Synthesis of Bioethanol from duckweed

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List of Acronyms

AFEX	Ammonia fiber explosion
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CAHP	Concentrated Acid Hydrolysis Process
CBP	Consolidated bioprocessing
CCD	Central Composite Design
CI	Confidence Interval
CRGE	Climate Resilient Green Economy
DAHP	Dilute Acid Hydrolysis Process
DDGS	Distillers Dried Grain and Soluble
DMC	Direct microbial conversion
DF	Degree of Freedom
GGE	Gasoline gallon equivalency
GHG	Greenhouse gas
GTP	Growth and Transformation Plan
FTIR	Fourier Transform Infrared Spectroscopy
NADH	Nicotinamide adenine dinucleotide
HMF	Hydroxymethylfurfural
SSCF	Simultaneous saccharification and co-fermentation
SSEF	Simultaneous saccharification and extractive fermentation
SSF	Simultaneous saccharification and fermentation
SHF	Separate enzymatic hydrolysis and fermentation
RSM	Response Surface Methodology
R^2	Regression Coefficiet

Abstract

Duckweed can be utilized to produce ethanol, butanol and biogas, which are promising alternative energy sources to minimize dependence on limited crude oil and natural gas. The advantages of this aquatic plant include high rate of nutrient (nitrogen and phosphorus) uptake, high biomass yield and great potential as an alternative feed-stock for the production of fuel ethanol, butanol and biogas.

This study involved ethanol production from duckweed and optimization of acid hydrolysis for maximizing glucose concentration and ethanol yield. The conversion of duckweed to ethanol can be achieved mainly by four process steps, pretreatment of duckweed to remove different contaminates, dried at 60 °C for 24h and ground to the particle size of 2 mm, After soaking in diluted acid hydrolysis of pretreated duckweed to convert starch into reducing sugar (glucose), fermentation of the sugars to ethanol using Saccharomyces cerevisiae and finally distillation. Duckweed washed in order to remove the different contaminates, hydrolysis was carried out.

In order to obtain ethanol yield, optimal value of factors for hydrolysis was determined by Response Surface Methodology (RSM) using Central Composite Design (CCD). The optimum combination of temperature, acid concentration, and time was determined. Twenty runs for each sample were carried out and analyzed using Design Expert 7.0 software, to investigate the effects of hydrolysis parameters on yield of ethanol. High yield of bioethanol 4.57ml/20g (0.18g/g) as obtained at the optimum, temperature of 100^0*C, acid concentration 0.8M and time 120min. ANOVA (statistical analysis) showed that an ethanol yield of 4.332211/*20g sample *(0.17g/g) was obtained at a temperature of 103.59* ^{0}*c ,0.91M and 2.45 hr. generally The production of bioethanol from duckweed could be an option for energy and other uses.*

Keywords: Bioethanol, duckweed, hydrolysis, fermentation and Saccharomyces cerevisiae

1. Introduction

1.1 Background

The world energy consumption has been increasing steadily with population growth and industrialization processes. Since 1900 Fossil fuels, e.g., Crude oil and natural gas are currently the predominant energy sources. However, crude oil and natural gas are limited resources that will be depleted sometime in the near future. Although there are debates about the exact year of peak oil production, it is generally believed that it will occur before 2025, after which a decline in worldwide crude oil production will begin (Campbell, 2013). Campbell & Laherrere (1998) also predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. An increasing demand for energy and inevitable depletion of fossil fuels has stimulated exploration for alternative energy sources.

Bio renewable energy is one of the important energy alternatives to reduce world dependence on notorious fossil based fuels. Unlike fossil fuels, bioethanol is a renewable energy source produced through fermentation of sugars, and it has been recognized as a potential alternative renewable energy source to petroleum derived transportation fuels. Developing bioethanol from renewable biomass would provide environmental and social benefits (Wyman, 1994).

The production of bioethanol and its consumption as a fuel could substantially lower CO_2 emissions compared with those from fossil fuels. The production of renewable biomass and its conversion to bioethanol could also generate jobs for local communities. Ethanol has been widely used as a gasoline additive worldwide. The production of ethanol fuel has been increasing over the last 10 years, and reached a level of 85.2 billion liters in the year 2012. The United States is the world's largest producer of bioethanol fuel, accounting for nearly 47% of global bioethanol production. Brazil is the world's largest exporter of bioethanol and second largest producer after the United States (Balat and Balat 2009). Using ethanol blended fuel for automobiles can significantly reduce petroleum use and greenhouse gas emissions.

Bioethanol can be produced via several processes based on the properties of the feedstock, i.e. sugar, starch and cellulose platforms (Cheng, 2010). Corn grain is currently the dominant feedstock for bioethanol production in the United States. However, using corn for fuel production is inevitably competing for limited cropland for food/feed production because corn is

also an important food/feed source (Endo *et al*., 2008). Moreover, intensive corn production has raised environmental concerns. Corn production has high requirements for agricultural inputs, and its cultivation causes more total soil erosion than any other crop. Sugarcane molasses is also the main feedstock for ethanol production in India. Sugarcane grows in tropical regions and its planting area is very limited. The same dilemma of food/feed versus fuel also exists in ethanol production using other feedstock containing abundant carbohydrates, such as sweet potato and cassava. It is evident that lignocellulosic biomass is of great potential importance for ethanol production because the material is abundant in many regions of the world. However, conversion of lignocellulosic biomass to bioethanol is difficult and prohibitively expensive because of the tight structure of the biomass (Sarkar *et al*., 2012). Among the three platforms for bioethanol production, the starch platform is currently the most widely used in the world because of availability of the feedstock and relatively mature technology (Cheng, 2010). However, almost all the current starch feed stocks (corn, rice, wheat, sweet potatoes, etc.) are important food/feed sources and need precious cropland to produce. Therefore, there is great interest in exploring novel starch crops that do not necessarily compete for cropland to make bioethanol production more sustainable.

Duckweeds are monocotyledonous, floating plants, and are the world's smallest and simplest flowering plants (Hillman and Culley, 1978). Each plant consists of little more than two, poorly differentiated fronds, a combination of leaf and stem. The tissue is composed principally of chlorenchymatous cells, separated by large intercellular spaces that provide buoyancy. The upper epidermis is cutinized and sheds water. In Lemna and Spirodella the roots are believed to be adventitious, are only a small proportion of overall plant weight and lack root hairs. The other two genera lack roots. Duckweed can be a novel starch source to supplement corn to starch for bioethanol production in the U.S. (Cheng and Stomp, 2009). Based on the duckweed species and the growing conditions applied, duckweed starch contents ranging from 3% to 75% dry weigh. Research has also shown that the starch content of duckweed can be substantially increased by manipulating growing conditions such as pH, phosphate concentration, and nutrient starvation (Cheng and Stomp, 2009), which makes duckweed a promising starch source and a potential feed-stock for bioethanol production.

1.2 Statement of problem

Duckweeds form large covers in waterways, which may negatively affect the water quality and cause odorous nuisance. It is recommended to remove these covers frequently, but disposal and composting may be costly. Using duckweeds for production of bioethanol and as animal feed can be a sustainable alternative.

The commercial production of fuel ethanol in the world relies mainly on the fermentation of sugar and starch, but production of ethanol from such “first generation” feedstock is often viewed as competing with food production and increasing prices of food. So the productions of bioethanol or biofuel from agricultural wastes and duckweed will be expected to cure the concern associate with food security.

Although there is renewed interest in ethanol, there are many challenges to overcome before there is widespread ethanol use and production. The main reason for conducting this research is to expand awareness of the challenges associated with increasing ethanol use and production in the Ethiopia. Some of the primary challenges includes the need to expand ethanol markets, the need to increase domestic ethanol production and to further develop the production from alternative feedstock rather than sugar cane molasses, to fully realize (economically and socially) the environmental benefits of ethanol versus gasoline. So this research aimed at studying alterative feedstock for bioethanol production.

1.3 Objectives

1.3.1 General Objective

The general objective of the study was to synthesize Bioethanol from Duckweed using dilute acid hydrolysis.

1.3.2 Specific objectives

The specific objectives of the study were:-

- To characterize duckweed and to determine glucose concentration after hydrolysis.
- Evaluate the effect of process variables (temperature, acid concentration and reaction time) on the yield of ethanol using response surface methodology.
- Optimize operating process variables in ethanol yield (temperature acid, concentration and time).
- To characterize the produced product.

1.4 Significance of the study

The main importance of this study was to enhance the importance of duckweed through the production of bioethanol through dilute acid hydrolysis. Bioethanol is a clear liquid alcohol that is made by the fermentation of different biological materials. This alcohol is known to have many uses, but one in particular is becoming more popular. The ethanol in the end is blended with gasoline to improve vehicle performance and reduce air pollution.

Bioethanol production from duckweed is considered a 2nd generation biofuel process since it has no direct conflict with human food, as the case of 1st generation biofuels produced from agricultural crops, such as corn, sugarcane and soybean oil. So that need to increase domestic ethanol production and to further develops the production from alternative feedstock rather than sugar cane molasses.

All energy sources have an impact on the environment. Concerns about the greenhouse effect and global warming, air pollution, and energy security have led to increasing interest and more development in renewable energy sources such as bio-fuel, solar, wind, geothermal, and hydrogen.

2. Literature Review

2.1 Introduction

Biofuel production has increased significantly in recent years, driven by a number of factors. The rapid development of the global economy has triggered a dramatic increase in fossil fuel demand and shortages, especially in large, rapidly developing countries, such as China, India, Brazil and Russia. These countries represent not only huge and growing commercial markets, but are also expected to have large and increasing fuel demands. Fuel demand has increased significantly in recent years and this trend is likely to continue. The price of crude oil has increased nearly 7-fold in the last 20years, from $15per barrel in 1994 to $109.2per barrel in 2013, and the highest price touched $147.7 in July 2008 (Bloomberg, 2013). This has had a profound effect on the price of oil-derived fuels. For instance, the price of unleaded petrol in UK has increased from £0.75 in 2000 to £1.30 in November 2013, a rise of 73.3% (Deloitte, 2013). Increasing costs of oil-derived fuels is a significant driver for the adoption of biofuels that can replace their oil-derived counterparts. Biofuels is likely to be desired by those countries in which fuels are predominantly relied on the import, because the biofuel production enables these countries to become energy independent. Another major driver is the increasing concern over greenhouse gas emissions associated with fossil fuels. Global greenhouse gas (GHG, including CO_2, NH_4, N_2O and fluorinated gases) concentrations have increased 32 % from 1990 to 2012, with CO_2 contributing 80 %. The increase in GHG emissions is mainly attributed to energy supply (26 %), industry (19 %) and transport (13 %). Biofuel potentially could reduce GHG emissions through the avoidance of fossil fuels. For instance, ethanol from corn could reduce GHG emission by 20 % relative to gasoline, excluding GHG change of land use (Searchinger *et al*, 2008). The exhaustion of fossil fuel has been considered as the third driver since peak oil appeared at 74 million barrels per day (mb/d) in 2008. Although the peak of the production of crude oil is expected to be delayed to 2042 and it could attain to 150 mb/d, the rate of production will enter the terminal decline eventually.

