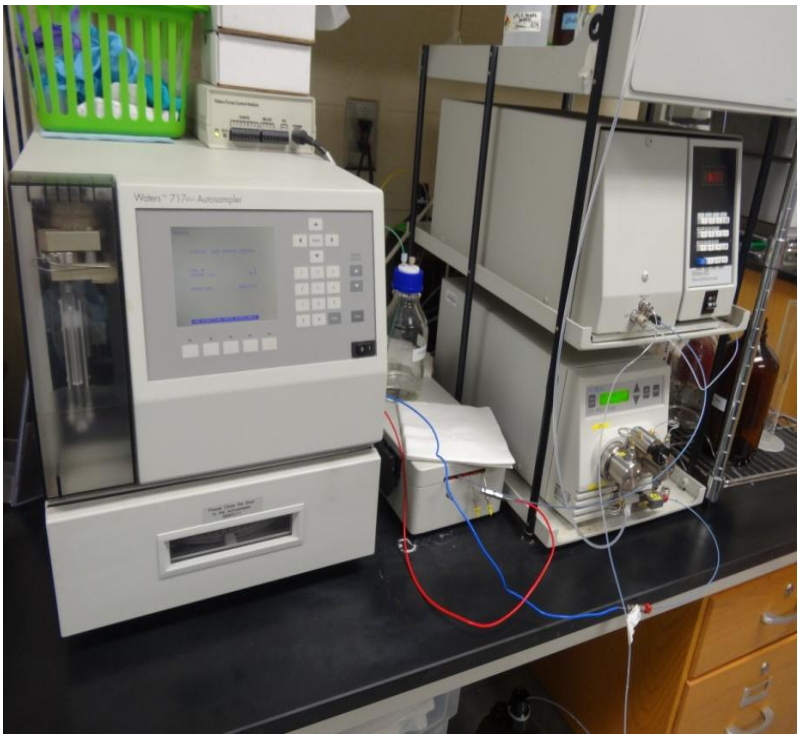

*Figure 17. H*igh Performance Liquid Chromatography, Waters, Milford, MA.

The ash content of the dried sample was determined in TGA shown in Figure 18. Sample sizes for the analysis were in the range of 5-15 mg and air at flow rate of 100 ml/min was used as a purge gas. During the analysis, air was used to combust the solid sample and the mass of final ash after combustion was determined.

Figure 18. Thermo gravimetric analysis equipment (TGA).

3.5 Ethanol Production from Fresh Biomass

The pretreated cattail, duckweed and algal cells rich in glucan was hydrolyzed and fermented into ethanol. Total solid concentration of biomass was determined by the oven dry method at105°C. Following enzymes were used to hydrolyze the pretreated cells: (a) cellulase, NS50013, 15 FPU/g-glucan; (b) β-glucosidase, NS50010, 20 µl/g-glucan; and (c) Hemicellulase, NS22002, 50µl/g-total solid. Cellulase concentration was varied according to experimental design. The optimum enzymatic hydrolysis temperature was 50°C and pH was 5.0 ± 0.1. Wheaton glass bottles of 150ml or 250 ml were used as bioreactors for all hydrolysis and

fermentation. 0.05 M buffer citrate was added to adjust the pH to 5.0±0.1 during fermentation of pretreated algae, duckweed, and cattail biomasses.

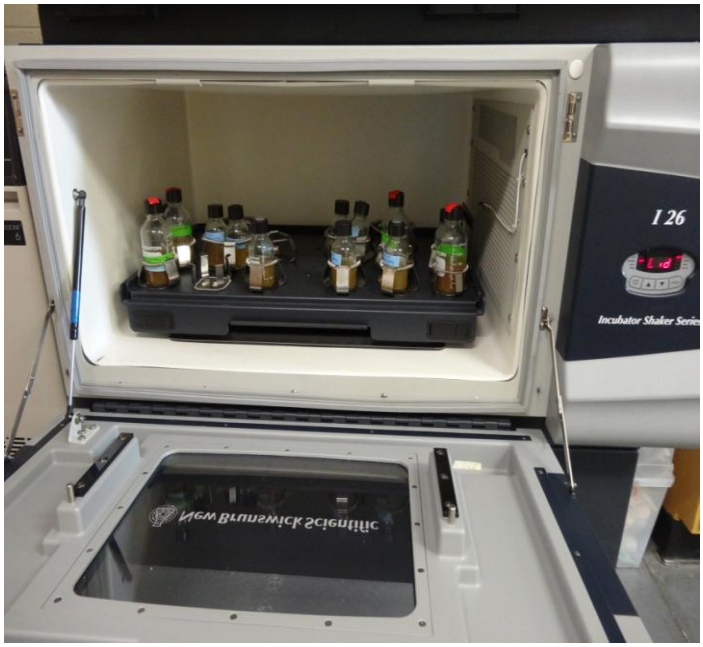

Figure 19. Fermentation experiments in Environmental incubator shaker (New Brunswick Scientific I 26).

E. coli was used for ethanol fermentation of pretreated biomass without any additional nutrition sources. Both Simultaneous Saccharification and Fermentation (SSF) and Separated Hydrolysis and Fermentation (SHF) were carried out for ethanol fermentation of algae and cattail.

3.5.1 Separated hydrolysis and fermentation (SHF). Separated hydrolysis and fermentation (SHF) was the process that enzymatic hydrolysis and *E. coli* fermentation were carried out in sequence at different times; in which hydrolysis was performed for more than three days until the sugar concentration did not increase anymore. Then it was followed by fermentation, so that both hydrolysis and fermentation could perform at their optimized conditions. Pretreated cattail, duckweed and algae were loaded to 250 ml of bottles and a citrate

buffer solution of 0.05 M was added to adjust the pH to 5.0 ± 0.1. These bottles were then autoclaved at 121°C for 15 min. The biomass slurries in bottles were cooled to a room-temperature and reweighed. Sterile DI water was added to compensate for the water loss during autoclave. Desired volumes of cellulase, β-glucosidase, and hemicellulose to each bottle were calculated based on the solid biomass concentration and the glucan content of biomass. The fermentor bottles were placed in an incubator shaker (Series 126, New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) at 150 rpm and 50°C. Sample aliquots of the fermentation broth were collected at designated times of 0, 3, 6, 12, 24, 48, 72, 96 and 120 h.

After enzymatic hydrolysis was completed when the concentrations of sugars were not increased anymore, the prepared *E. coli* was added aseptically to the hydrolyzed broth and fermentation was started in the shaker at 200 rpm and 37°C. Appropriate sampling times were 0, 3, 6, 12, 24, 48, 72, 96 and 120 h. All the samples from hydrolysis and fermentation were filtered through a filter at a 0.45 µm filter into HPLC vials and stored in a refrigerator at 4°C.

The glucose yield expressed as % of the theoretical yield (%digestibility) by using the following formula (LAP-008):

$$\%Yield_{Glucose} = \frac{[Glucose] + 1.053\ [cellobiose]}{1.111 f\,[Biomass]} \times 100\%$$

where, [Glucose] – Residual glucose concentration (g/L); [Cellobiose] – residual cellobiose concentration (g/L); [Biomass] – Dry biomass concentration at the beginning of the fermentation (g/L); f-cellulose fraction in dry biomass (g/g); 1.111- Conversion factor from cellulose to glucose.

SHF was carried out for fresh algae and cattail biomass with different ultrasonic pretreatment conditions.

3.5.2 Simultaneous Saccharification and Fermentation (SSF). Simultaneous Saccharification and fermentation was conducted, in which the *E. coli* was added at the same time as enzyme instead of being added after the hydrolysis. Optimum temperature for *E. coli* fermentation is 37°C, where 50°C is the optimum hydrolysis temperature for the enzymes. The compromised temperature between the optimum hydrolysis and fermentation temperatures was chosen at 37°C. The fermentation was carried out in a similar manner to separate hydrolysis and fermentation in the shaker at 150 rpm and 37°C.

The ethanol yield as % of the theoretical yield by using the following formula:

$$\%Yield_{\text{Ethanol}} = \frac{[Ethanol]_f - [Ethanol]_o}{0.568 f [Biomass]} \times 100\%$$

Where, $[\text{Ethanol}]_f$ –Ethanol concentration at the end of the fermentation (g/L); $[\text{Ethanol}]_o$-Ethanol concentration at the beginning of the fermentation (g/L); [Biomass] – Dry biomass concentration at the beginning of the fermentation (g/L); f- Cellulose fraction of dry biomass (g/g); 0.568 – Conversion factor from cellulose to ethanol (LAP-008).

SSF was carried out for all of the three fresh biomasses. For algae, Cellulase concentrations, *E. coli* concentrations and fermentation temperatures were varied to observe the effects on ethanol yield during the SSF process. Table 5 shows the values of these parameters that were used in this study for algae SSF.

Table 5

Parameters and Levels of Algae SSF

Parameters	Levels
Enzyme concentration	7.5, 15 and 60 FPU cellulase
E. coli concentration	0.5 g/L and 1 g/L
Fermentation temperature	37°C and 40°C

3.5.3 Dilute Sulfuric Acid Hydrolysis and fermentation. Only fresh duckweed was used for dilute sulfuric acid hydrolysis. Around 15 grams of homogenized fresh duckweed was mixed with 150 mL of dilute sulfuric acid in 250 mL Wheaton glass bottles at three concentration levels: 1.2, 1.5, 1.8% (vol%) and placed in an autoclave for 25 min at 121°C. After autoclaving, pH value of the mixture was adjusted to 7.0 using 1.0 M ammonium hydroxide in preparation for ethanol fermentation. This hydrolyzed biomass was used as a fermentation medium for *E. coli* without any addition of enzymes. Fermentation was carried out in a similar manner described above in the shaker at 200 rpm and 37°C. Appropriate sampling times were 0, 3, 6, 9, 12, 24, 48, 72, 96 and120 h.

3.5.4 Protein content determination of algae samples before and after SSF. Fresh algae and algae fermented residues were centrifuged. The collected solids were dried at 50°C for 2 days. Elemental analysis of the dried sample of fresh algae and fermented algae residue were done with a Carbon-Hydrogen-Nitrogen-Sulfur (CHNS) analyzer (Model 2400, Perkin-Elmer Inc.) as shown in Figure 20.

Figure 20. Elemental analyzer (Model 2400, Perkin-Elmer Inc.)

The instrument oxidizes the carbon, hydrogen, nitrogen and sulfur into CO_2, H_2O, N_xO_y and SO_2 at 980°C, and then these gases are carried with helium gas to a detector. The measured gas profile was compared with known gas standards to determine the concentrations of individual gas components. The results are then reported as a weight percentage of samples. Protein content was calculated by timing Nitrogen % by mass by 6.25 (Kjeldahl method).

3.6 Acetic Acid Fermentation

3.6.1 Pretreatment of biomass. About 50.0 g of dried ground cattail was stirred into a 0.5 L of 4% NaOH solution and left at room temperature for 24 hours. The mixture was then centrifuged at 3400 rpm for 30 min, the supernatant was discarded and the pellet was rinsed with water six times and twice with the 0.05 M citric acid buffer to lower down the pH to 5.0. Samples were centrifuged and supernatants discarded between rinses.

3.6.2 Preparation of fermentation medium. Reinforced Clostridial Medium was used first to investigate the xylose fermentability of *Clostridium*. This medium contained 10.0 g/L peptone, 10 g/L of beef extract, 3.0 g/L of yeast extract, 5.0 g/L of dextrose, 5.0 g/L of sodium chloride, 1.0 g/L of soluble starch, 0.5 g/L of cystein HCl, 3.0 g/L of sodium acetate and 0.5 g/L of agar. The final pH of the medium was 6.8 ± 0.2.

Two fermentation media were prepared to study the fermentability of *Clostridium* first. These fermentation mediums were then used for biomass fermentation. Fermentation medium-1 was prepared using the method developed by Borden, Lee, and Yoon's [80]. Component names and concentration are given in Table 6.

Certain weight percent of glucose and xylose or the equivalent of pretreated biomass was added to the medium for fermentation. Fermentation medium-2 was prepared using the components given in Table 7.

Table 6

Component Names and Concentrations of Fermentation Medium-1

Components	Concentration
Yeast Extract	5 g/L
$(NH_4)_2SO_4$	1 g/L
$MgSO_4.7H_2O$	0.25 g/L
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.04 g/L
$NiCl_2.6H_2O$	0.00024 g/L
$ZnSO_4.7H_2O$	0.00029 g/L
Na_2SeO_3	0.000017 g/L
Cystein.HCl	0.25 g/L
Citrate Buffer	0.1 N

Table 7

Component Names and Concentration for Fermentation Medium-2

Components	Concentration
Yeast Extract	5 g/L
Peptone	10 g/L
NaCl	5 g/L
Cystein.HCl	0.5 g/L
Phosphate Buffer	0.1 N

Certain weight percentage of glucose and xylose or the equivalent of pretreated biomass was added to the medium.

3.6.3 Fermentation of pure xylose and pretreated biomass. It was reported that *M. thermoacetica* or *C. thermoaceticum* needs to be acclimatized to a xylose environment to obtain high yields of acetic acid. To maintain viability for xylose fermentation, it is necessary to grow

the organism in xylose and glucose media alternately. For this reason, pure xylose fermentation was carried out.