2.2 Types of biofuels

Bioethanol, biodiesel and biogas are three major biofuels. Bioethanol as a general transport fuel has been developed for over 30 years since it was a natural extension of brewing technology. The combustion value of bioethanol has been measured by gasoline gallon equivalency (GGE) value, in which 1.5gallons of bioethanol generates the equivalent energy of one gallon of gasoline. Biodiesel is the product of the trans-esterification of plant oils (Sims *et al*, 2008). The particulate emissions from biodiesel are less harmful than conventional diesel. Biogas (methane) is generated from the anaerobic digestion of organic wastes, such as animal manure and sewage. This mature technology is applied on a small domestic scale in India and China (Sims *et al*, 2008). It has also been systematically developed for industrial production in Germany and Denmark (Sims *et al*, 2008) and more recently in the United Kingdom.

2.3 Bioethanol

The ethanol obtained from biomass based waste materials or renewable sources is called as bioethanol. It can be used as a fuel, chemical feedstock, and solvent in various industries. It has certain advantages as petroleum substitutes, viz., alcohol can be produced from a number of renewable resources, alcohol as fuel burns cleaner than petroleum this aspect is environmentally more acceptable. It is biodegradable and thus, keeps a check on pollution and it is far less toxic than fossil fuels (Domínguez-Bocanegra *et al*., 2014). The fermentation reaction, symbolized by the simple equation is actually very complex, Ethanol or ethyl alcohol (C_2H_5OH) is an important organic chemical because of its unique properties, and therefore can be used widely for various purposes.

Under ordinary conditions, ethanol is a volatile, flammable, agreeable odor, clear, colorless liquid, miscible in both water and non-polar solvents. In dilute aqueous solution, it has somewhat sweet flavor, but in more concentrated solutions it has a burning taste. Ethanol, CH_3CH_2OH, is an alcohol, a group of chemical compounds whose molecules contain a hydroxyl group, ^-OH, bonded to a carbon atom. The word alcohol derives from Arabic al-kuhul, which denotes a fine powder of antimony used as an eye makeup. Alcohol originally referred to any fine powder, but medieval alchemists later applied the term to the refined products of distillation, and this led to the current usage (Wondal, 2012).

Ethanol has been made since ancient times by the fermentation of sugars. All beverage ethanol and more than half of industrial ethanol is still made by this process. Simple sugars are the raw material. Zymase, an enzyme from yeast, changes the simple sugars into ethanol and carbon dioxide. The fermentation reaction, symbolized by the simple equation is actually very complex and impure cultures of yeast produce varying amounts of other substances, including glycerin and various organic acids.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad (2.1)$$

In the production of beverages, such as whiskey and brandy, the impurities supply the flavor. In addition to simple sugars starches from potatoes, corn, wheat, and other plants can also be used in the production of bioethanol by fermentation; however, the starches must first be broken down into simple sugars. An enzyme released by germinating barley, diastase, converts starches into sugars. Thus, the germination of barley, called malting, is the first step in brewing beer from starchy plants, such as corn and wheat (Shakhashiri, 2009).

2.3.1 Uses of Bioethanol

The main use of ethanol is as a motor fuel and fuel additive. Ethanol and other alcohols can be used to power motor vehicles instead of gasoline. In almost all cases the ethanol is mixed with gasoline. Efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no GHG (Altintas *et al*., 2002).

A number of market segments are available in the ethanol industry serving a wide range of uses in the medical sectors, pharmaceuticals, beverages, industrial, household, and transport uses. The market potential for bioethanol is therefore not just limited to transport fuel or energy production but has potential to supply the existing chemicals industry and house hold uses. However, the most prevalent use of bioethanol in Ethiopia is as a transport fuel in spark ignition engine vehicles and the current amount of ethanol fuel blended with gasoline is 10% and the government is working to increase the share. The government is also working to start export in two years of time and to substitute household cooking fuel in the future (Yacob, 2013).

The major uses of ethanol are in alcoholic beverages. Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Most alcoholic

beverages can be broadly classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. Fermented beverages can be broadly classified by the foodstuff from which they are fermented. Beers are made from cereal grains or other starchy materials, wines and ciders from fruit juices, and meads from honey. Fermented beverages may contain up to 15–20% ethanol by volume, the upper limit being set by the yeast's tolerance for ethanol, or by the amount of sugar in the starting material.

Absolute ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62% (Ayele, 2011).

2.3.2 World fuel ethanol production

USA and Brazil have been the leading countries in the production of ethanol from corn starch and sugarcane respectively, and the amount of ethanol produced by these two countries together in 2013 was 19,567 billion liters, accounting for 84% of the world's production in that year.

Table 2.1World fuel ethanol production by country or region

World Fuel Ethanol Production by Country or Region (Million Gallons)									
Country	2007	2008	2009	2010	2011	2012	2013	2014	2015
USA	6,521	9,309	10,938	13,298	13,948	13,300	13,300	14,300	14,806
Brazil	5,019	6,472	6,578	6,922	5,573	5,577	6,267	6,190	7,093
Europe	570	734	1,040	1,209	1,168	1,179	1,371	1,445	1,387
China	486	502	542	542	555	555	696	635	813
Canada	211	238	291	357	462	449	523	510	436
Rest of World	315	389	914	985	698	752	1,272	1,490	1,147
World	13,123	17,644	20,303	23,311	22,404	21,812	23,429	24,570	25,682

Data Source: F.O. Licht, cited in Renewable Fuels Association, Ethanol Industry Outlook 2008-2014 reports. Available at www.ethanolrfa.org/pages/annual-industry-outlook

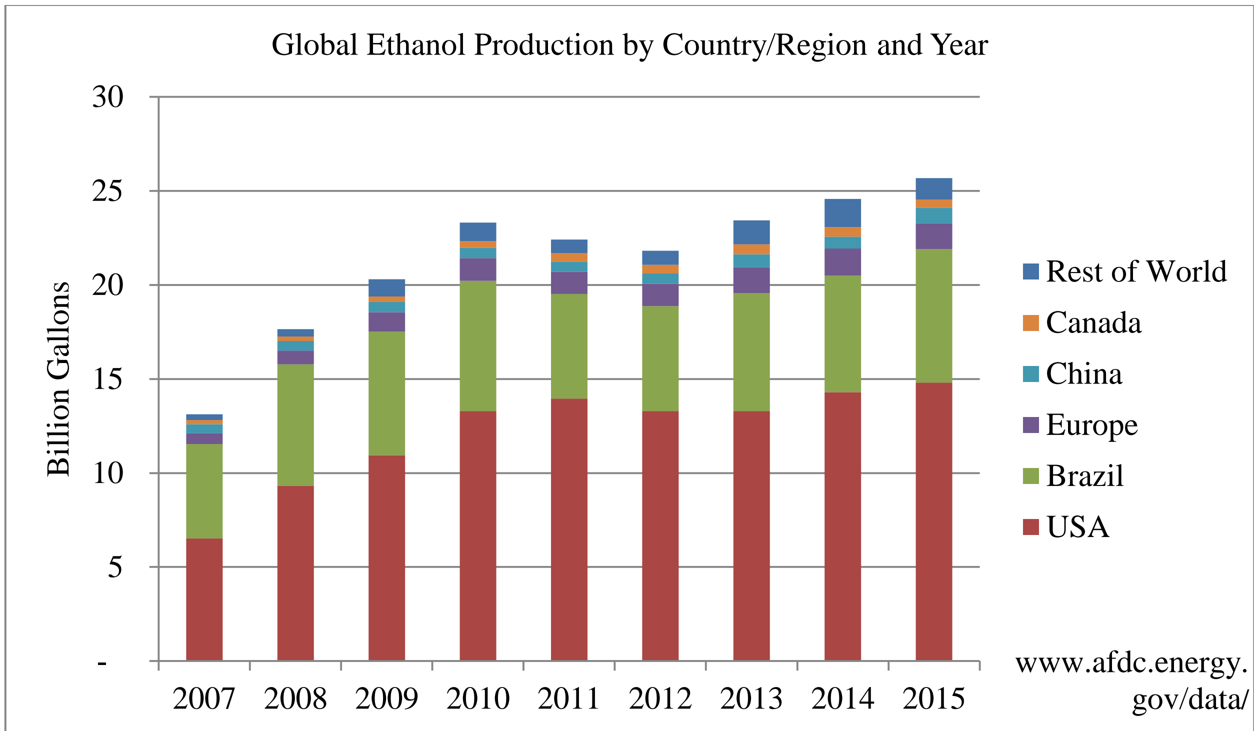


Figure 2.1 Global ethanol productions by country/region and year

2.3.3 Ethanol production in Ethiopia

Findings reveal that bioethanol production from molasses in Ethiopia can be very viable. However, the viability and competitiveness of biodiesel production in Ethiopia will largely depend on price of feedstock. Although most of the companies registered had the intention of pursuing large-scale commercial development, especially those companies registered for the cultivation of energy crops for biodiesel production, only very few of them are in operation.

Ethanol production in Ethiopia is linked with sugar factories and aimed for import substitute of petroleum products, enhance agricultural development and agro processing, job creation, and export earnings. However, only a small fraction of the potentials is utilized yet and an alternate 5% and 10% ethanol blend has accessed in the capital city of the country. Moreover, at present only two of the sugar factories, Finchaa and Metehara, are producing bioethanol (Gebreegziabher *et al.*, 2014).

Previously, there was only one biofuel factory in Ethiopia, a power alcohol plant that has been producing bioethanol as a byproduct at Finchaa Sugar Factory. Finchaa has a distillery, an ethanol plant annexed to its sugar mill with a capacity of 12 million liters per year. The plant was

commissioned in 1998 and produces ethanol from sugarcane molasses, it had a stock of about four million liters of bioethanol at the end of December 2001. However, although the government had issued a directive allowing Finchaa to produce and sell fuel alcohol to oil companies, who would in turn blend it with gasoline and distribute it to motorists, it could not sell its fuel alcohol on the market at that time. The major reasons for the refusal of the oil companies appeared to be the need for rehabilitating the existing old fuel stations and lack of interest in investing in a fuel sales operation that gives them little profit. This was also viewed as a lack of understanding and absence of commitment to alleviate one of the major problems of the country.