Pre-cultured *C. thermoaceticum* (ATCC49707) was used in the anaerobic fermentation of acetic acid using xylose in clostridial medium first. For this purpose, 0.2, 0.5 and 1% of xylose solution with original clostridial medium (originally contains 0.5% glucose) were prepared. Fifty mL of each of these xylose containing media were loaded into 150 ml serum bottles. The anaerobic condition was achieved by purging the system with carbon-dioxide gas using the set up as shown in Figure 21 for 30 min, which was filtrated through a 0.2µm filter first, bubbled from bottom to the top of the fermentors and finally exited to the atmosphere through another needle and filter. The bottles were then sealed and autoclaved at 121°C for 15 min. The bottles were kept at 59°C to gain the suitable temperature for the microorganism before adding *Clostridium*. Three milliliter of pre-cultured *C. thermoaceticum* was added to100 ml of fermentation medium. For pretreated biomass SSF, enzymes were also added. The bottles were kept at 59°C which is the best growth temperature for *C. thermoaceticum*. Samples were collected for HPLC analysis using 3 ml syringes, needles and 0.45 µm nylon filter.

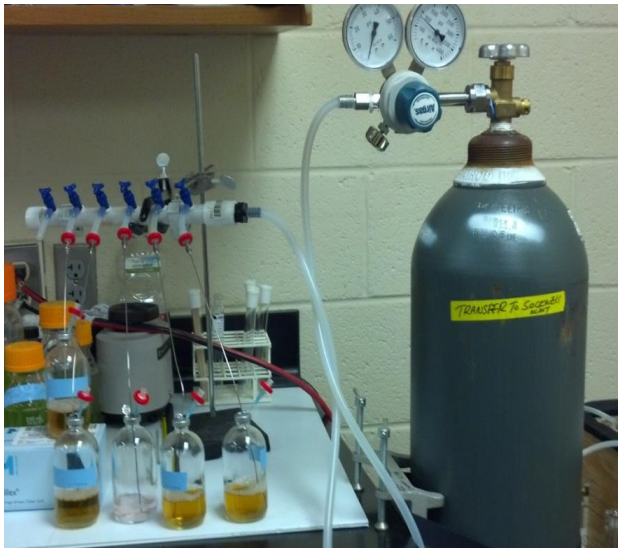

Figure 21. Experimental set-up to flush CO_2 in acetic acid fermentation medium.

Two fermentation media described in previous section were also used for the fermentation of both pure xylose and pretreated biomass fermentation of *C. thermoaceticum* in a similar fashion (see Figure 22).

Figure 22. Clostridium fermentation in oven.

3.7 Microalgae Culture in Medium Containing Biomass Juice

Juice of cattail was prepared by using a laboratory blender. Cattail juice was separated by filtration and stored in -20°C temperature for further use. Bristol medium was prepared using each of the following components at 10 ml/L: $NaNO_3$ (2.94 mM), $CaCl_2 \cdot 2H_2O$ (0.17 mM), $MgSO_4 \cdot 7H_2O$ (0.3mM), K_2HPO_4 (0.43 mM), KH_2PO_4 (1.29 mM) and NaCl (0.43 mM). Alfalfa and cattail juice was added to Bristol medium and DI water to prepare 1, 2, 5 and 10% (v/v) solution of juice and the medium. Ten ml of seed microalgae culture was added to each 100 ml of culture medium.

Only alfalfa juice culture medium was used in photobioreactor as shown in Figure 23. Three tubes of the photobioreactor were used for 1, 5 and 10% alfalfa green juice containing Bristol medium and one for proteose medium. A total of 80 ml volume was used for each tube including 5 ml seed culture inoculation. Duplication of each experiment was conducted simultaneously. Temperature was set at 25°C and light intensity at 600 $\mu molm^{-2}s^{-1}$.

Figure 23. Photobioreactor used to culture microalgae in biomass juice.

Similarly, 1, 2, 5 and 10% (v/v) biomass juice containing DI water medium were prepared. Both green juice with Bristol medium and de-ionized water medium were used to grow microalgae in room temperature. Total 100 mL of medium with inoculated algae culture in 150 mL wheaton glass bottle was used to culture microalgae in room temperature. They were kept in the lightbox with light intensity of 600 $\mu molm^{-2}s^{-1}$ unit (see Figure 24).

Figure 24. Microalgae culture in biomass juice containing medium at room temperature in the lightbox.

After inoculation, 40 mg/L chloramphenicol was used in each case for the growth of the algae. Optical density was measured for all microalgae culture every other day by spectrophotometer as shown in Figure 25 at 680 nm.

Figure 25. Spectrophotometer used to measure optical density.

CHAPTER 4

Results

4.1 Composition of Raw Biomass

Preliminary compositional analyses were conducted on the biomass raw materials without any pretreatment and the results are shown in Table 8.

Table 8

Compositions of Untreated Biomass

Raw biomass	Glucan % by mass (dry basis)	Xylan % by mass (dry basis)	Lignin % by mass (dry basis)	Ash % by mass (dry basis)	Moisture content % of fresh biomass
Algae	7.18%	9.58%	2.9%	7%	97%
Duckweed	12%	13.8%	3%	16%	92.4%
Cattail	29.5%	18.7%	20.7%	4.7%	75%

Algae and duckweed both had a low lignin content compared to cattail. However cattail had more glucan and xylan than algae and duckweed. Moisture contents of both fresh algae and duckweed were very high, which were above 90% and the moisture content of fresh cattail biomass was 75%. The moisture content of aquatic indicates that using fresh biomass would be advantageous with respect to energy required to dry them before fermentation.

4.2 Fermentation of Algae

4.2.1 Simultaneous saccharification and fermentation of algae. Cellulase concentrations, *E. coli* concentrations and cultivation temperatures were varied to observe the effects on ethanol yield during the SSF process. Table 5 shows the values of these parameters that were used during SSF of algae in this study.

4.2.1.1 Effect of enzyme concentration on SSF. Three cellulase concentrations (7.5, 15 and 60 FPU/ g glucan) were selected to observe the effects of cellulase concentration on SSF.

For all three cases, 50 ul-xylanase /g-glucan and 20 ul- β-glucosidase/g-glucan were used. While changing enzyme concentration, *E. coli* concentration of 0.5 g/L was used and fermentation temperature was set at 37°C. Figure 26, 27, and 28 show the glucose and ethanol profiles during fermentations with three different enzyme concentrations. As shown in figures, the glucose concentration decreased rapidly with the ethanol concentration increased from zero to a high level. Ethanol concentration was not increasing after 48 hours. Enzymatic hydrolysis was the limiting step in SSF process, because SSF temperature was only 37°C but the optimal enzymatic hydrolysis temperature was 50°C, thus the enzyme activities were limited by the temperature.

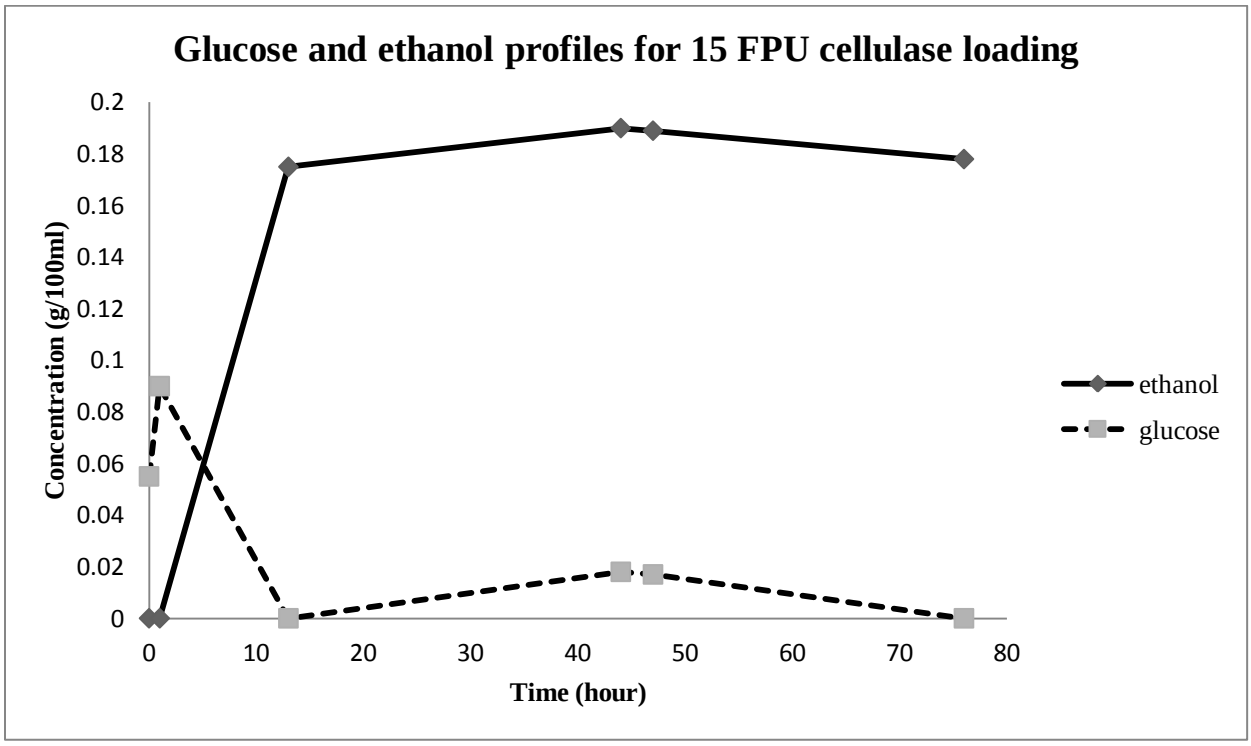

Figure 26. Concentrations of ethanol and glucose in a 150 ml fermentor during the SSF process using 15 FPU/g glucan cellulase.

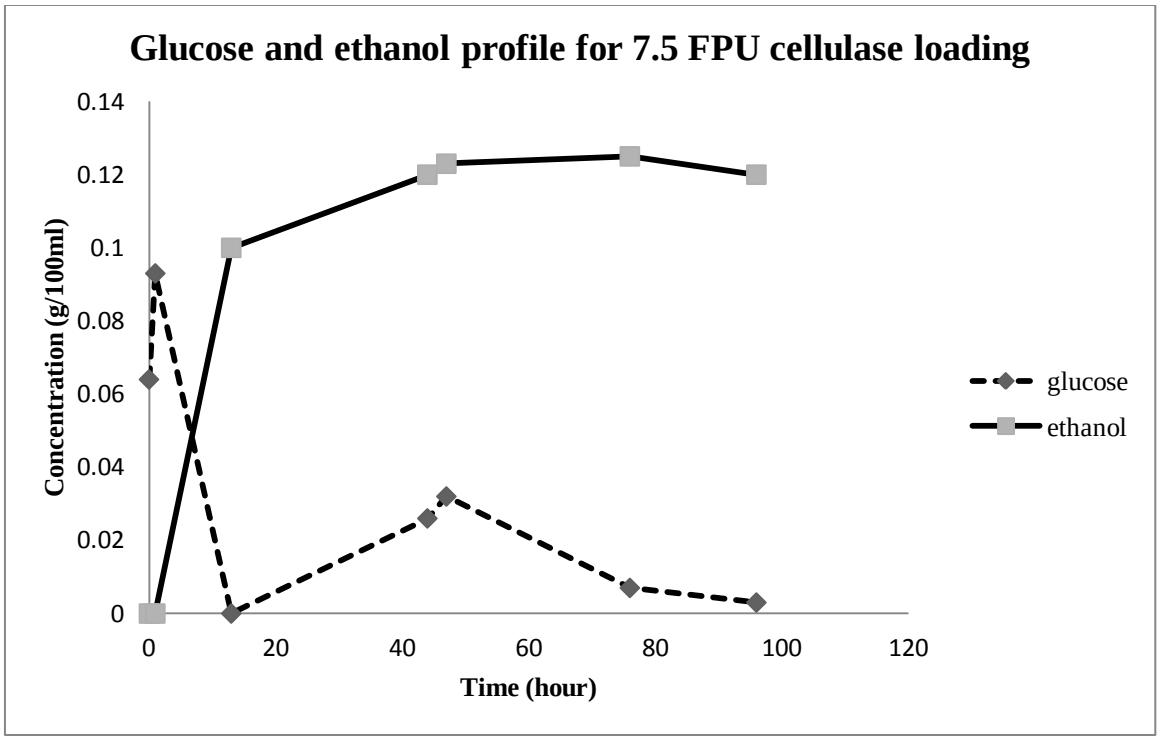

Figure 27. Concentrations of ethanol and glucose in a 150 ml fermentor during the SSF process using 7.5 FPU/ g glucan cellulase.

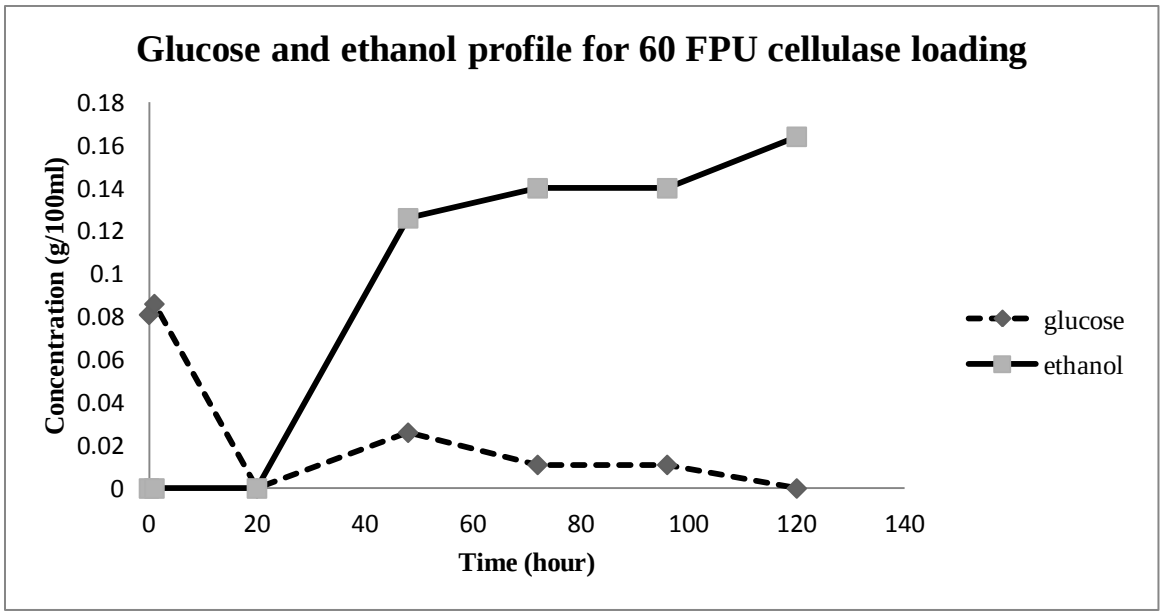

Figure 28. Concentrations of ethanol and glucose in a 150 ml fermentor during the SSF process using 60 FPU/g glucan.

Figure 29 shows the ethanol yields in SSF of algae for different enzyme concentrations. Ethanol yield increased to 77.7% from 58.9% of the theoretical yield (i.e., the maximum yield), when enzyme concentration increased from 7.5 to 15 FPU/ g-glucan.

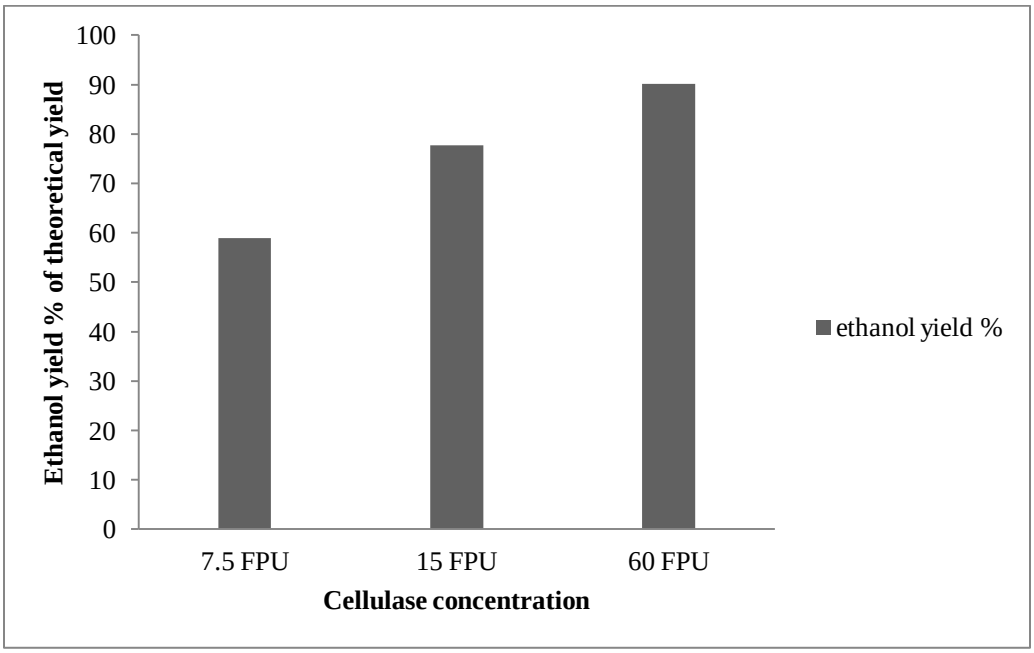

Figure 29. The effects of three different enzyme concentrations on the ethanol yields for algae SSF.

Ethanol yield increased close to 90% of the theoretical yield, when a cellulase dosage of 60 FPU/g gulcan was used. But as SSF temperature was 37°C where optimum enzyme hydrolysis temperature was 50°C, the enzyme activity was limited with respect to concentration. Enzymes are costly so future study would focus on optimization and higher temperature SSF.

4.2.1.2 Effects of E. coli concentration on SSF of microalgae. Two *E. coli* concentrations of 0.5 g/L and 1 g/L were used to observe the effects of *E. coli* concentration on the SSF process. Figure 30 shows that the *E. coli* concentration had only a slight influence on ethanol yields. This means that *E. coli* concentration of 0.5 g/L was high enough to perform the

conversion of available sugars under the specified SSF conditions. And higher *E. coli* concentration will also increase the bioprocess cost.

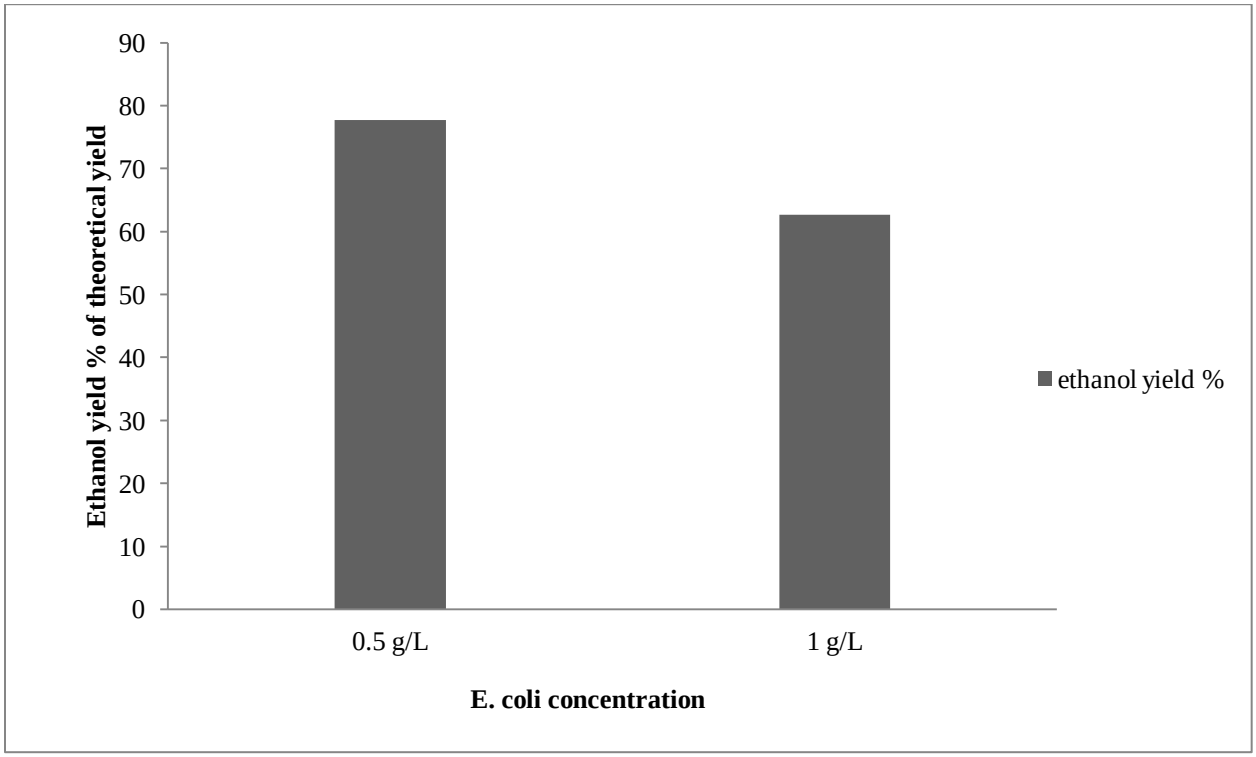

Figure 30. The effects of *E. coli* concentrations on the ethanol yields for algae SSF.

4.2.1.3 Effects of temperature on SSF of microalgae. Optimal *E. coli* fermentation temperature is 37°C where optimal enzyme hydrolysis temperature was 50°C. Temperature above 40°C is unsuitable for *E. coli* to grow. Considering this, two temperatures of 37°C and 40°C were used to observe the effects of *E. coli* concentration on SSF. Cellulase concentration used was 15 FPU/ g glucan. Figure 31 shows that increasing temperature decreased the ethanol yield from 77.7 to 50.9% of the theoretical yield. This means that 37°C temperature is the most suitable temperature to perform the conversion work under the specified SSF conditions.

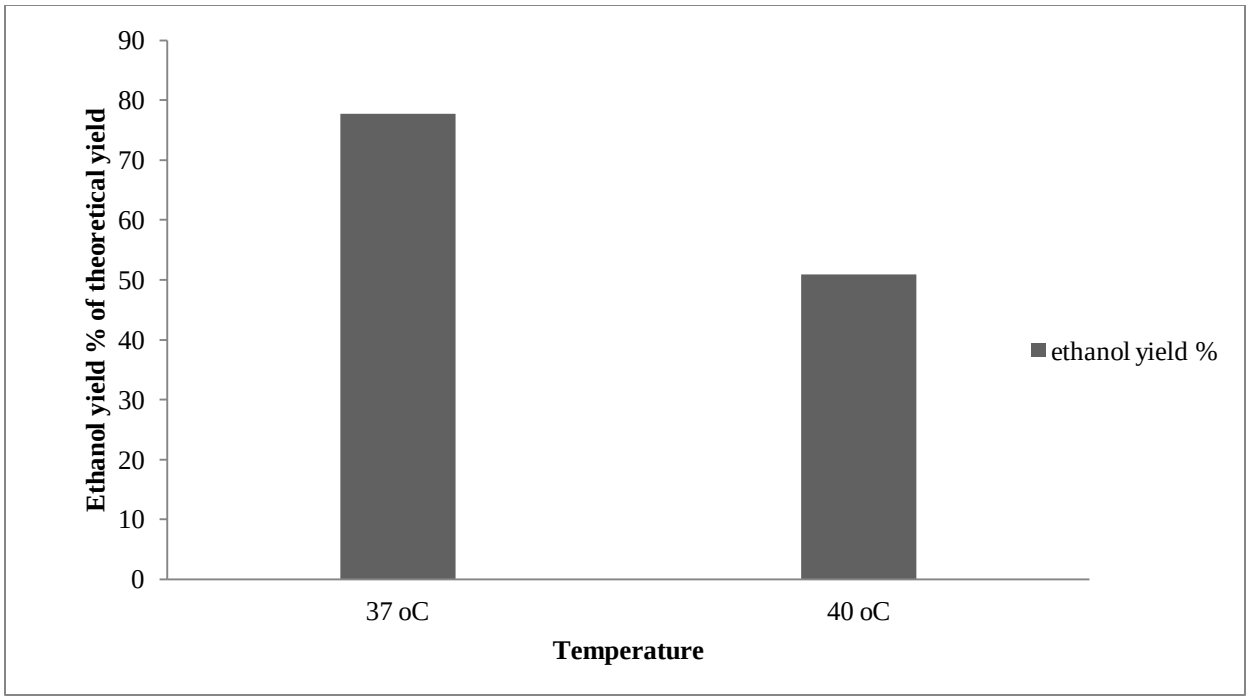

Figure 31. The effects of the cultivation temperature on ethanol yields for algae SSF.

4.2.1.4 Effects of biomass concentration on SSF of microalgae. Biomass concentration was increased from 0.5% to 3% for SSF of algae to investigate the effects of biomass concentration on SSF. Ethanol yield for 3% biomass concentration was 60% of theoretical yield compared to 77.7% for the 0.5% biomass concentration during SSF when other fermentation conditions were the same. Therefore, the increase in biomass concentration decreased the ethanol yield.

4.2.2 Separate hydrolysis and fermentation of algae. Separate hydrolysis and fermentation (SHF) was also carried out using fresh algae. Enzymatic hydrolysis and fermentation of the fresh algae was conducted after 30 min and 60 min ultrasonic pretreatment. Biomass concentration was 0.5% and enzyme concentration was 7.5 FPU during the enzymatic hydrolysis. Figure 32 shows the changes of the ethanol yield during the SHF process. It shows that ethanol yield increases with the increase in pretreatment time. The ethanol during the SHF process of algae without any pretreatment was 36.4% of the theoretical yield. The 60 min

ultrasonic pretreatment can increase the ethanol yield to 56.8%. However, the ultrasonic pretreatment for 60 min consumes more energy and produces a substantial amount of heat, which increases the bioprocessing cost. Ten-min ultrasonic pretreatment of algae was used for all SSF experiments. It was found that the ethanol yield at 58.9% of theoretical value was achieved during SSF using the same biomass and enzyme concentrations.