However, the interest in biofuels development has been revitalized with the recent hike in oil prices. Several local and international private and public biofuels companies, developers have registered in the country since 2006. For example, by 2010 there were more than 82 registered biofuel investor, most of which were registered for the cultivation of energy crops for biodiesel production. In the case of bioethanol, however, there are only a few developers in the country, most of which are publicly owned sugar factories that intend to produce bioethanol as a byproduct of sugar production.

Given rising world prices of fossil oil, the biofuels industry has developed a very significant national presence. Accordingly, there are biofuels investment activities in different regions of Ethiopia with a focus on bioethanol and biodiesel production. Besides, Ethiopia embarked on a 5% blend of bioethanol in transport fuel in 2008, which was doubled to 10% a few years later. Official reports also indicate that, by blending more than 38.2 million liters of bioethanol with gasoline, the country has been able to save 30.9 million US dollars on oil imports since 2008 (Gebreegziabher *et al*., 2014).

Although the recently launched Climate Resilient Green Economy (CRGE) strategy of Ethiopia envisages 5% biodiesel blending in transport fuel by 2030, biodiesel blending in transport fuel has not yet started in Ethiopia. As part of the planned large scale expansion in the sugar industry that is stipulated in Ethiopia’s national Growth and Transformation Plan (GTP), the country was also aims to produce 181,604 cubic meters of bioethanol from sugar byproducts, from molasses toward the end of the GTP period 2010/11-2014/15. In addition, constructing bioethanol plants

in conjunction with existing and upcoming sugar factories is underway (Gebreegziabher *et al*., 2014).

Meanwhile, this effort of the corporation will also boost the current 11.1 million liters of annual ethanol production capacity of the country to 181.6 million liters and the current 100 MWh annual electrical energy generated through cogeneration to 607 GWh. To this effect, the corporation is working to finalize expansion projects of the existing sugar factories as well as construction of the largest factory at Tendaho in addition to the ten new sugar factories. In addition to this, research to develop high yield sugarcane varieties and establishment of well-equipped breeding station including construction of four tissue culture laboratories and green house to enhance the productivity of sugarcane that range between 105 and 145 to 155 tons per hectare is in progress. Therefore, this effort will enable the country to export 623,000 tons of raw sugar and 623,000 tons of white sugar which in total makes the amount 1,246,000 tons as a source of export earnings by the end of the GTP period. It also allows using fuel ethanol to substitute imported gasoline through blending and to replace imported kerosene from abroad by the extra ethanol produced in order to minimize the foreign currency the country spent on oil import (Yacob, 2013).

The current biofuel development strategy in the country emphasizes the production of bioethanol from sugar beet, sugar cane, sweet sorghum and others, and biodiesel from jatropha, castor bean plants, and palm.

Table 2.2 Summary of sugar factories production plan in Ethiopia

Year	Description	Unit	Sugar Factories					
			Wonji	Metehara	Fincha	Tendaho	New	Total
2012	Annual sugar Production	Million Tons	0.075	0.137	0.11	-	-	0.322
	Annual Ethanol Production	m^3	-	12500	8000	-	-	18000
	Grid Power Generation	MW	-	-	-	-	-	0
2015	Annual sugar Production	Million Tons	0.174	0.137	0.27	0.62	1.049	2.25
	Annual Ethanol Production	m^3	10299	25500	20000	55400	70405	181604
	Grid Power Generation	MW	17	11	-	73	-	101
2030	Annual Sugar Production	Million Tons	0.174	0.137	0.27	0.62	3.067	4.268
	Annual Ethanol Production	m^3	10299	25500	20000	55400	278942	390141
	Grid Power Generation	MW	17	11	-	91	-	119

Source: Wondal, 2011

2.4 Feedstock for Bioethanol Production

Ethanol can be produced synthetically from petroleum or by the microbial fermentation of sugars. The three main groups of raw materials for the production of ethanol by fermentation are sugar, starch, and lignocellulose (Lin and Tanaka, 2006).

Sugar-containing raw materials include sugarcane, sugar beets, fruits and sweet sorghum. The advantage with the sugar-based raw materials is that they can be converted into ethanol directly without hydrolysis. A disadvantage is that many of these raw materials are considered to be a human food resource and will therefore be too expensive to use for ethanol production (Badger, 2002).

Starch-based materials that are commonly used for ethanol production include corn, potatoes, cassava, and various cereal grains. Starch is a biopolymer and defined as a photopolymer consisting only one monomer, D-glucose. During bioethanol production from starch, it is necessary to break down the chains of this carbohydrate for obtaining glucose syrup, which can be converted into bioethanol by yeasts. This type of feedstock is the most utilized for bioethanol production in North America and Europe. Starch can be converted to fermentable sugar by a method called the hydrolysis technique (Sanchez *et al,* 2007).

Lignocellulose-based feedstocks that can be considered for ethanol production are wood residues, agricultural residues, and the spent sulfite liquor from pulp and paper mills. The advantage of using lignocellulose as raw material for ethanol production is due to its abundance and relatively cheap (Wheals *et al*., 1999).

In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production is difficult or relatively recalcitrant to hydrolysis (Gray *et al*., 2006). Because of its complex structure, this resists degradation. The basic structure of all lignocellulosic biomass consists of cellulose $(C_6H_{10}O_5)_x$, hemicelluloses $(C_5H_8O_4)$ m, and lignin $[C_9H_{10}O_3.(OCH_3)_{0.9-1.7}]_n$. Production of ethanol from lignocellulosic waste materials, such as sawdust from the forestry industry and BSG from Beer industries have a benefits from the fact that the energy input for the overall process can be kept low. Energy-related expenses for planting, fertilization and harvesting can be avoided if waste materials are used. If lignocellulosic waste materials are used, there will not be any competition for the limited agricultural land available, which instead might be needed for food production (Sun and Cheng, 2002).

2.4.1 Starch Based Feedstock

Starch is the most widespread carbon reserve stored in plants and is of significant importance in the industry for food, chemical, and enzymatic uses. Starch is synthesized in plants as a result of photosynthesis, and the composition of starch varies with different species.

Starch consists of glucose, as the monomeric building unit, which is a raw material for other chemicals. Starch, the main component of many agricultural products, e.g., Corn, potatoes, rice and wheat, is deposited in plant cells as reserve material for the organism. Natural starches are processed to yield different end products which find many industrial applications (Dincbas and Demirkan, 2010).

2.5 Aquatic plants

Aquatic plants are considered as a potentially ideal biofuel feedstock due to their significantly common advantages ,aquatic plants are generally low in lignin but with a high proportion of carbohydrate, aquatic plants do not compete with food crops for arable land, they commonly have higher productivity because of their fast proliferation, many types of aquatic plants could be cultured in waste water system for decontamination, In addition, abundant nutrients such as protein, vitamin and minerals generated from biofuel production are potentially useful as feed for animals (Wolverton and McDonald, 1979).

2.5.1 Duckweed

Duckweed is scientifically known as the taxonomical family of *Lemnaceae*. It is the smallest flowering plant that free floats on the surface of still or slow moving bodies of fresh water. It lives in nutrient rich environments, grows exponentially in size, and has the potential to completely cover the surface of a water body in less than 24 hours (Verma & Suthar, 2015). One of the particularities of duckweed is its ability to absorb nutrients, heavy metals phenols, pesticides, dioxins, and pathogens from the water and it often used as a remediation mechanism for basins with poor water quality (Leng, 1999).

They are classified under the *Lemnaceae* family which consists of about 40 species in five genera, Spirodela, Lemna, Landolita, Wolffiella and Wolffia (Michael *et al*., 2008). The species are easily differentiated by size, i.e., the fronds of Lemna species typically average between 6 - 8 mm while those of the Wolffia species are about 2 mm or less in diameter (Cheng and Stomp, 2009). In the winter months, the duckweed survives the low temperatures by forming a starchy survival frond known as a turion, which sinks to the bottom of the pond and remains dormant until spring. The increase in temperatures in spring triggers their return to normal growth (Zirschky and Reed, 1988).

Plant growth and reproduction is mainly affected by the availability of macronutrients such as nitrogen, phosphorus and potassium in addition to micronutrients, temperature, light, wave action and plant density. Duckweed is reported to be tolerant to a wide range of pH from 3 - 10 with an optimum range of 5 – 7. The plants can also grow in a wide range of temperatures from 6 – 33°C with an optimum temperature range of 18 – 30°C (Culley *et al*., 1981).

Zirschky and Reed (1988) noted that duckweed growth can be limited by very high metal concentrations, presence of ethylene as well as filamentous algae or fungus. Duckweed growth is also known to be highly sensitive to wind and wave action, as the wind blows the duckweed to the sides of the ponds where it piles up and subsequently dies. The effect of wind on duckweed systems not only affects the growth of the plants but also harvesting of the plant biomass. Wind effects on harvesting were clearly on harvesting duckweed by skimming (Iqbal, 1999).

There is a growing concern of introducing organic pollutants, personal care products and pharmaceuticals into the food chain through duckweed based wastewater treatment systems (Reinhold, 2007).the removal of EDC (estrone, 17α-ethinyl estradiol, and 17ß-estradiol) in wastewater using duckweed and algae based systems. They realized removal efficiencies comparable to those of conventional activated sludge systems. It was also noticed that duckweed systems had a higher efficiency at removing estrogens compared to algae systems. However the main removal mechanism was attributed to sorption and subsequent degradation by microorganisms. L. minor uptake of organic pollutants (fluorinated phenols) was reported to be rapid with a pseudo first order uptake rate of $0.2 – 0.84\ d^{-1}$ in a study carried out by (Reinhold, 2007).

Moreover, duckweed is considered to have a high ductility and can be used in the manufacturing of many products because of its high protein and starch content (Verma & Suthar, 2015). Particularly, in the past years businesses have increasingly developed an interest towards this plant, specifically in regards to bioenergy production (Verma & Suthar, 2015). On the other hand, if uncollected, duckweed blocks sunlight from passing through the water, resulting in a reduction of aquatic life.

Duckweed is composed of water, mineral elements, and organic matter. Fresh duckweed fronds have been reported to contain 87 to 97% water depending on the species (Cross, 2006). The cell wall is rich in cellulose and also contains 20.3% pectin comprising galacturonan, xylogalacturonan, rhamnogalacturonan; 3.5% hemicellulose comprising xyloglucan and xylan, and 0.03% phenolics. In addition, essential fatty acids (0.6%, α-linolenic and linoleic/linoelaidic acid) and p- coumaric acid (0.015%) respectively are the most abundant fatty acids and phenolics in whole duckweed.

The usefulness of duckweed for bioethanol depends on the starch content of the duckweed. The more starch, the more bioethanol can be produced. Various studies found this starch content varied between duckweed species and circumstances from 3 to 75% of its dry weight (DW). The carbon content of duckweeds is one of the highest in the plant kingdom, but it is dependent on growth conditions, vary from 14.1% to 43.6% (Landolt and Kandeler, 1987). The starch in duckweed increases when its growth environment is not optimal. Thus, duckweed can be manipulated to increase starch levels. This can be achieved by, for example, depositing freshly harvested duckweed into nutrient poor water leads to nutrient stress of the duckweed, to which the duckweed responds by producing starch to store glucose. Another method might be to use temperature differences because they are expected to increase starch levels because lower temperature leads to a lower respiration level Furthermore, it is also possible to increase starch production by increasing the salinity of the water (Xu *et al*., 2012).

Duckweed grows faster than most other plants and can double its biomass every 16–24 h under appropriate environmental conditions. Cheng *et al*. (2002) reported that the growth rate of duckweed cultivated in swine lagoon liquid could reach 29 g (dry based) m^{-2} day^{-1}, which could be translated to 106t (dry based) ha^{-1} $year^{-1}$ if the duckweed could grow for 365 days $year^{-1}$. This yield is much higher than most starch crops such as corn (7.84 $tha^{-1}year^{-1}$), wheat (3.15 tha^{-1} $year^{-1}$) and barley (3.70 $tha^{-1}year^{-1}$), indicating that duckweed has great potential as an alternative starch crop and therefore for bioethanol production.

Figure 2.2 Duckweed

2.5.2 Water hyacinth

Water hyacinth (*Eichhornia crassipes*), another aquatic plant family exhibiting rapid vegetative growth, is native of the Amazon basin. Water hyacinth is a free-floating plant and grows as much as 1 metre in height, with broad, thick, glossy, ovate leaves and violet flowers. Water hyacinth has become a controversial plant due to its invasiveness since it was introduced to Europe, Asia, Australia, North America and Africa. The habitat of water hyacinth can range from tropical to subtropical or warm temperature desert to rainforest zones and it tolerates a range of warm temperatures (21.1 - 27.2 °C) and pH value (5.0 -7.5) (Bolenz *et al*, 1990). Under appropriate conditions, it exhibits a fast growth rate. Its ability to remove nitrogen, phosphorus and iron from waste water system has been noted. This plant is therefore considered for the decontamination of waste water. Dry matter of 60-110 tonnes/ha per year has been harvested in wastewater system. Also, a weekly dry weight yield (71 % w/w) was harvested at a lagoon with a nutrient increase (29 % w/w DM) through May to October (Wolverton and McDonald, 1979).

Figure 2.3 Water hyacinth plant

2.5.3 Algae

Algae are a group of simple, autotrophic eukaryotic organisms that includes unicellular and multicellular organisms. Their habitats range from small ponds to the sea and they exhibit a number of reproductive kinetic (Parfrey *et al*, 2006). For unicellular algae, they appear to obey Kleiber's 3/4-power rule and a very high dry matter yield is therefore potentially achieved from these very small organisms. Green algae also parasitically live in other aquatic plants where they

normally stay under cuticles or sometimes under epidermis of the host plant (Nelson, 2008). In addition, the ability of diverse biofuel (bioethanol and biodiesel) production has been demonstrated. Each algal cell has been compared to a small ethanol production plant. The essential elements including water, sunlight, CO_2 and nutrients can be converted to ethanol through the metabolism cycles (photosynthesis, pyruvate, and ethanol synthesis) in algae plant.

Biodiesel production from algae has also been studied and reported as having some conspicuous advantages: rapid growth rate, surprising space efficiency, high yield, sulphur free fuel, non-toxicity and high biodegradability. However, biofuel production from algae is more costly than using other energy crops due to the requirement for artificial addition of light, CO_2, water and inorganic salts (Chisti, 2007). Biofuel production from algae is commonly considered as the 3rd generation biofuel.

2.6 Starch Composition

Starch is a well-known polymer of glucose, linked by glycosidic bonds. The two major types of molecules in starch are amylose and amylopectin. Amylose, the minor linear polymer in potatoes, consists mainly of α-1, 4 linked D-glucopyranosol residues. It contains up to 6,000 glucose units.

Amylose (AM) and amylopectin (AP) together form insoluble, semi-crystalline starch granules with an alternating grow ring structure. The granule size, shape, crystallinity, and internal molecular organization vary with the botanical source. Normal native starches have 20-30% AM and 70-80% AP by weight. AM is an essentially linear macromolecule with mostly α-(1-4)-linked D-glucose pyranosyl units and less than 0.5% of the glucoses in α-(1-6) linkages. AM has a molecular weight in the range of 105 to 106, corresponding to between 500 and 6,000 glucose units. AM is not essential for the construction of starch granules, as the starch granule morphology not varying greatly with widely differing AM contents. AM chains are thought to be predominantly in a single helical state, although a small proportion of them may participate in double helices with AP branches or are entangled within the complex architecture of the starch granule. AM occurs as two different forms in native starch granules; free AM and lipid-complexes AM, which contribute differently to starch functionality and digestibility, especially for cereal starches (Wang and Copeland, 2015).

2.7 Starch Conversion

Starch, unlike lignocellulose biomass, is more easily convertible to ethanol and can be described in three basic steps. Initially, the starch must be converted to a useable fermentable sugar. This can be achieved through a variety of different processes, including acid hydrolysis, high temperature and pressure extrusion, and enzymatic hydrolysis. Once the starch is converted to simple sugars, it must then be fermented using biocatalysts such as yeast or bacteria to produce ethanol. Finally, the aqueous ethanol solution must be distilled to extract its pure form. The need for alternative starch crops will create a demand for the development of processes to efficiently convert different forms of starch to ethanol.

2.8 Production process

2.8.1 Pretreatments

An effective pretreatment is characterized by several criteria, it should avoid the need for reducing the size of biomass particles, should preserve the hemicellulose fractions, should reduce formation of inhibitors that hinder growth of fermentative microorganisms, and minimize energy demands and limit cost (Mosier *et al*, 2005). Various pretreatments are being investigated for their effectiveness in subsequent enzymatic saccharification. The material treated by effective single or combined pretreatment is more accessible and susceptible to enzymatic saccharification and more fermentable sugars are obtained for subsequent fermentation. However, the application of pretreatment is likely to vary from material to material. Thus, utilization of an appropriate pretreatment is an important prerequisite of enzymatic saccharification and fermentation. Acid, alkaline, thermal and combination approaches may be applied and these can differ with respect to pH, temperature, retention time and material type.

Physical pretreatment

Physical pretreatments aim to degrade the cellulose crystallinity to improve biomass digestibility by changing the physical character of materials using pyrolysis and mechanical comminution including dry, wet and vibratory ball mills (Millet *et al*, 1976).

Mechanical comminution is a combination process involving chipping, grinding and milling which reduces the material size down to 0.2 - 2 mm (Sun and Cheng, 2002). Dry and wet mill processes are economically effective when applied in starch-to-ethanol production. Vibratory

ball milling has been used to effectively generate smaller particles than other mechanical comminution methods (Millet *et al*,1976).pyrolysis is a thermal-physical pretreatment associated with higher temperature and pressure and the decomposition of polysaccharides, it results in over 80 % conversion to reducing sugars (Sun and Cheng, 2002). However, to achieve a conspicuous enhancement of enzymatic saccharification for lignocellulosic materials, the particle size is necessarily reduced to less than 0.05 mm. The energy demands of pure physical Pretreatments are fairly uneconomic for lignocellulosic materials and their utilization is consequently restricted or must be associated with other chemical or thermal pretreatments (Waldron, 2010).

Chemical pretreatment

Chemical pretreatments assist in specifically removing unwanted compounds, such as lignin or hemicellulose compounds (Waldron, 2010), and their application is therefore changed based on the nature of the biomass. Ozonolysis can effectively remove 60 % lignin (e.g. In wheat straw) and also avoid the generation of toxic products at room temperature conditions. Dilute acid hydrolysis (using H_2SO_4, HCl) has been successfully developed to achieve high sugar yield and avoid the toxic, corrosive issues trigger by concentrated acid hydrolysis. This acid hydrolysis is also utilized to successfully convert xylan to xylose under moderate conditions. Alkaline hydrolysis can also remove lignin by saponification, which causes biomass swelling, increasing the internal surface area and decreasing the extent of polymerization and crystallinity of cellulose. However, the neutralization of chemical hydrolytic products is necessary prior to downstream enzymatic saccharification and fermentation. Although chemical hydrolysis significantly improves the digestibility of materials, the cost is usually higher than other pretreatments (Sun and Cheng, 2002).

Physical-chemical pretreatment

Physical-chemical pretreatment is an efficient process possessing the advantages of both individual physical and chemical pretreatments, in which fine pretreated materials for downstream process are obtained and the cost and energy demands are also dramatically decreased. Steam explosion a recognized thermal hydrolytic method is one typical physical-chemical pretreatment and is extensively applied to enhance ethanol production for lignocellulosic biomass (Glasser and Wright, 1997). The process explodes biomass by sudden decompression following high pressure and temperature conditions. Recent studies tend to use

lower temperatures combined with longer retention time. Steam exploded cellulose is more accessible to cellulase (Schwald *et al*, 1989). The advantages of steam explosion are the high yield of glucose and xylose attributed to the considerable lignin transformation and hemicellulose degradation. However, disadvantages are seen as the high energy demand, a consequence of high pressure and temperature requirements, and the formation of fermentation inhibitors. Addition of H_2SO_4 or CO_2 into steam explosion could improve the degradation of hemicellulose and enzymatic hydrolysis and decrease the formation of inhibitory compounds (Sun and Cheng, 2002). Ammonia fiber explosion (AFEX) is another classic physical-chemical pretreatment. It involves exposing lignocellulosic biomass to steam explosion with additional liquid ammonia. AFEX does not effectively decompose lignin and hemicellulose but generates only trace levels of inhibitors (Waldron, 2010).