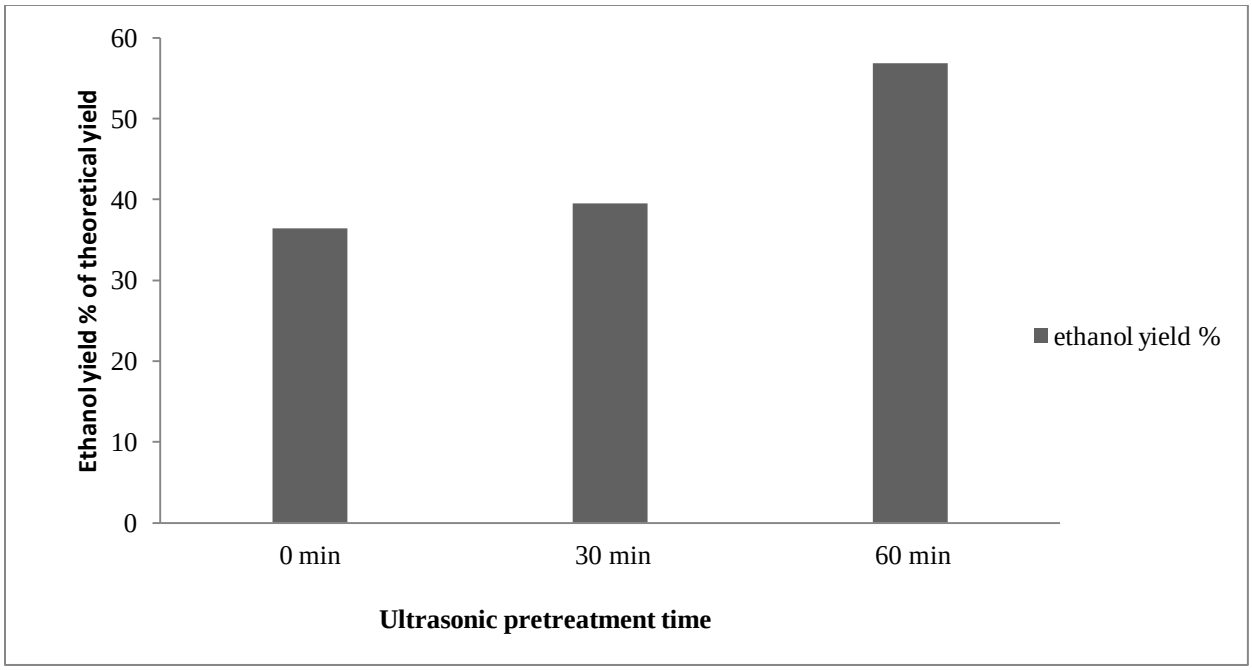

Figure 32. Effects of the ultrasonic pretreatment conditions on ethanol yields for algae SHF.

So higher ethanol yield was achieved with SSF experiment of algae compared to SHF experiment under the same fermentation condition

4.3 Protein Content of Algae and Algal Residue after SSF

Another objective of this research was to investigate the effect of enzyme hydrolysis and ethanol fermentation on the protein content of algae during SSF. Fresh algae and algal residues after SSF were centrifuged. The solid residues were collected and dried at 50°C for 2 days. Elemental contents of C, H, N and S of these dried algae and fermented residues were

determined by an elemental analyzer. Protein content was calculated by multiplying N% by a factor of 6.25.

Table 9 shows the result obtained from elemental analyzer (i.e., elemental C, H, N and S content of algae and fermented residue at different fermentation conditions). Figure 33 shows effects of change of protein content by fermentation of algae with different cellulase loadings. It shows that protein content increased by all fermentation experiments compared to that of the pure algae. The highest protein content was 71.81% which was obtained from the SSF of algae with 15 FPU cellulase loading while the protein content of fresh algae was 62.56%.

Figure 34 shows effects of change of protein content by fermentation of algae with different fermentation conditions. The protein content was increased for all fermentation experiments. The highest protein content determined was 71.81% from the SSF of algae with 15 FPU cellulase loading. So the removal of carbohydrates during SSF increases the protein content of algal biomass. These nitrogen enriched fermented residue can be used as organic fertilizer.

Table 9

C, H, S, N % Obtained from Elemental Analysis and Calculated Protein Contents of the Algal Residues Collected from Different Fermentation Conditions

Fermentation condition	C% (by mass)	H% (by mass)	S% (by mass)	N% (by mass)	Protein content % by mass
Fresh algae without fermentation	42.86	6.37	1.21	10.01	62.56
Fermentation with 7.5 FPU cellulase concentration	48.87	7.04	1.38	10.73	67.06
Fermentation with 15 FPU cellulase concentration	49.81	7.10	1.33	11.49	71.81

Table 9

(Cont.)

Fermentation condition	C% (by mass)	H% (by mass)	S% (by mass)	N% (by mass)	Protein content % by mass
Fermentation with 60 FPU cellulase concentration	49.27	6.86	1.31	10.84	67.72
Fermentation with 1g/L *E. coli* concentration	49.98	6.94	1.30	11.30	70.59
Fermentation with 0.5g/L *E. coli* concentration at 40°C	50.10	7.00	1.32	10.98	68.62

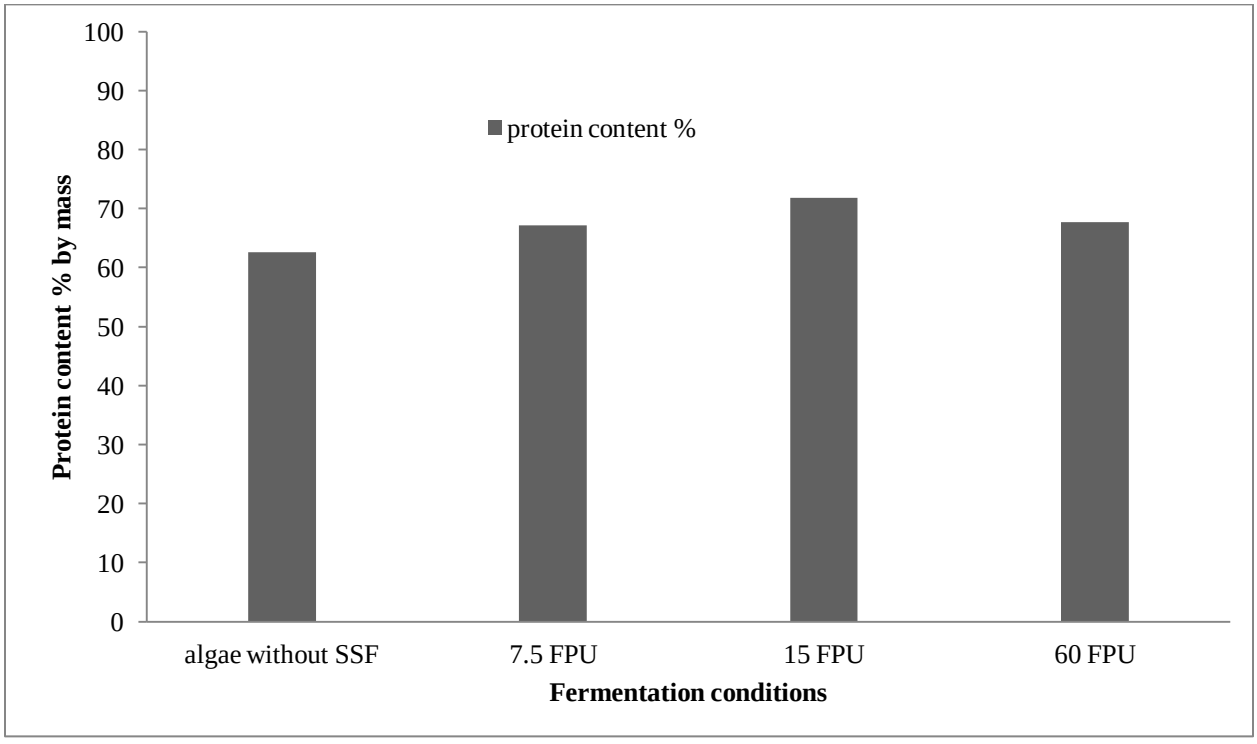

Figure 33. Effects of change protein content by SSF of algae with different cellulase concentrations.

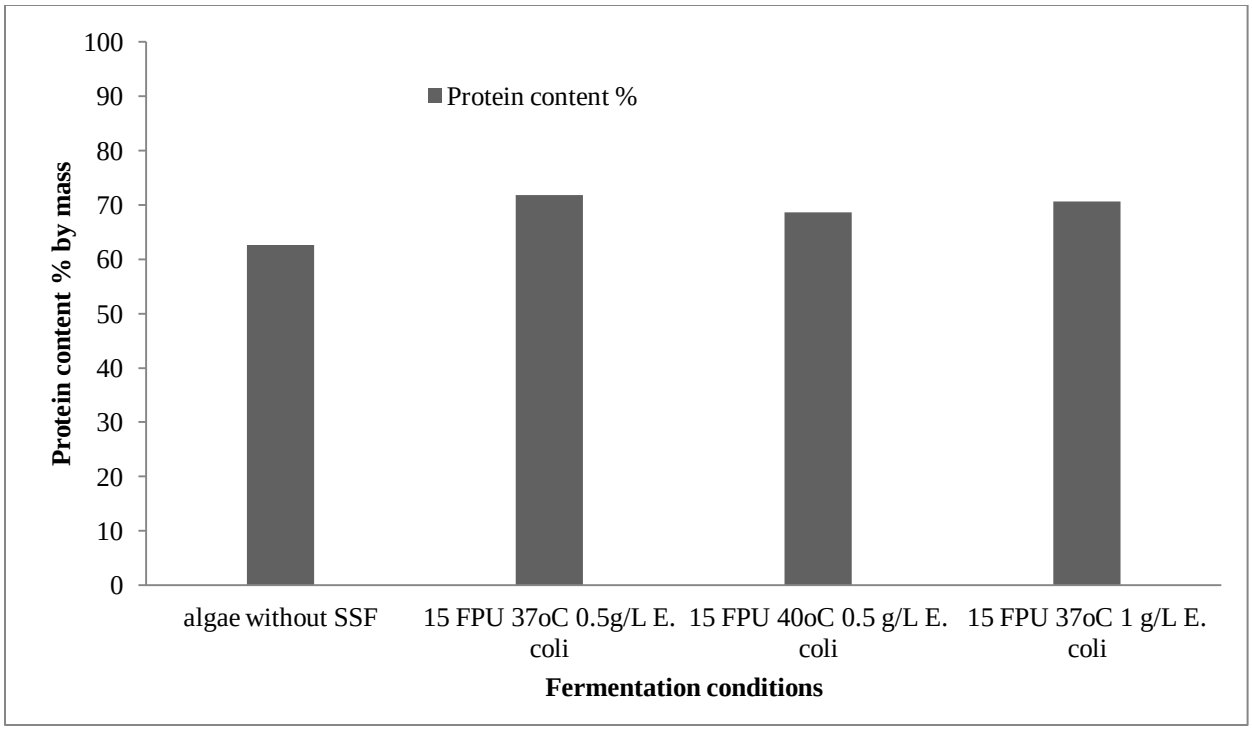

Figure 34. Effects of change protein content by SSF of algae with different fermentation conditions.

4.4 Simultaneous Saccharification and Fermentation of Duckweed

Fresh duckweed was collected and washed with water before SSF in a 150 ml fermentor. The duckweed undergoes a series of pretreatments. First only homogenized duckweed was used for fermentation. Then homogenized and autoclaved duckweed was used for fermentation. Homogenized duckweed was subjected to ultrasonic treatment and then autoclaved. These pretreated duckweeds were used to analyze the change in ethanol yield for three levels of pretreatments. Autoclaving was done at 121°C for 15 min. Ultrasonic homogenizer was used for 10 min at 25% amplitude and then autoclaved before SSF was carried out. Biomass concentration of 1% (w/v) was used for all duckweed fermentation experiments.

Figure 35 shows the ethanol and glucose profiles in SSF of duckweed. It follows a similar trend as algae. The glucose concentration decreased rapidly while the ethanol

concentration increased from zero to a high level. Figure 36 shows the comparison of ethanol yields using the three different pretreatment methods. With the increase of pretreatment levels, the ethanol yield increased from 35 to 90% of the theoretical value. It is also clear that autoclaving can increase the ethanol yield substantially, while 10 min of ultrasonic treatment can only further increase the ethanol yield slightly. So the combination of thermal treatment by autoclaving and ultrasonic pretreatment is an effective pretreatment method for fresh duckweed fermentation.

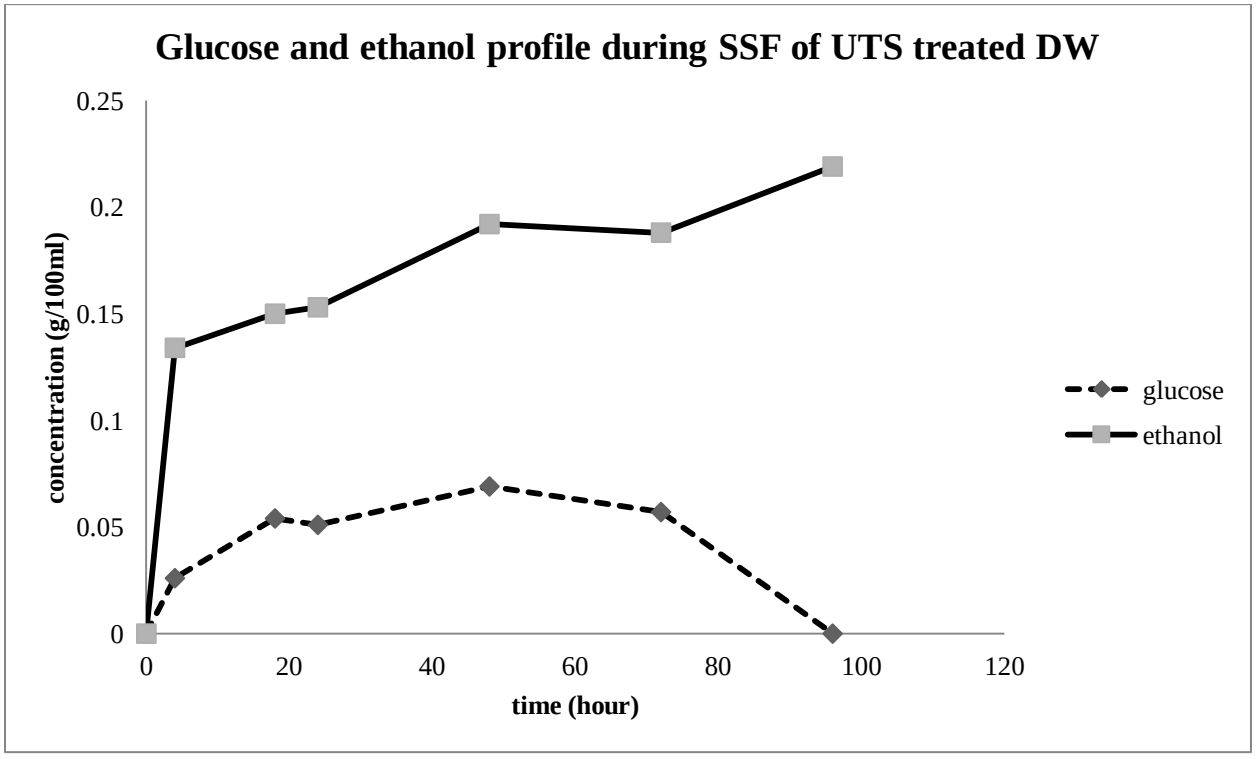

Figure 35. Ethanol and glucose profiles for SSF of ultrasonically treated duckweed.

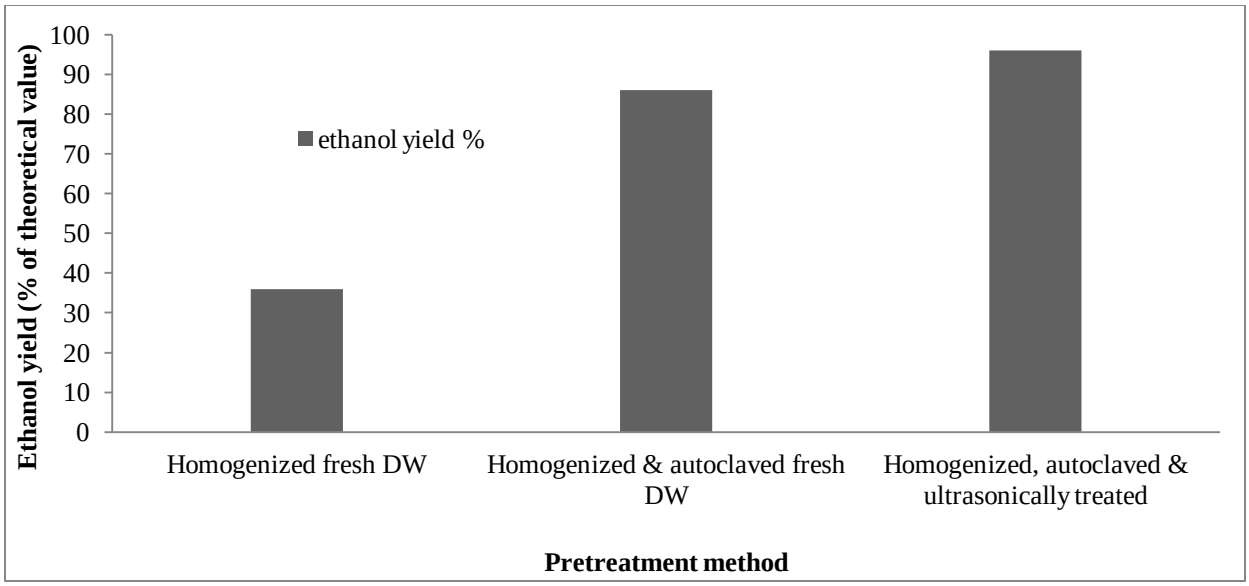

Figure 36. The effects of three different pretreatment methods on ethanol yields for duckweed SSF with 1% (w/v) biomass concentration.

4.4.1 Effects of biomass concentration on SSF of duckweed. Biomass concentration was increased from 1% to 3% for SSF of duckweed to investigate the effects of biomass concentration on SSF. For 3% biomass concentration, ethanol yield was only 30% of theoretical value. But under the same fermentation condition, ethanol yield for the SSF of duckweed at 1% biomass concentration was 90% of theoretical value. Therefore, the increase in biomass concentration decreased the ethanol yield significantly.

4.5 Acid Hydrolysis and Fermentation of Duckweed

Three different concentrations of dilute sulfuric acid were used to hydrolyze fresh duckweed in the autoclave at 121°C for 25 min. Hydrolyzed slurry was neutralized by ammonium hydroxide to a pH value of 7.0 to use this slurry directly for fermentation. Figure 37 shows the highest ethanol concentration achieved after fermenting acid hydrolyzed fresh duckweed for 96 hours. Biomass concentration used was 1.5% (w/v). According to Figure 37 the increase of acid concentration had almost no influence on ethanol concentration. The highest

ethanol concentration of 0.425g/100ml was obtained from the duckweed hydrolyzed by 1.2% acid.

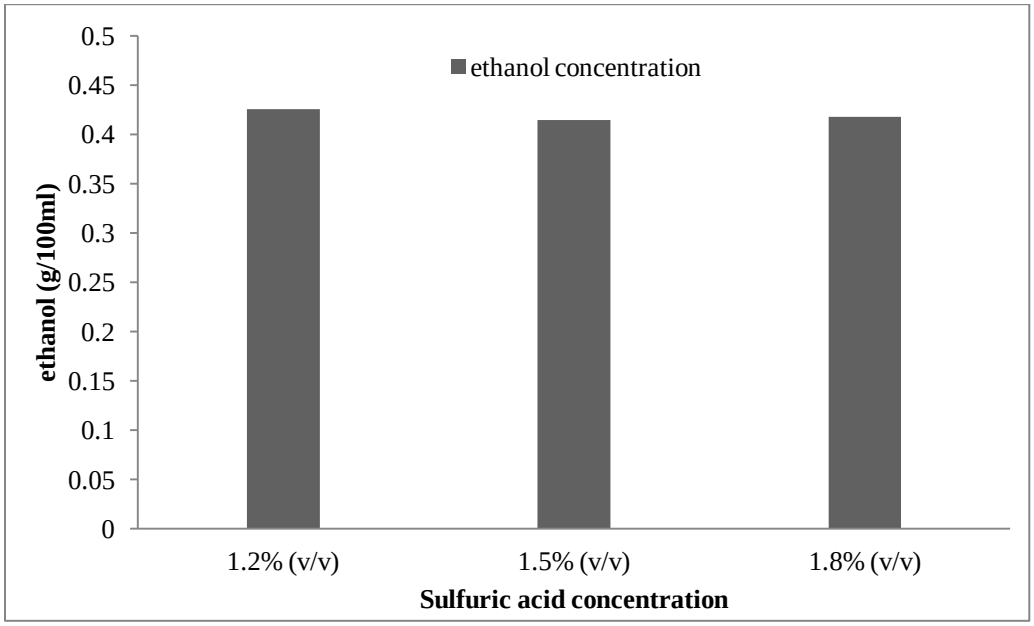

Figure 37. The effects of three different acid concentrations on ethanol concentration of fresh duckweed fermentation.

4.6 Fermentation of Cattail

4.6.1 Separate hydrolysis and fermentation of cattail. To fractionate cattails, fresh cattails were first homogenized with DI water and then filtered to separate the juice and solid part. As cattail has a high amount of lignin, pretreatment was needed to separate the cellulose from lignin. This parameter relates to ultrasonic power, meaning that increase in amplitude leads to increase in power input. Biomass concentration of 1% (w/v) was used. Ultrasonic pretreatment was done with three different amplitudes-45%, 55% and 60% and pretreatment time was set to 15 min. Separate hydrolysis was carried out with cellulase of 7.5 FPU/g glucan to analyze the efficiency of the pretreatment. Figure 38 shows the glucose profile during the enzymatic hydrolysis of ultrasonically treated fresh cattail.

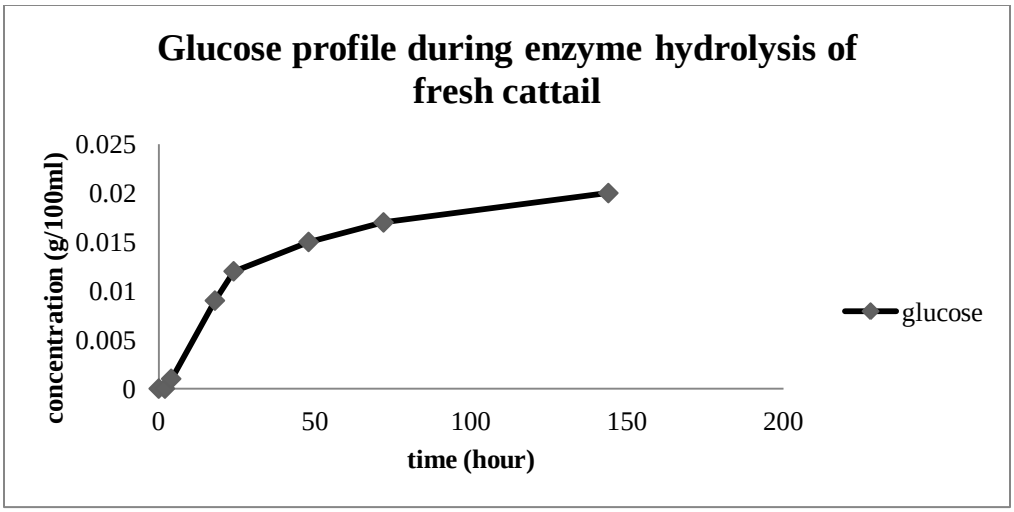

Figure 38. Glucose profile for separate hydrolysis of ultrasonically treated (45% amplitude and 15 min) cattail of 1% (w/v) biomass concentration with 7.5 FPU cellulase concentration.

E. coli fermentation was conducted after enzymatic hydrolysis where similar trends were found for ethanol and glucose profiles as algae and duckweed fermentation.

Figure 39 shows the ethanol yields of separated hydrolysis and fermentation of fresh cattail for different pretreatment conditions of ultrasonic treatment.

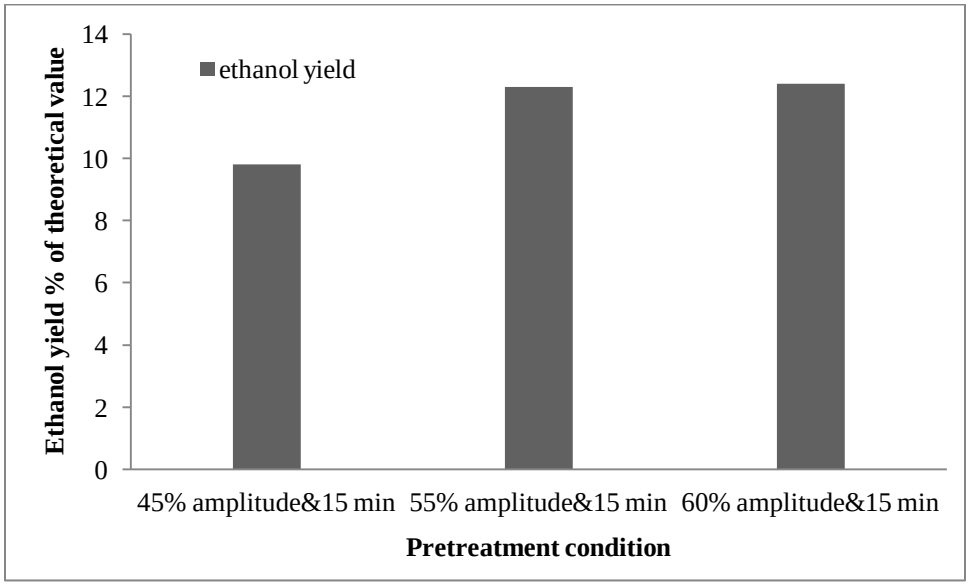

Figure 39. Glucose and ethanol yield with different ultrasonic pretreatment condition of fresh cattail.

Figure 39 shows that the increase of amplitude during ultrasonic treatment has slight influence on ethanol yields. But higher amplitude will result in higher energy consumption, and it also produces heat which can increase bioprocess cost.

4.6.2 Simultaneous saccharification and fermentation of cattail. Fresh cattail was homogenized with DI water and then filtered to separate the juice and solid part. That separated solid part was used for SSF in 150 ml fermentors. Around 1% biomass concentration (w/v) was loaded for each fermentation experiment where buffer citrate with a pH value of 5.0 was used as a fermentation medium. Ultrasonic pretreatment was done for 10 min at 30% amplitude and then the slurry was autoclaved before fermentation. Enzyme concentration used was 15 FPU cellulase concentration/g-glucan.

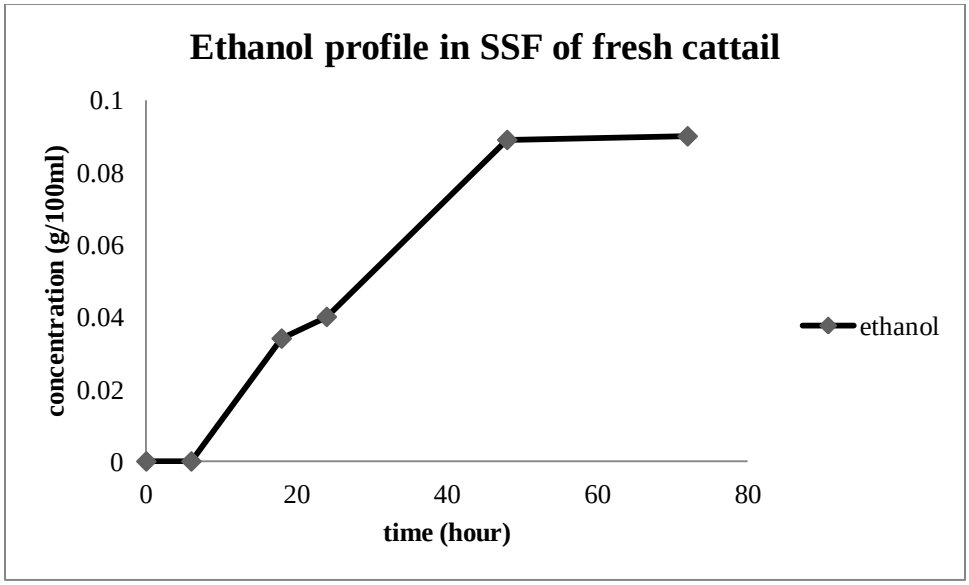

Figure 40. Ethanol concentration during SSF of fresh cattail.