Biological pretreatment

Microorganisms have been studied and used for decomposition of plant cell wall material. Fungi are employed in the decomposition of lignocellulosic materials due to the many saccharifying enzymes they produce. Diverse fungi involving brown-, white- and soft-rot fungi have been used to target different compounds. For instance, brown rot fungi mainly degrade cellulose, while white and soft rots can degrade both cellulose and lignin. Schwald *et al*, (1989) showed that these crustaceans potentially possess all of the enzymes for lignocellulose digestion due to the absence of the gut microbes. Although biological pretreatment requires low energy at moderate conditions, the extent of decomposition is less (Sun and Cheng, 2002).

2.8.2 Acid Hydrolysis

The pretreatment techniques that can be used include acid, alkaline, biological pretreatment, wet Oxidation, organosolv, ozonolysis, ultrasound pretreatment and hydrogen peroxide with metal salts pretreatment. These methods are significantly different from one another in terms of reaction conditions, process efficiency and complexity (Ramadoss and Muthukumar, 2015).

Acids have been used to catalyze the hydrolysis of starch in starch cookers operating at temperatures of 50 to 150^0C, a process referred to as acid hydrolysis (Wondale, 2012). Acid hydrolysis aims the mass production of fermentable sugar from starch. Two main developing or

commercialized acid hydrolysis processes of starch; Dilute Acid Hydrolysis Process (DAHP) and Concentrated Acid Hydrolysis Process (CAHP) have been introduced.

CAHP is operated at the condition of high acid concentration (above 1M HCl) and at low temperature (below 70 ^{0}C), which takes a long reaction time. The neutralization and the separation of acid are the main problems in CAHP.

DAHP is carried in a few second or minute at high temperature (between 150 and 200 ^{0}C) and dilute acid (below 3wt %). DAHP involves the undesired byproducts from degradation, reversion and retrogradation while it has some merits on the hydrolysis rate and alleviation of the neutralization. Starch hydrolysis has been also performed at moderate temperature between 90 and 120°C for analytical application. This takes about 3h to complete the reaction in the case of 0.7M HCl (Hong and Bae, 1994).

Dilute Acid Hydrolysis

Among the chemical hydrolysis methods, dilute-acid hydrolysis is probably the most commonly applied. It is a method that can be used either as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolyzing starch to the sugars. The first established dilute-acid hydrolysis process was probably the Scholler process. This was a batch process, in which the material was carried out at a temperature range of (50 – 150)^{0}C between 30 minutes and 4 hours. (0.2 – 1.0)M strength of H_2SO_4 acid was used and pH values will be range of 4.5 – 5.2 was considered during acid hydrolysis (Taherzadeh and Karimi, 2007).

Batch reactors have been the most widely used reactors for kinetic study of hydrolysis and for laboratory and pilot study of ethanol production. A main drawback of dilute-acid hydrolysis processes is degradation of sugars in hydrolysis reactions and formation of undesirable byproducts. This is not only lowers the yield of sugars, but also several of the byproducts severely inhibit the formation of ethanol during the fermentation process. Potential inhibitors are furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, acetic acid, formic acid, uronic acid, 4 hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, formaldehyde, etc.

Most dilute acid processes are limited to a sugar recovery efficiency of around 60 to 75%. The biggest advantage of dilute acid processes is their fast rate of reaction, which facilitates continuous processing. The biggest disadvantage is their low sugar yield. For rapid continuous

processes, in order to allow adequate acid penetration, feed-stocks must also be reduced in size so that the maximum particle dimension is in the range of a few millimeters (Wondale, 2012).

Concentrated Acid Hydrolysis

Hydrolysis by concentrated sulfuric or hydrochloric acids is a relatively old process. Concentrated acid processes are generally reported to give higher sugar yield e.g. 90% of theoretical glucose yield and consequently higher ethanol yield, compared to dilute acid processes. Furthermore, the concentrated acid process can operate at low temperature (e.g. 40 ^{0}C), which is a clear advantage compared to dilute acid processes. However, the concentration of acid is very high in this method e.g. 30-70%, and dilution and heating of the concentrated acid during the hydrolysis process make it extremely corrosive. Therefore, the process requires either expensive alloys or specialized nonmetallic constructions, such as ceramic or carbon-brick lining. The acid recovery is an energy demanding process. In addition, when sulfuric acid is used, the neutralization process produces large amount of gypsum. Furthermore, the environmental impact strongly limits the application of hydrochloric acid. The high investment and maintenance costs have greatly reduced the potential commercial interest of this process (Taherzadeh and Karimi, 2007).

Concentrated acid process uses relatively mild temperatures and the only pressures involved are usually only those created by pumping materials from vessel to vessel. The primary advantage of the concentrated process is the high sugar recovery efficiency, which can be on the order of over 90%. Unfortunately, it is a relatively slow process and cost effective acid recovery systems have been difficult to develop. Without acid recovery, large quantities of lime must be used to neutralize the acid in the sugar solution (Wondale, 2012).

2.8.3 Fermentation

Ethanol fermentation has been used in brewing for thousands of years. Fermentation is a process in which microorganisms metabolise sugars under low oxygen conditions to produce high-energy compounds (adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) and ethanol, acids and CO_2 are produced as waste products (Edward, 1922). Many types of sugars (glucose, fructose, sucrose, maltose, galactose and xylose) are metabolised by variouss yeasts in ethanol fermentation. However, different sugars resources

may require optimization of the yeast strains employed. For example, glucose is consumed by Saccharomyces *cerevisiae* and xylose is consumed by Pichia stipitis to produce ethanol.

The fermentation process can be utilized to convert duckweed biomass into ethanol. The feasibility of this process mainly depends on the amount of starch present in the plant biomass. Starch is a polymer of glucose consisting of two structural components known as amylose and amylopectin: amylose is a linear polymer whose glucose residues are connected by α-1, 4 linkages while amylopectin is a larger branched polymer consisting of both α-1, 4 and α-1, 6 linkages Starch can therefore be hydrolyzed to fermentable monomeric glucose sugars by the use of enzymes (Cheng and Stomp, 2009).

Ethanol fermentation methodologies

Although ethanol fermentation has been applied in the brewing industry for thousands of years, novel fermentation methods are still being developed. Separate enzymatic hydrolysis and fermentation (SHF) is a process in which enzymatic hydrolysis and fermentation are carried out independently to maintain the optimal conditions for both processes (Erdei *et al* 2012). The significant disadvantage of SHF is that the accumulation of sugar products (cellobiose, glucose, and xylose) in enzymatic saccharification process hinders the breakdown of cellulose and subsequently reduces ethanol yields. Simultaneous saccharification and fermentation (SSF) in a combination of saccharification and fermentation potentially it decreases inhibition by sugars accumulation and greater ethanol yield and might result (Tomás-Pejó *et al*, 2008). Tomás-Pejó *et al* also stated that SSF could improve ethanol production from SHF under the same conditions. SHF has a high initial ethanol yield in the first 3 hours, but SSF could achieve higher total ethanol yield than SHF. Additionally, SSF also requires less energy input than SHF as the process duration is considerably reduced.

SSF has been expanded to semi-simultaneous saccharification and fermentation (SSSF), simultaneous saccharification and co-fermentation (SSCF) and simultaneous saccharification and extractive fermentation (SSEF). SSSF is the process between SHF and SSF and includes a pre-hydrolysis prior to SSF to provide the optimal conditions for both enzymes and yeast in SSF. SSCF can convert both hexose and pentose to ethanol by using multiple microorganisms. SSEF aims to reduce the influence of alcohol accumulation on yeast by periodically removing ethanol from the reaction chamber and enabling yeast to remain in a highly active state (Moritz and Duff,

1996). However, all these methods encounter the inhibition problem at higher substrate concentrations which decreases the activity of yeast. Fed-batch fermentation can reduce inhibition and achieve high ethanol yields at very high substrate concentrations (65.5 % w/v). Direct microbial conversion (DMC) which is also known as "consolidated bioprocessing" (CBP), has been developed by Lynd *et al* (2005). DMC/CBP refers to an integrated process where enzymatic hydrolysis of polysaccharides and hexose/pentose fermentation are all carried out in one bioreactor using selected bacteria that secrete cellulase enzymes.

The cost of this fermentation method is very low for industrial production with reasonable profit margins, but the ethanol yield of DMC is generally lower than SHF and SSF (Lynd et *al*, 2005).

Detoxification

Due to the potential inhibition generated from pretreatment and enzymatic hydrolysis of lignocellulosic biomass, detoxification (reduction in inhibitor concentration) needs to be considered. Taherzadeh *and* karimi (2007) stated that four approaches can be applied to minimizing the presence of inhibitors, reducing the production of inhibitors in pretreatments, removing or decomposing the inhibitors, conversion of inhibitors to compounds that can be tolerated by yeast, developing novel yeast strains to enhance their tolerability of inhibitors. A number of detoxification methods have been developed and reported including physical, chemical and biological methods. The application of detoxification varies according to the nature of inhibitors but sugar loss may also need to be considered.

Co-products

Several attractive by-products are produced during the ethanol fermentation process. In the anaerobic conditions, methane and CO_2 are produced from degrading organic acids (formic and acetic acids). CO_2 from fermentation can be extracted and reused as an industrial source for dry ice, carbonating beverages, etc. Moreover, the residual biomass contains a number of potential nutrients, such as protein, minerals and vitamins, which could be extracted for animal feed.

2.8.4 Factors Affecting Fermentation

Microorganisms for ethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature, pH, alcohol tolerance,

growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance.

Effect of sugar concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. The amount of ethanol produced is proportional to the amount of sugar added; thus, high sugar concentrations are desired. However, too high sugar concentrations can inhibit metabolism due to increased osmotic pressure. Very low levels of sugar may limit the rate of ethanol production Hence, each fermentation process will have an optimal glucose or equivalent sugar concentration. (Dunn, 1959)

Effect of temperature

Temperature has an important factor on the growth rate of the microorganisms and the rate of ethanol production. Wine and beer fermentations are generally conducted below 20°C, whereas higher temperatures (30-38°C) are being examined for industrial alcohol production by yeast cultures. Too high temperature kills yeast, and low temperature slows down yeast activity and growth. Thus, specific range of temperature is required (Sofer and Zaborsky, 1981). Operating at greater temperatures is desirable for the following reasons:

- High fermentation temperature increases growth rate and productivity exponentially
- Plant capital cost is less due to higher productivity per unit volume of ferment or vessel and cooling equipment investment is lowered.
- Operating costs are less since less energy is required to maintain desired fermentation temperature and recover the ethanol.
- Contamination risk is less as fewer organisms exist at high temperatures.