Figure 40 shows only the ethanol profile during SSF. The ethanol yield was only 8% of the theoretical value (maximum ethanol yield). Such a low ethanol yield can be caused by higher lignin content of cattail biomass compared to duckweed and algae. So ultrasonic pretreatment was not sufficient for the pretreatment of fresh cattail for ethanol fermentation.

4.7 Comparison of Ethanol Yield for The SSF of Fresh Algae, Duckweed and Cattail

Figure 41 shows the comparison of ethanol yields achieved in SSF of fresh algae, duckweed and cattail at same fermentation conditions—all of them were pretreated by ultrasonic for 10 min and autoclaved at 121°C for 15 min. Biomass concentration used was 1% (w/v) and 15 FPU/g-glucan cellulase concentration was used. Duckweed and cattail were needed to be homogenized before ultrasonic pretreatment where fresh algae were directly used for pretreatment after harvesting.

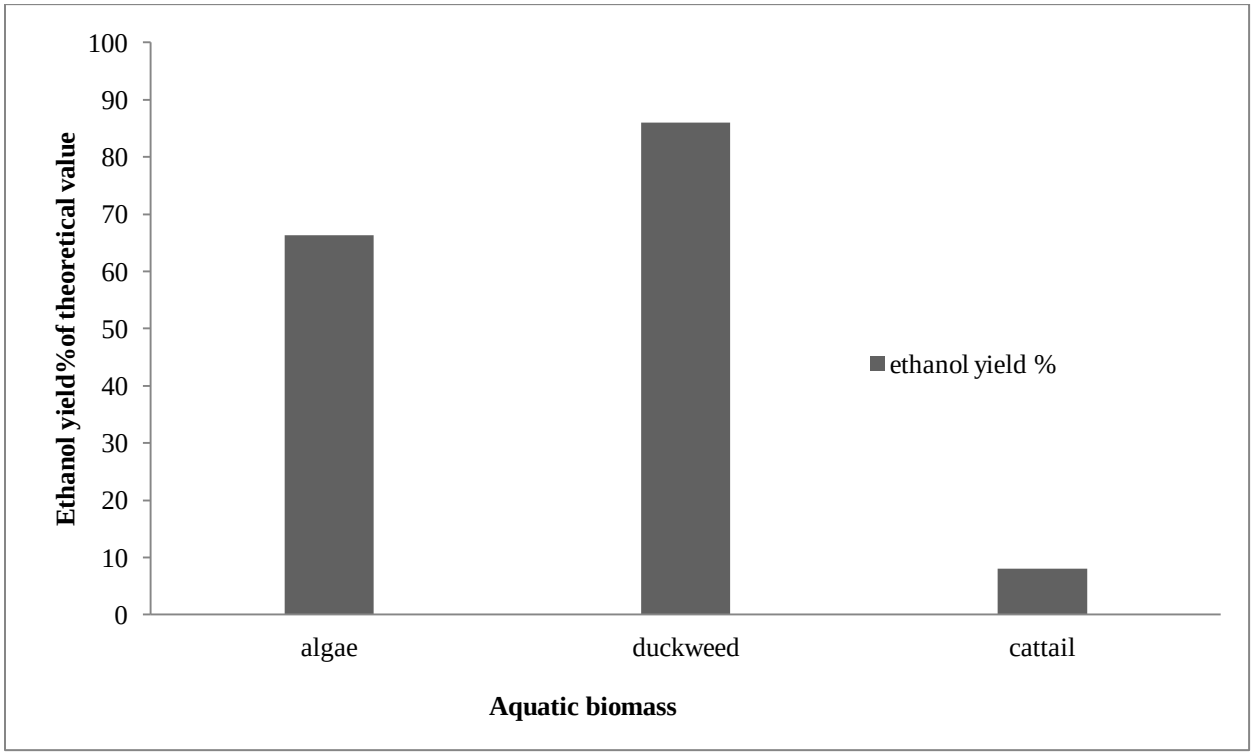

Figure 41. Comparison of ethanol yields achieved during SSF of fresh algae, cattail and duckweed in same fermentation condition.

According to Figure 41 in same condition, the highest ethanol yield was 86%, which was achieved by SSF of fresh duckweed while the SSF of fresh algae achieved a 66.31% ethanol yield. Ethanol yield for cattail is very low compared to algae and duckweed. The main reason for such low ethanol yield for cattail was that cattail has a high lignin content. As both algae and

duckweed have a little amount of lignin, moderate pretreatment can make celluloses more accessible for enzyme hydrolysis. Strong pretreatment like chemical pretreatment is needed to achieve higher ethanol yield from fresh cattail.

4.8 Microalgae Culture in Biomass Juice

The growth curves of microalgae *Chlorella sp.* were determined by measuring the optical density. Two types of media containing different concentration of biomass juice were used as the growth medium. One is Bristol medium and other is DI water. The original culture medium of the algae was proteose medium which is modified Bristol medium with 1g/L proteose peptone.

4.8.1 Microalgae culture in Alfalfa juice

4.8.1.1 Microalgae culture in Alfalfa juice with Bristol medium. An objective of this research was to investigate the usefulness of liquid juice separated from biomass as a culture medium for microalgae. The liquid juice from fresh biomass contains proteins, free amino acids, organic acids, dyes, enzymes, hormones, other organic substances and minerals. So it can be a good source to prepare culture medium for algae. To investigate this assumption first collected alfalfa juice was added into Bristol medium and DI water medium to prepare 1, 2, 5 and 10% concentration. Microalgae *Chlorella* sp. was grown in these media at a room temperature in the light-box and at 25°C in the photobioreactor. Antibiotic chloramphenicol was used to reduce contamination caused by bacterial growth in the culturing medium. Original culture medium for *Chlorella* sp. (i.e., proteose medium was also used to culture microalgae in the same volume to compare the growth with juice contained medium).

Figure 42 shows the growth curves that were obtained at room temperature and 600 μmol $m^{-2}s^{-1}$ light intensity. *Chlorella* could grow in all of the juice media. The growth of microalgae

was increased with the increase of juice concentration. Microalgae grew in a medium with 5% juice at a similar rate as the proteose medium. Microalgae grew faster in the medium containing 10% juice than the proteose medium. Highest OD value achieved was 1.2 by 10% juice compared to the highest OD value of 1.0 for the proteose medium. But for the medium containing 5% and 10% juice, contamination was observed after 10 days.

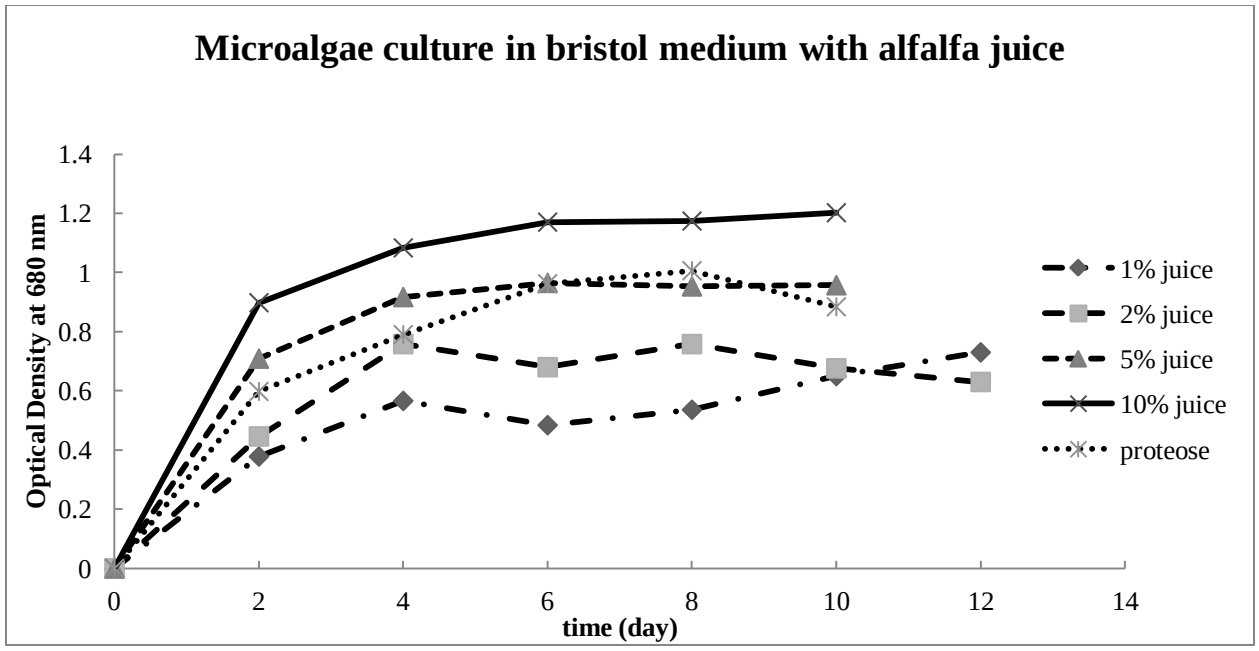

Figure 42. Growth curves of Chlorella sp. in Bristol medium with alfalfa juice at room temperature and 600 μmol $m^{-2}s^{-1}$ light intensity.

4.8.1.2 Microalgae culture in Alfalfa juice with DI water medium. Figure 43 shows the growth curves of *Chlorella* sp. in DI water containing alfalfa juice at a room temperature. Contamination occurred very quickly in all DI water media. As a result smooth curve were not obtained. The graph shows a high growth rate between day 0 and day 4 in all juice media. After day 4, the decrease and fluctuations of growth rates indicate contaminations. Although 1 ml/L chloramphenicol dissolved in ethanol was used as antibiotic to reduce contamination, ethanol can

accelerate contamination of yeast. Another antibiotic ampicillin dissolved in DI water was used for the following culturing experiment.

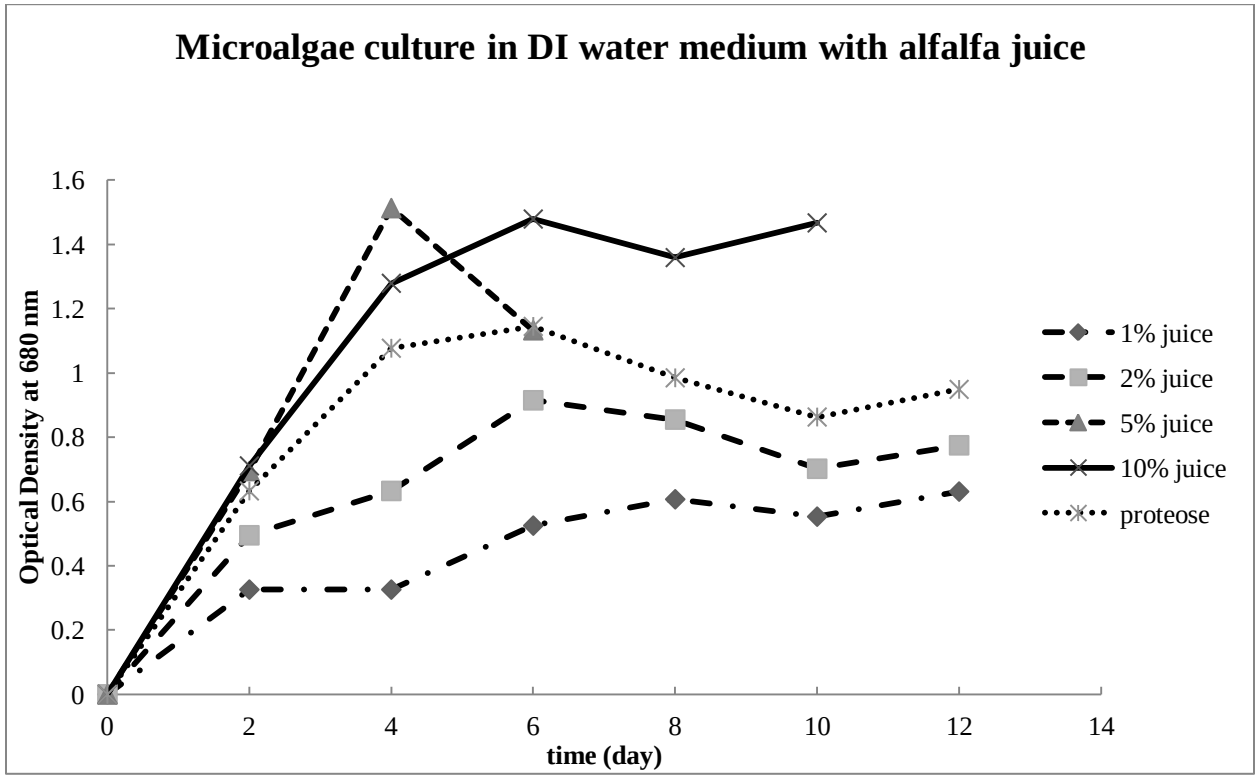

Figure 43. Growth curves of Chlorella sp. in DI water medium with alfalfa juice.

4.8.1.3 Microalgae culture in Alfalfa juice with bristol medium in photobioreactor.

Figure 44 shows the growth curves of *Chlorella* sp. in Bristol medium containing alfalfa juice at 25°C and 600 $\mu molm^2s^{-1}$ light intensity in the photobioreactor. From Figure 37, it was observed that in the 5% juice medium microalgae grew in a similar rate as the proteose medium between day 0 and day 4. After that contamination was observed. It also shows a similar growth of *Chlorella* sp. in both 5% and 10% juice. Highest OD value achieved was 1.74 by 10% juice where highest OD value for proteose medium was 1.45 Å.

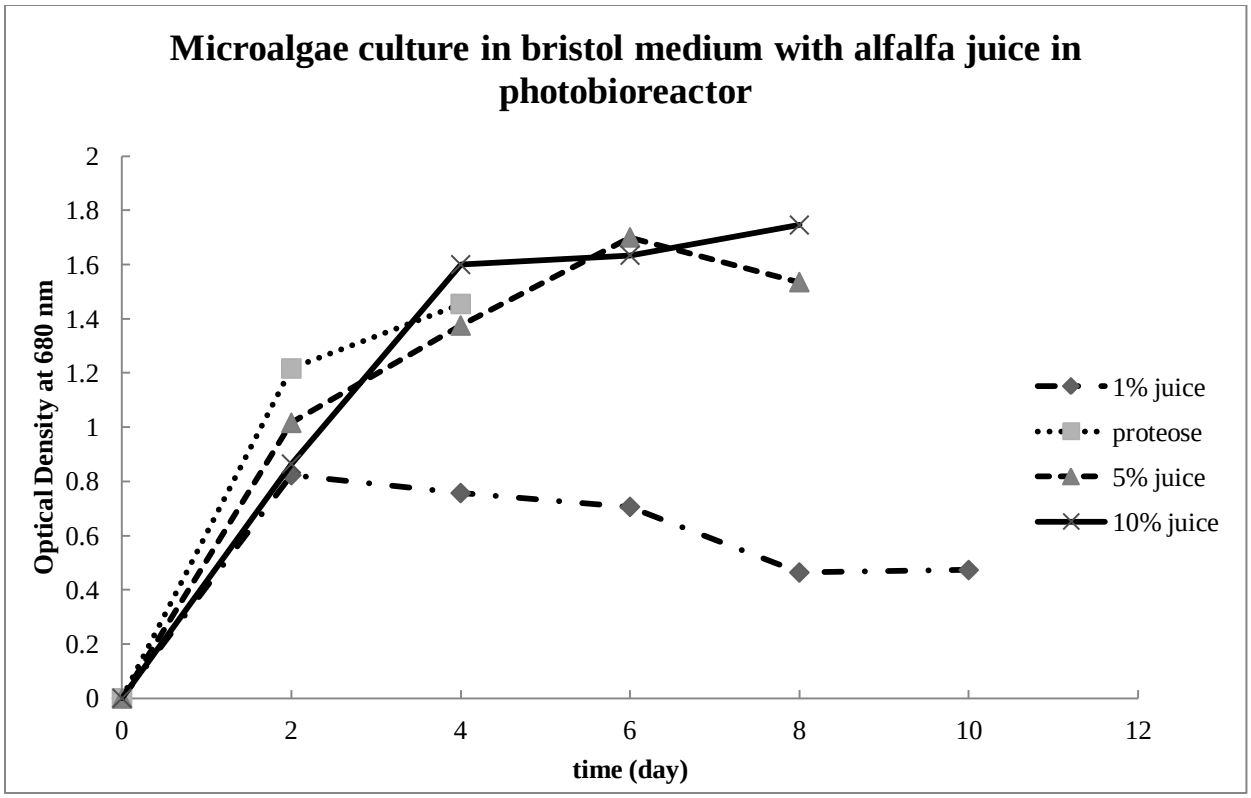

Figure 44. Growth curves of *Chlorella sp.* in Bristol medium with alfalfa juice at 25°C and 600 $\mu molm^{-2}s\text{-}^{1}$ in the photobioreactor.

4.8.2 Microalgae culture in cattail juice. Similar to the alfalfa juice cattail juice containing Bristol medium and DI water medium were used to grow microalgae.

4.8.2.1 Microalgae culture in cattail juice with Bristol medium. Figure 45 shows the growth curves of Chlorella sp. in Bristol medium with cattail juice that was obtained at room temperature and 600 $\mu molm^{-2}s^{-1}$ light intensity. Chlorella grew in all of the juice media. The growth rate of microalgae increased with increasing juice concentration. The highest growth was observed in 10% juice medium. In 1% and 2% juice, the similar growth patterns of microalgae were observed.

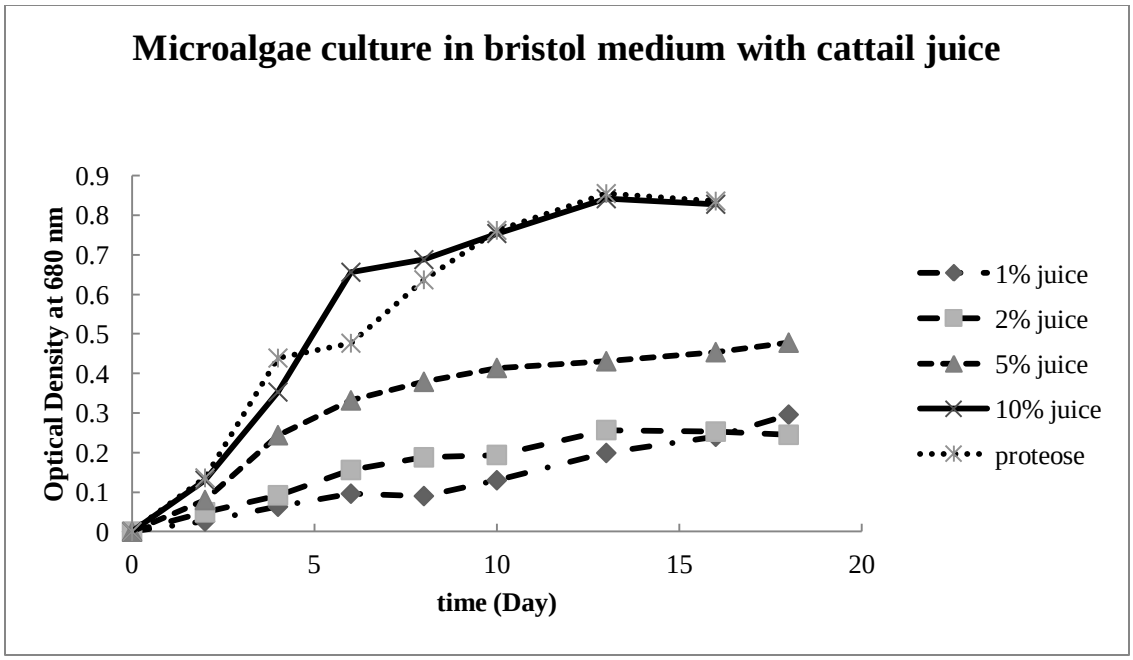

Figure 45. Growth curves of Chlorella sp. in Bristol medium with cattail juice at room temperature and 600 $\mu molm^{-2}s^{-1}$ light intensity.

Contamination observed in 10% juice medium after 14 days. No contamination was observed for 1, 2 and 5% juice concentration after 14 days. Another antibiotic ampicillin dissolved in water was used instead of chloroamphenicol to reduce the contamination and to avoid adding ethanol in medium. Highest OD value achieved was 0.84 Å by 10% juice where highest OD value for proteose medium was 0.85 Å.

4.8.2.2 Microalgae culture in cattail juice with DI water medium. Figure 46 shows the growth curves of *Chlorella* sp. in DI water medium with cattail juice. The growth curves were obtained at room temperature and 600 $\mu molm^{-2}s^{-1}$ light intensity. The growth rate of microalgae increased with the increase of juice concentration. The highest growth rate was observed in 10% juice medium. In 1% and 2% juice, a similar growth pattern of microalgae was observed. Highest OD value achieved was 0.78 Å by 10% juice where highest OD value for proteose medium was 0.85 Å.

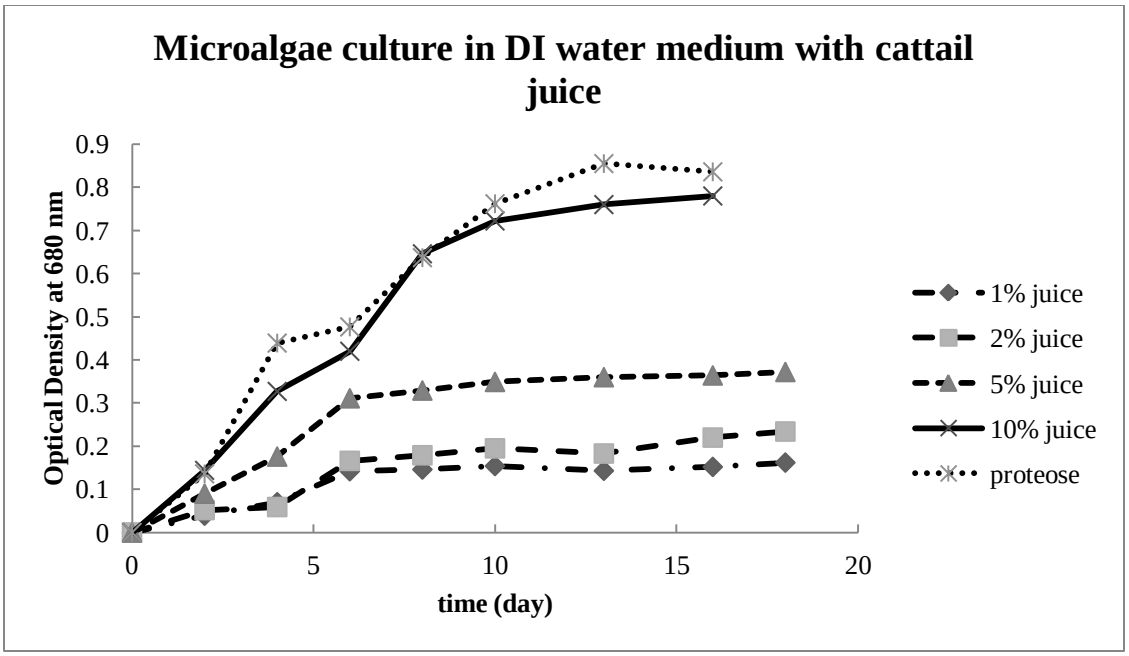

Figure 46. Growth curves of *Chlorella sp.* in DI water medium with cattail juice at room temperature and 600 $\mu molm^{-2}s^{-1}$ light intensity.

Contamination was observed in the 10% juice medium after 14 days. According to Figure 46 the optical density of microalgae (or the growth of microalgae) in DI water medium was generally lower than that of Bristol medium, as Bristol medium contained more minerals. When comparing to the media with alfalfa juice, microalgae growth in the media with cattail juice was relatively slower. One assumption is that the solid concentration of alfalfa juice was higher than cattail juice. As cattail juice was obtained by homogenizing fresh cattail with DI water at 1 to 5 ratio, where alfalfa juice was obtained using 1 to 3 ratio. However, contamination occurs rapidly in higher juice concentration than in lower juice concentration. Further study regarding microalgae growth in juice containing medium is needed to optimize the juice concentration with microalgal growth.

4.9 Acetic Acid Production

4.9.1 Pure xylose fermentation by clostridium.

4.9.1.1 Pure xylose fermentation in original clostridial medium. The strain of *Clostridium thermoaceticum* (ATCC 49707) was obtained from the American Type Culture Collection. The freeze-dried culture of *C. thermoaceticum* was rehydrated by using the Reinforced Clostridial Medium (BD 218081, Becton, Dickinson and company) according to the instructions of ATCC. The medium composition is given in Chapter 3. It contains 0.5% glucose. To check the xylose fermentability of the bacteria, pure xylose was added to the original medium. Two different concentrations of xylose were used 0.2% and 0.5%. Table 10 shows the result of pure xylose fermentation by *Clostridium*.

As shown in Table 10, *Clostridium thermoaceticum* can utilize xylose and produce acetic acid up to 1% concentration. Figures 47 and 48 show that *Clostridium* can utilize xylose faster in the medium containing 0.2% xylose than the medium containing 0.5% xylose. The highest acetic acid concentration of 0.998 g/100 ml was achieved by 0.5% added xylose fermentation while the highest acetic acid concentration of 0.952 g/100 ml was obtained by 0.2% added xylose fermentation.

Table 10

Pure Xylose Fermentation by Clostridium thermoaceticum

Xylose concentration added(g/100ml)	Time (hour)	Glucose (g/100ml)	Xylose (g/100ml)	acetic acid (g/100ml)
0.5	0	0.888	0.887	0.658
	48	0.858	0.577	0.986
	72	0.844	0.558	0.998
	96	0.840	0.556	0.998

Table 10

(Cont.)