Effect of pH

A very important factor for cellular growth is external pH. Most alcoholic yeast fermentations are conducted around pH 4.5, although this may not be the optimal pH for growth or ethanol production. Yeast cultures can grow over a wide range from 3 to 8 with an optimum for growth generally in the slight acidic range. Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures. Thus, the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth, and physicochemical effect on

waste product pathways. Low pH values in yeast fermentation help to inhibit growth of contaminating bacterial cultures. Bacterial cultures generally have a pH optimum around 7-7.5, with less tolerance than yeast to acid conditions (Sofer and Zaborsky, 1981)

Osmotic tolerance

The semi-permeable membrane surrounding the cell must be able to withstand wide osmotic pressure changes in extracellular fluids that impact the relative osmotic pressure difference. If not, the cells may be severely damaged or even killed. The cells may burst in a hypotonic solution, when the solution becomes more dilute than the intracellular fluid. If hypertonic, the cells will shrink from the osmotic pressure difference. Osmotic pressure limits can be one of the factors that restrict maximum substrate concentration (Sofer and Zaborsky, 1981).

2.8.5 Distillation

The concentration of ethanol produced by fermentation is far too low for direct use as transportation fuel, and distillation is required to generate concentrated and purified ethanol from the mixed fermentation products. Distillation has been applied in brewing and separating fossil fuel products for many years. The principle of distillation depends upon the boiling points of components in the mixture (Uragami *et al*, 2004), lower boiling components preferentially vaporise at lower temperatures. In bioethanol distillation, ethanol vaporises before water as it has a lower boiling point (78.5°C). The ethanol vapour passes to the condenser and the condensed liquid is eventually collected in a clean container.

Simple distillation requires components which have a large difference in boiling points in order to achieve high purities of the separated components (Doherty and Perkins, 1978). However, simple distillation could not purify ethanol with 100 % effectiveness because vapour is enriched in ethanol but moisture always remains. This situation is called an azeotrope. Azeotrope means the components of mixture at certain proportion have similar boiling point which leads the expected component to be no longer separated by distillation (Uragami *et al*, 2004).

The azeotrope of ethanol is 95.6 % (v/v). To make matters worse, if the ethanol distillation proceeds over time, the purified ethanol will collect more water, which negatively affects final ethanol concentration (Uragami *et al*, 2004).

Fractional distillation is a technique developed from simple distillation that enables a number of components possessing similar boiling points to be separated (Kneil and Scarsdale, 1952). The presence of azeotrope in ethanol distillation limits the purity of final distilled ethanol less than 95.6 %. However, the use of fractional distillation can obtain higher pure ethanol than 95.6 %. The principle of fractional distillation is according to a temperature gradient (coolest in the top and hottest at the bottom) generated by the distance from the heat source (Kneil and Scarsdale, 1952). When the mixed vapour (ethanol and water) ascends through the temperature gradient, ethanol remains in the vapour and water condenses back to refluxing liquor. After several cycles of vaporisation and condensation, ethanol is purified and relatively water free. Industrial distillation is the process of repeated vaporisation and condensation in a huge refluxing distillation column (Kneil and Scarsdale, 1952).

2.8.6 Dehydration

Ethanol from distillation process is sent to the molecular sieves column for further dehydration to produce 99.9 percent v/v ethanol. After distillation, about 5 percent of water remains in ethanol. Especially, this water is a big problem for fuel ethanol because the presence of this amount of water enhances the molecular polarity of ethanol when it is mixed with gasoline. Consequently, they separate into two phases, ethanol phase and gasoline phase. It is easy to imagine that this inhomogeneous fuel is not acceptable. Thus, dehydration can be another issue (Onuki, 2005). For the ethanol to be usable as a fuel, water must be removed. Most of the water is removed by distillation, but the purity is limited to 95-96 percent due to the formation of a low boiling water-ethanol azeotrope. For blending with gasoline, purity of 99.5 to 99.9 percent is required, depending on temperature, to avoid separation. Currently, the most widely used purification method is a physical absorption process using molecular sieves and another method is zoetrope distillation.

3. Material and Methods

The experimental work was done in laboratory of Addis Ababa Institute of Technology, School of Chemical and Bio-Engineering (in the Reaction, Research, Environmental and Biological lab) and College of Natural Science of Addis Ababa University School of Physics and Chemistry Department.

3.1 Material and Equipment's

The materials that were used: Plastic bags, Oven (GALLENKAMP), Balances (ADAM, PW 124) ,PH meter (pH meter 3310, JENWAY) ,Thermostats ,Vessels Rack, Autoclave, Density meter (DMA4100M), spectrophotometer(SPECTRO UV-VIS DOUBLE BEAM PC 8 SCANNING AUTO CELL UVD -3200), Shaker, Fermentation and distillation set ups and the chemical were used Sulfuric Acid, Sodium Hydroxide Benedict's reagent, Yeast extracts (Agar), Urea , Sugar, Mg $SO_{4,}7\ H_2O$ preparation ,Peptone and Yeast *Saccharomyces cerevisiae*

3.2 Procedure methods

Duckweed was collected from the Addis Ababa science and technology university, Addis Ababa, Ethiopia (they collected from Hawassa and Bahirdar and multiply by preparing of pond). The sample was packed plastic bags and transported to Addis Ababa institute technology (AAiT), School of Chemical and Bio Engineering laboratory. The Duckweed was washed in order to remove unwanted matter and dried at 60°C for 2 days until constant weight were found. Then the dried sample was sieved and milled the over size in to appropriate particle.

3.2.1 Acid Hydrolysis

The aim of this experiment was to convert starch to glucose. Diluted acids transform starch into glucose. Samples based on the duckweed were put in Erlenmeyer flask. The ratio between duckweed and acid solution was 1:10 W/V. The mixture of duckweed and acid solution made 500 ml in Erlenmeyer flask.

Procedures for Acid Hydrolysis

- Different concentration of sulfuric acid was prepared and added to the non-soluble component from pretreatment steps in the order of experimental design for all experiment.

- The duckweed was then hydrolyzed in the reactor at a temperature of 80, 100 and 120° C, acid concentration 0.6, 0.8 and 1M and time of 1, 2 and 3hr.
- After hydrolysis, the solid part was separated from the liquid in the hydrolyze by vacuum filtration unit to remove the non-fermentable component.
- The filtered hydrolyzate was neutralized with 2M NaOH until the pH became in a range of 4.5-5.2

(a) (b) (c)

Figure 3.1(a) Raw materials ready for hydrolysis (b) autoclave (c) sample after hydrolysis

3.2.2 Filtration

The monomeric sugar was separated in pressurized filter or bag filter. Then the sugar solution (hydrolyzate) was neutralized and introduced into fermentation. The solid part which obtained from this filtration process was dried and weight before use for another purpose.

3.2.3 pH Adjustment

Before addition of any micro-organism to the above prepared samples, pH of these samples has to be adjusted. Otherwise the micro-organism was dying in high acidic or basic state. A pH of around 4.5-5.2 is maintained.

Procedures in pH adjustment

- First the pH meter was calibrated by using buffer solution.

- The hydrolyze solution is acidic, so it needs highly basic solution to bring the pH in the range of 4.5-5.2.
- Sodium hydroxide solution was added drop wise to the other flask with constant stirring until the pH reaches to a range of 4.5-5.2.
- If suppose the pH goes beyond 4.5-5.2 concentrated sulfuric or hydrochloric acid was added drop wise to maintain the pH in the range.

Figure 3.2 adjustment of PH after the hydrolysis

3.2.4 Sugar content determination

Total reducing sugar of the hydrolyze sample was determined using Spectrometer, which measures the intensity of red light. Standard curve was plotted from known concentration of standard glucose and benedict solution reagent in digital spectrophotometer at 540nm.so that concentration sugar yield content of hydrolyzate which obtained from hydrolysis was determined using digital spectrophotometer (SPECTRO UV-VIS DOUBLE BEAM PC 8 SCANNING AUTO CELL UVD -3200) by measuring absorbance vs. sugar concentration at 540nm wave length. Quantitative benedict solution and standard glucose solution was used for assays to plot the calibration curve. Benedict’s solution is designed to detect the presence of reducing sugars. In hot alkaline solutions, reducing sugars reduce the blue copper (II) ions to brick red copper (I) oxide precipitate. As the reaction proceeds, the color of the reaction mixture changes progressively from blue to green, yellow, orange and red. When the conditions are carefully controlled, the color developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of glucose-equivalent concentration sugars present in a sample can be obtained.

Calibration plot for glucose standard

The content sugar concentration was determined by using UV-visible spectrophotometer at 540 nm wavelength of glucose absorbance and the quantification was made from calibration curve using glucose as standard and calculation was performed by equation of the linear regression obtained from calibration curve.

Stock glucose solution was made by dissolving 100g of glucose in 100 ml of distilled water. From the stock solution dilution series solution was prepared at different concentration of 8, 6, 4, 2, 1 and 0%; Pipette 0.5 ml from each of the dilution series into labeled test tubes, each containing 5 ml of Benedict's solution and then mix by shaking. Then all the labeled test tubes were heated at 90^0C water bath for 5 minutes. The test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent. After filtered the precipitate, % absorbance was measured using spectrophotometer at 540nm. Calibration curve was plotted to show the % of absorbance of blue light by the standard glucose solution.

Determination of the total reducing sugar in the hydrolyze

In order determine the sugar content of the hydrolyze sample from the autoclave, pipette 0.5 ml of each of the samples into labeled test tubes, to each containing 5 ml of Benedict"s solution, Mix by shaking. All the labeled test tubes were heated to 90^0C in water bath for 5 minutes. Then test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent. After filtered the precipitate; % absorbance was measured using spectrophotometer at 540nm. The concentration of sugar in each samples were read from the calibration curve of the standard glucose solution.

$$\text{CTRSUS} = \frac{\text{absorabonce of unkown sample} - \text{y intercept}}{\text{slpoe}} \tag{3.1}$$

Where

CTRSUS = Concentration of total reducing sugar of unknown sample

3.2.5 Fermentation

The fermentation process was carried out in shaker incubator, at 30 °C, with stirring at 180 rpm, for a 72h. The prepared hydrolyzates were adjusted to pH of 4.5-5.2 which is optimum for

Saccharomyces cervisiae using 2M sodium hydroxide solution. Before conducting fermentation, preparation of media for the yeast is a must. In order to prepare the media the favorable condition for yeast growth must be established to supply the required amount of nutrients.