Xylose concentration added(g/100ml)	Time (hour)	Glucose (g/100ml)	Xylose (g/100ml)	acetic acid (g/100ml)
0.2	0	0.884	0.634	0.659
	48	0.859	0.1	0.937
	72	0.831	0.0	0.951
	96	0.83	0.0	0.952

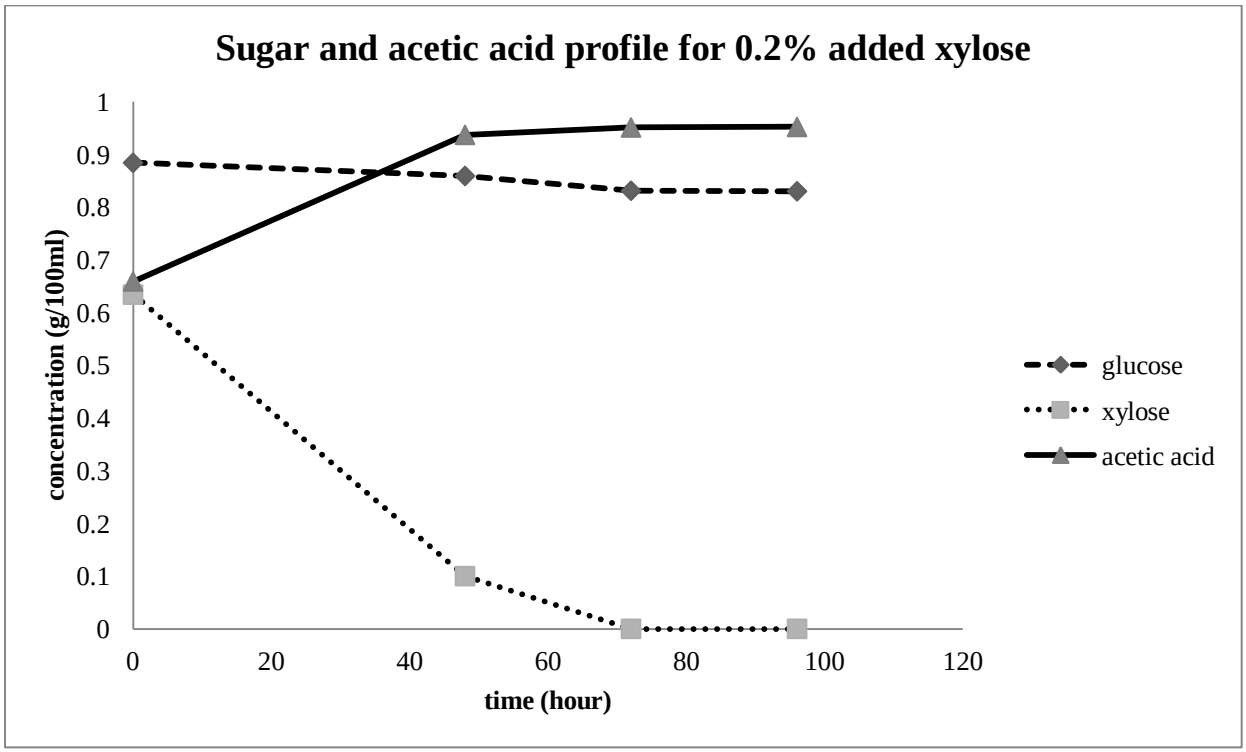

Figure 47. The profiles of sugars and acetic acid during the fermentation of *Clostridium* in the medium containing 0.2% xylose.

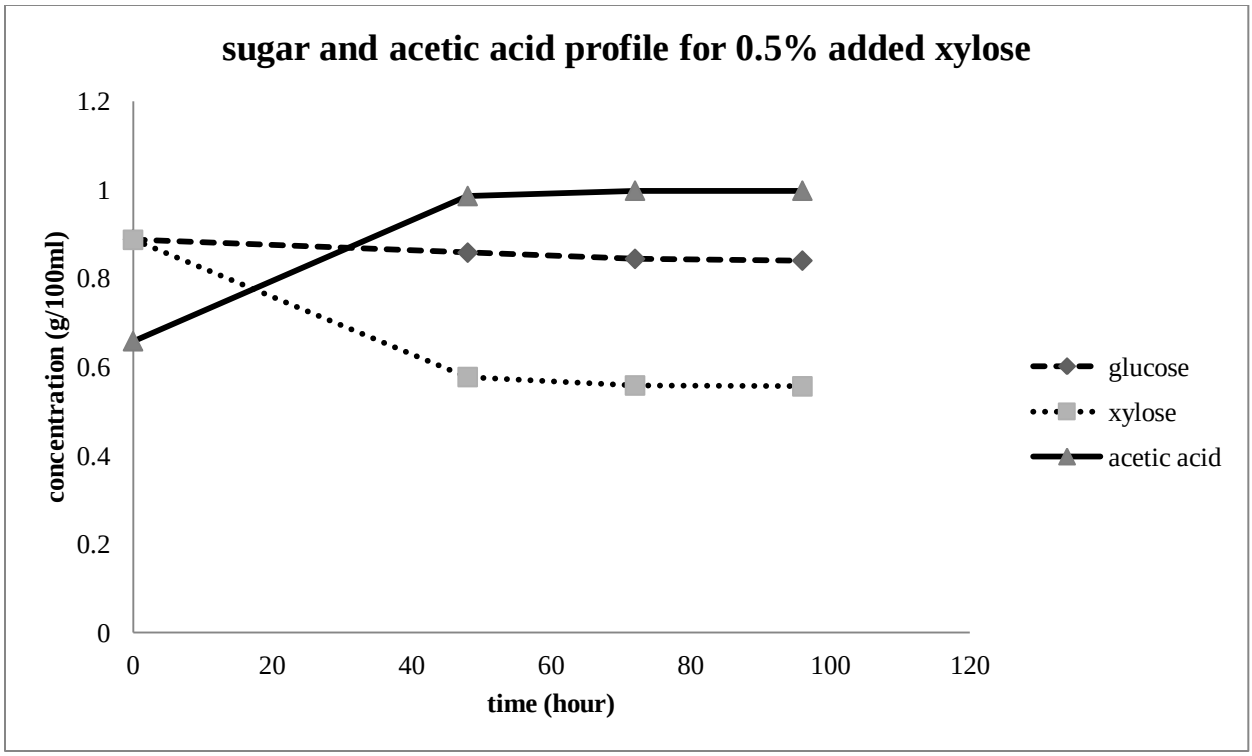

Figure 48. The profiles of sugars and acetic acid during the fermentation of *Clostridium* in the medium containing 0.5% xylose.

One possible reason for the low acetic acid production is the low acetate tolerance of the bacteria. As acetate concentration increases, the pH value decreases but the bacteria cannot produce acetic acid at a low pH value. Optimum pH for acetic acid production is between 5.5 and 6. It has been reported that the decrease of the pH value to 5.5 has detrimental effect on acetic acid production rate. Another reason is initial low pH of the fermentation medium. As 3 ml/100ml medium clostridium was added to fermentation broth, it contained some acetic acid. The clostridium cells should be washed with peptone before adding it to fermentation broth. But as clostridium was strictly anaerobic this washing procedure could not be done without contacting air.

4.9.1.2 Pure xylose fermentation in fermentation medium. Two fermentation media were designed for *Clostridium* which was used for biomass fermentation. Fermentation

medium-1 was designed with the components which were reported as cellulosic biomass fermentation medium components for *Clostridium*. But buffer citrate of pH value 5.0 was used which was optimal pH for the enzymes. No acetate production was observed using this medium at pH 5.0 with addition of pure xylose and glucose.

Fermentation medium-2 was designed with phosphate buffer which has a pH value of 6.6. Pure xylose at different concentrations was added to check the fermentability of *Clostridium* in this new medium. Only the medium with the additional of 0.5% pure xylose shows the growth of C*lostridium* and production of acetic acid.

According to the data represented in Table 11, *Clostridium* can grow and produce acetic acid in fermentation medium-2. Though it took long time to produce acetic acid it could be acclimatized in this fermentation medium by alternately fermenting it in this designed medium and the original medium. The highest acetic acid concentration achieved was 0.67g/100ml. But as initial acetic acid concentration is 0.433g/L, only 0.237 g/100ml was produced by 0.5% added xylose fermentation.

Table 11

0.5% Pure Xylose Fermentation by Clostridium thermoaceticum in Designed Fermentation Medium

Xylose concentration added(g/100ml)	Time (day)	Glucose (g/100ml)	Xylose (g/100ml)	acetic acid (g/100ml)	Acetic acid production (g/100ml)
0.5	0	1.08	0.861	0.433	0
	2	1.089	0.855	0.433	0
	4	1.09	0.705	0.579	0.146
	6	1.093	0.653	0.628	0.195
	8	1.087	0.623	0.65	0.217
	10	1.087	0.6	0.67	0.237

4.9.2 Biomass fermentation by *Clostridium.*

4.9.2.1 Biomass fermentation by Clostridium in fermentation medium-1. Pretreated cattail biomass was used to ferment in fermentation medium-1. Initially, pH 5.0 was used for fermentation. But no clostridium growth as well as acetic acid production was observed. The pH value for fermentation medium-1 was increased to 5.5 to check the fermentability of *Clostridium* of treated cattail biomass to acetic acid first. Three different enzyme concentrations were used 7.5 FPU, 15 FPU and 60 FPU/g glucan cellulase concentrations. Biomass concentration used was 1% (w/v). Only the experiment with 60 FPU cellulase concentration showed clostridium growth and acetic acid production at pH 5.5.

Table 12 shows highest acetic acid concentration of 0.675 g/100ml was achieved by fermentation. But as initial acetic acid concentration 0.483g/100ml, only 0.212 g/100ml acetic acid was produced by clostridium fermentation of cattail biomass which gives acetic acid production of only 0.212g acetic acid/g of biomass. Though g acetic acid/g biomass production is low, this result confirms that *Clostridium* can use lignocellulosic biomass to produce acetic acid.

Table 12

Pretreated Cattail Biomass Fermentation by Clostridium thermoaceticum in Designed Fermentation Medium-1 at pH 5.5 and 60 FPU/g Glucan Cellulase Concentration

Time (day)	Glucose (g/100ml)	Xylose (g/100ml)	acetic acid (g/100ml)	Acetic acid production (g/100ml)
0	0.466	0.420	0.483	0.000
2	0.468	0.475	0.468	0.005
4	0.482	0.437	0.482	0.019
6	0.702	—	0.675	0.212
8	0.701	0.436	0.675	0.212

4.9.2.2 Biomass fermentation by Clostridium in fermentation medium-2. Pretreated cattail biomass was used to ferment in fermentation medium-2. Phosphate buffer was used and pH 6.6 was used for fermentation. Biomass concentration used was 1% (w/v). Though clostridium growth was observed using this medium with pure xylose, no *clostridium* growth as well as acetic acid production was observed while using pretreated biomass.

Different combinations of enzyme concentrations and pH was used to grow *Clostridium* using pretreated cattail biomass, but unfortunately no *clostridium* growth as well as acetic acid production was observed while using pretreated biomass using this fermentation medium.

4.10 Conclusions

For the fermentation of algae after ultrasonic treatment, SSF resulted higher ethanol yield than SHF. The highest ethanol yield was obtained at 37°C with 0.5g/L *E. coli* concentration and 15 FPU cellulase/g glucan. In this condition SSF of 0.5% (w/v) biomass resulted in 77.7% ethanol yield. Increasing biomass concentration to 3% decreased the ethanol yield to 60%. Increasing fermentation temperature and *E. coli* concentration also decreased the ethanol yield. For scaling up, the optimization of the whole process is needed. Protein content of fresh algae during SSF was increased by around 10%. So fermented residue of fresh algae residue can be useful as a protein source or organic fertilizer. For the fermentation of fresh duckweed at 1% (w/v) solid concentration, combined heat treatment and ultrasonic treatment could achieve 96% ethanol yield. Increasing the biomass concentration decreased the ethanol yield significantly. Ultrasonic pretreatment was not effective for fermentation of fresh cattail as the highest ethanol yield obtained was only 12%. However, cattail juice alone could be a good medium for microalgae culture. Depending on the concentration, it can be a supplement to an original culture medium of microalgae which can be further used for ethanol fermentation. For acetic

acid production from *Clostridium* fermentation, further research will be needed to increase the fermentability of lignocellulosic biomass to acetic acid and to optimize the process. A considerable amount of research will need to be pursued before algae, duckweed and cattail can be used as viable bio-fuel and biochemical feedstock.

CHAPTER 5

Discussion and Future Research

In recent years, growing attention has been devoted to the conversion of biomass into fuel ethanol, considered as the cleanest liquid fuel alternative to fossil fuels. Significant advances have been made towards the technology of ethanol fermentation using various biomass sources. Most of attention has focused on the cellulose, hemicelluloses, and lignin present in dry lignocellulosic biomass materials so far where biomass handling and pretreatments are costly. However, few research activities have been reported on fermentation of fresh biomass and ethanol production. In this research, feasibility of using fresh aquatic biomass for ethanol production was studied. Future research should focus on optimization of fermentation conditions with various pretreatment methods.

Since a kilogram of protein is generally much more valuable than an equal weight of carbohydrate, aquatic biomasses can be used as a good candidate for green biorefinery in respect of high value protein recovery. Effect of fermentation on protein content of algae biomass was investigated in this research. Effect of SSF on oil extraction from fermented algae should be also studied as algae oil extraction is one of the more costly processes which determines the sustainability of algae-based biodiesel. Hydrolysis and fermentation can break the algal cell walls to enhance the release of oil from the biomass matrix of microalgae. So future research should focus on enhancing oil and protein extraction of algae by fermentation. Similarly, effect of fermentation on protein content of duckweed should also be investigated.

In this study three green biomass sources including algae, duckweed and cattail were selected as biomass for ethanol production due to their fast growth rate, availability and high protein, lipid and cellulose content. Though ethanol yield achieved was low for cattail, higher

ethanol yield were achieved by algae and duckweed. Further study is needed to increase the growth rate and optimize the growth condition of microalgae at low cost by using cattail juice. Cattail biomass was also used for acetic acid production by *Clostridium* and 0.212g acetic acid/g biomass was obtained. Different pretreatment methods and fermentation conditions should be used to increase and optimize the acetic acid yield by this bacteria.

In addition to cellulosic ethanol production from algae and duckweed, there are several previously unaccounted for benefits to algae and duckweed. This feature would be beneficial to farmers with swine lagoons or potentially integrated into a waste water treatment facility. Thus, when viewed as part of a bigger, integrated system designed to treat waste water, to generate protein rich animal feed, and to produce cellulosic ethanol, aquatic biomasses become interesting biomass source.

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