Culture media preparation

The aim this step of the experiment is to culture the microorganism that used for fermentation. Yeast was the microorganism used for conversion of glucose to ethanol. Before conducting fermentation, the media for the preparation of yeast was prepared. For preparing one liter of media, the following ingredient were add, dextrose 20g, yeast extract 10g,Urea 5g, $MgSO_4.7H_2O$ 5g, peptone 20g and add to 1000ml distills water to make up one liter media (Izmirlioglu and Demirci, 2012).

(e) (f)

Figure 3.3 (e) Ingredient for culture preparation (f) Cultured media

From the above prepared media 100ml media prepared as above, 0.5g of yeast, *Saccharomyces cerevisiae* (arif instant) was added using a 250ml conical flask.

Sterilization

The aim of this step is to remove contaminant microorganisms which could share the glucose concentration prepared for ethanol production from yeast. Consequently, all the equipment that were used for fermentation purposes were sterilized. The sterilization was carried out at a temperature of 121^0C for 15 minutes using autoclave.

Figure 3.4 autoclave for sterilization

Procedure for Fermentation

To perform the experiment, the sample was conditioned to temperature of 30^0C before fermentation step is started. This is the temperature at which all fermentation experiments are carried out. Then the pH was checked before adding the cultured media and it was maintained by 2M of NaOH. The pH 4.5-5.2 was optimum for yeast culture. The cultured media and the hydrolyzate sample were mixed with the proportion of 1:10 in Erlenmeyer flask. At the end the mixture was placed in the shaker incubator at a temperature of 30^0C and 180 rpm for 72 hours. And after 72 hours of fermentation, the samples would take out and distilled.

3.2.6 Distillation

Distillation was the final step in the production of ethanol from duckweed. The aim of this step of the experiment was to purify the alcohol. Distillation is the method used to separate two liquid based on the difference of their boiling points. In this experiment simple distillation set up was used at a temperature of 82^0C for 4 hours on water bath. Water bath was used in order to control the temperature at set point.

The fermented product is separated by distillation into alcohol and stillage. Byproduct of distillation which is called stillage can be useful for animal feed by drying or can be used as fertilizer. The remaining sugars and starch do not go into the effluent because the stillage can be dried. The dried residue is called Distillers Dried Grain and Soluble (DDGS), which is an excellent ingredient for animal feed. During fermentation grain starch gets utilized and the other components such as protein remain unutilized in the stillage. The dried stillage, DDGS, is not

only rich in protein and fat, but also rich in vitamins produced by yeast during fermentation. (Sheorain, 2000)

3.3 Experimental analysis

3.3.1 Determination of Moisture Content

First, the empty dish was weighed. Then accurately weight of duckweed was into an empty dish. The sample were dried in an oven at 105^0C; weighing each 2 h until constant weight was obtained and finally the weight were taken and compared with the initially recorded weight. The percentage weight in the duckweed was calculated using the formula.

$$\text{moisture content \%} = \frac{W_1 - W_2}{W_1} * 100 \quad (3.2)$$

Where W_1 is original weight of the sample before drying

W_2 is weight of the sample after drying

3.3.2 Determination total organic Carbon

First the empty crucible weighed, and then accurately weight of duckweed dried in oven at 105^oC for 24 hrs, in a pre-weighed crucible and ignites the material in a furnace at 550^0C for 6-8 hrs. Then sample was cooling to room temperature and keep in Desiccator for 12 hrs. Weigh the contents with crucible. Calculate the total organic carbon by the following formula

$$\text{ToM} = \frac{\text{sample weight @105c} - \text{sample weight @550c}}{\text{sample weight @105c}} * 100 \quad (3.3)$$

Then the total organic carbon was determined using the following formula

$$\text{Toc} = \frac{\text{ToM}}{1.8} \quad (3.4)$$

3.3.3 Determination of concentration and density of ethanol

The concentration and density of ethanol was determined by using a density meter. A highly accurate Digital Density Meter such as the DMA4100M Plus provides the easiest means to measure the density of an alcohol/water mixture and automatically converting this measurement into an alcohol concentration. The measurement is fast, accurate, and highly reproducible. The DMA4100M Plus is loaded for measurements by injecting a small 3ml to 5ml sample with a syringe. The DMA4100M (Density Meter) will then accurately measure the sample's density and automatically perform the conversion of density, to alcohol concentration using one or more of

the officially recognized alcohol tables. To be sure the equipment measuring accurately by using the density of the sample it possible to find the ethanol concentration from the Perry Chemical Engineering Handbook other reference to check alcohol percentage.

3.3.4 Yield of ethanol

99.9% bioethanol yield from each fermented sample was determined as follows;

Volume of 99.9% Ethanol yield (VAE) = $\frac{VHE*\rho HE*XE}{\rho AE}$ (3.5)

Where: V_{HE} = volume of hydrous ethanol formed.

V_{AE} = volume of anhydrous ethanol formed

ρ_{AE}= density of anhydrous ethanol which is 0.789g/ml

ρ_{HE} = density of hydrous ethanol which of the sample

X_E = mass fraction of ethanol (% alcohol by weight)

3.3.5 FT-IR Spectrum for Ethanol

Infrared spectrometer passes infrared radiation through a sample of an unknown compound and uses a detector to plot percent transmission of the radiation through the molecule versus the wavenumber of the radiation. A downward peak on the plot represents absorption at a specific wavenumber. In sum, IR spectroscopy is useful in determining chemical structure because energy that corresponds to specific values allows us to identify various functional groups within a molecule. An IR spectrum usually extends from radiation around 4000 cm^{-1} to 600 cm^{-1} and can be split into the functional group region and the fingerprint region. The fingerprint region is different for each molecule just like a fingerprint is different for each person. Two different molecules may have similar functional group regions because they have similar functional groups, but they always have a different fingerprint region.

To describe the functional group of ethanol FT-IR analysis were done in Natural Science College of Addis Ababa University, Department of Chemistry. The samples were examined and the result was checked by comparing with standard ethanol graph.

3.4 Design of the Experiment

Data analysis was carried out by DESIGN EXPERT version 7.0 software (central composite design) to evaluate the effects of the process variables; temperature (80,100 and 120) ^{o}C, reaction

time (60min, 120 min and 180 min) and acid concentration (0.6M and 0.8M and 1M). (100^oC, 120 min and 0.8M) temperature, acid concentration and time respectively were used center point. A 2^3 full factorial experimental design with 20 experiments were employed, which includes 8 trails for factorial design, 6 trails for axial points and 6 trails for replication of the central points to estimate error based on the pattern generated through software. The response variable was sugar concentration after hydrolysis and ethanol yield after fermentation. This design of the experiment helps us to optimize of process parameters using Response Surface Methodology (RSM). Significance of the result was set from analysis of variance (ANOVA).

Table 3.1 low, center and high values of factors

Factor name	Unit	low	Center point	High
Temperature	^oC	80	100	120
Acid- concentration	M	0.6	0.8	1
Time	Hr	1	2	3

4. Results and Discussion

4.1 Moisture Contents of the Samples

Accurately weighting of two different samples which have different weights (10g, 15g) were prepared to determine the moisture content at 105°C, Heat in an oven for about 24 hours at 105^0C to until obtained constant weight. And the moisture content of the sample was obtained. The moisture content of the sample was calculated using Equation 3.2 above.

Table 4.1 Moisture content determination

Drying time, hrs	0	14	16	18	20	22	24
Weight of $sample_1$ (g)	10	7.95	5.55	4.92	3.53	1.14	1.12
Weight of $sample_2$ (g)	15	11.9	9.75	6.99	4.98	1.98	1.52

The moisture content of the duckweed sample with 10g, 15g was 88.8% and 89.89% w/w, respectively. Thus, the average moisture content of the two samples was 89.24%w/w. According to (Cross, 2006) Fresh duckweed have been reported that contain 91% w/w moisture content, so the value for moisture content obtained during this study was less than the moisture content value obtained by the previous works, but this difference was not significant.

4.2 Estimation of Organic Carbon

Weigh 10g of sample dried in oven at 105°C for 24 hrs. In a pre-weighed crucible and ignite the material in a furnace at 550^0C for 6-8 hrs. Cool to room temperature and keep in Desiccator for 12 hrs. Weigh the contents with crucible. Then using the equation 3.3 calculation the total organic matter is 77.41% then calculation of the total organic carbon was calculated using the equation 3.4 is around 43.01%. The result obtained by this experiment was supported by other studies like (Landolt and Kandeler, 1987)

Table 4.2 Estimation of carbon content

Weight of sample	Weight of sample at 105^0c	Weight of sample at 550^0c
10g	9.3g	2.1g

4.3 Total Reducing Sugar Determination

The wavelength and the corresponding concentration of sample was determined using equation 3.1. From table 4.3 it is clear that the absorbance and concentration of sugar has inverse relationship. The sample which have less sugar concentration will remain with high amount of CU (II) oxide, blue color which is unreacted with the Benedict's reagent due to this absorbance of the sample increases, but in the sample which have high sugar concentration CU (II) oxide is completely react with the Benedict's reagent and form CU (I) oxide red color precipitate and absorbance of the sample decreases.

Table 4.3 Concentration of standard glucose and its absorbance

Std.Glucose concentration (g/ml)	0	0.1	0.2	0.4	0.6	0.8
Absorbance	0.4525	0.4199	0.3541	0.2411	0.1135	0.0312

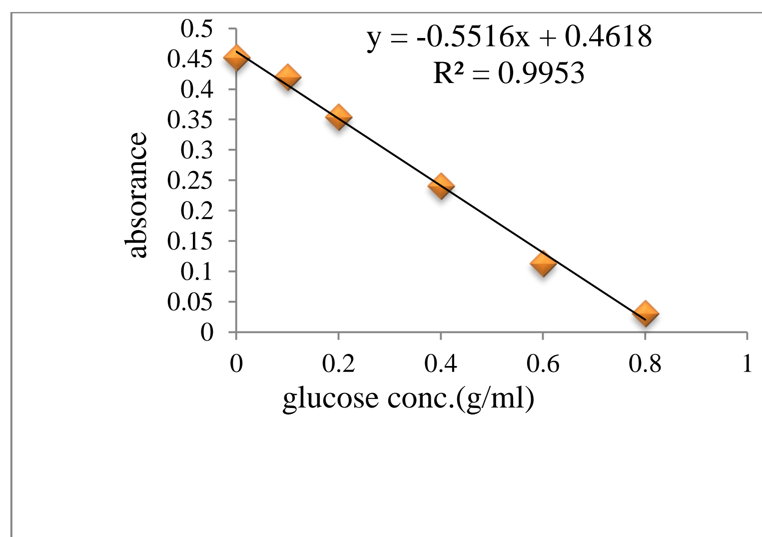


Figure 4.1 Calibration curve of standard glucose concentration

Table 4.4The yield of total concentration sugar of samples

Run No	factor A Tempratue	factor B acid concentration	factor C time	Observance	Con. Of glucose (%v/v)
1	100.00	0.80	2.00	0.2233	43.23
2	80.00	1.00	1.00	0.2638	35.9
3	100.00	0.80	2.00	0.2211	43.62
4	120.00	0.60	1.00	0.2539	37.68
5	80.00	1.00	3.00	0.2544	37.6
6	100.00	0.46	2.00	0.281	32.7
7	100.00	0.80	2.00	0.2169	44.39
8	80.00	0.60	3.00	0.2627	35.56
9	100.00	1.14	2.00	0.2256	42.8
10	100.00	0.80	0.32	0.2630	36.04
11	100.00	0.80	2.00	0.2238	43.15
12	100.00	0.80	2.00	0.2179	44.21
13	120.00	1.00	3.00	0.22759	42.52
14	66.36	0.80	2.00	0.2838	32.27
15	80.00	0.60	1.00	0.2789	33.14
16	120.00	1.00	1.00	0.2522	38
17	120.00	0.60	3.00	0.2499	38.4
18	100.00	0.80	3.68	0.2349	41.12
19	133.64	0.80	2.00	0.2803	32.9
20	100.00	0.80	2.00	0.2372	40.7

4.4 Evaluate the effect of hydrolysis process variables (temperature, acid concentration and time) in the yield of ethanol

The yield of ethanol was determined using equation 3.5. The ethanol density and concentration of ethanol was determined using density meter.

Table 4.5 Density and concentration of sugar

Run no	Volume of hydrous ethanol(ml) after distillation	Ethanol Con. (v/v %)	Density Of hydrous ethanol(g/ml)	Yield of anhydrous ethanol(ml/20g)
1	9.2	36.2	0.9431	4.02
2	9.0	31.7	0.9421	3.4
3	9.7	38.2	0.9523	4.38
4	8.4	35.9	0.9515	3.63
5	8.9	33.4	0.9482	3.57
6	8.2	30.3	0.95382	3
7	9.7	35.12	0.9516	4.1
8	8.2	32.90	0.9651	3.3
9	9.3	35.1	0.9516	3.94
10	9.7	29.6	0.9653	3.51
11	9.8	36.2	0.9472	4.25
12	9.7	39.3	0.9372	4.57
13	9.7	33.4	0.9482	3.88
14	8	31.0	0.9521	2.95
15	9.2	29.7	0.9612	3.28
16	9.3	34.4	0.9534	3.86
17	8.7	37.2	0.9471	3.86
18	9.9	32.6	0.9565	3.92
19	9.6	29.4	0.9542	3.23
20	9.6	40.12	0.9349	4.56

From the figure 4.2 below the maximum concentration of glucose and yield of ethanol have direct relationship that means the run which has maximum sugar content has maximum yield of ethanol. The same is true in the run which has less sugar content also less yield of ethanol. This situation indicates the efficiency of fermentation process. The fermentation parameters are fixed for all runs.

The maximum sugar concentration 44.21%v/v and yield of ethanol 4.57 ml/20g duckweed (0.18g/g) were obtained at run 12 (100°C, 0.8M and 2hr).The minimum sugar concentration 32.27 %w/w and yield of ethanol 2.95 ml/20g duckweed were obtained at run 14 (66.36^{o}C, 0.8M and 2hr). This low value of the ethanol and glucose concentration may be as a result of temperature is not sufficient to convert the starch to glucose.

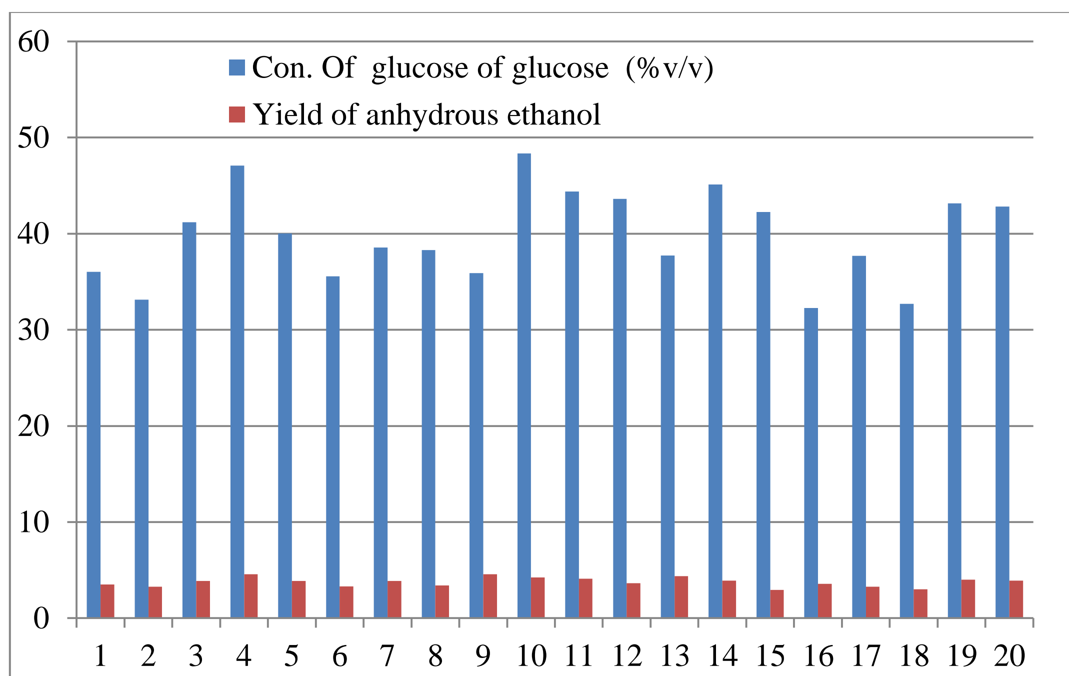


Figure 4.2 ethanol yield and sugar concentration from duckweed

4.5 Statistical Analysis of the Experimental Results

4.5.1 Analysis of variance (ANOVA)

Table 4.6 Analysis of variance (ANOVA) for Response Surface Quadratic Model

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob >F	
Model	3.75	9	0.42	6.45	0.0037	Significant
A-temperature	0.34	1	0.34	5.24	0.0451	
B-acid con…	0.36	1	0.36	5.59	0.0397	
C-C	0.093	1	0.093	1.44	0.2570	
AB	2.450E-003	1	2.450E-003	0.038	0.8496	
AC	4.500-004	1	4.500E-004	6.960E-003	0.9352	
BC	4.500-004	1	4.500E-004	6.960E-003	0.9352	
A^2	2.13	1	2.13	33	0.0002	
B^2	0.90	1	0.90	13.98	0.0039	
C^2	0.39	1	0.39	5.98	0.0345	
Residual	0.65	10	0.065			
Lack of Fit	0.38	5	0.076	1.42	0.3538	not significant
Pure Error	0.27	5	0.053			
Cor Total	4.40	19				

The model chosen for this analysis was quadratic model. In order to check whether the quadratic model is the right model or not, it was crucial to perform analysis of variance (ANOVA). The probability, P-values was used to check the significance of each coefficient of regression model equation. The P-values of corresponding coefficient should be less than or equals to 0.05. This is the bench mark for checking the significance of the proposed model. The Model F-value of 6.45 implies the model is significant.There is only a 0.37% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not

counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 1.42 implies the Lack of Fit is not significant relative to the pure error. There is a 35.38% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good , we want the model to fit.

4.5.2 Development of regression model equation

The application of RSM gives an empirical relationship between the response function and the independent variables. The mathematical relationships between the response, ethanol yield and the independent variables temperature (A), acid concentration (B) and time (C) in terms of coded and actual factors can be determined by Design Expert software 7.0. The model equation that correlates the response (ethanol yield) to the hydrolysis process variables in terms of coded factor and actual factors for duckweed is shown by equation 4.1 and 4.2.

Final equation in terms of coded factors for duckweed

Final Equation in Terms of Coded Factors

$$\text{ethanol yield} = +4.31+0.16*A+0.16*B+0.083*C-0.017*A*B+7.500E\text{-}003*A*C -7.500E\text{-}003*B*C -0.38*A^2-0.25*B^2-0.16*C^2 \quad (4.1)$$

Where ; A tmeprtature

B acid concentratation

C time

Final Equation in Terms of Actual Factors

$$\text{ethanol yield} = -11.91220+0.20300*\text{temperature} +11.34122*\text{acid concentration} +0.73029*\text{time} -4.37500E\text{-}003*\text{temperature}*\text{acidconcentration}+3.75000E\text{-}004*\text{temperature}*\text{time} - 0.037500*\text{acid concentrations} *\text{time}-9.61852E\text{-}004*\text{temprature}^2 -6.25976*\text{acidconcentration}^2-0.16377*\text{time}^2 \quad (4.2)$$

The results showed that the concentration of yield of ethanol produced from duckweed was dependent on the linear terms, on the quadratic terms and also on the interactions of variables. On the basis of the coefficients in equations (4.1) and (4.2) it was evident that the produced ethanol produced increases with the increase in temperature (A), acid concentration (B) hydrolysis time (C) until reach the optimum point.

Table 4.7 Model statistics for duckweed

Std. Dev.	0.25	R-Squared	0.8530
Mean	3.76	Adj R-Squared	0.9427
C.V. %	6.78	Pred R-Squared	0.9128
PRESS	3.29	Adeq Precision	7.526

The "Pred R-Squared" of 0.9121 is in reasonable agreement with the "Adj R-Squared" of 0.9427. "Adeq Precision" measures the signal to disturbance ratio due to random error. A ratio greater than 4 is desirable. Here ratio of 7.526 indicates an adequate signal. Therefore, this model can be used to navigate the design space.

The regression coefficients and the corresponding 95% CI (Confidence Interval) High and Low were presented in table 4.8 below. If zero was in the range High and Low 95% Confidence interval, the factors has no effect. From the 95% CI High and Low values of each model term, it could be concluded that the regression coefficients of temperature, acid concentration have highly significant effect in ethanol production.

Table 4.8Regression coefficients and the corresponding 95% CI High and Low

Factor	Coefficient Estimate	df	Standard Error	95%cl Low	95% CI High	VIF
Intercept	4.31	1	0.10	4.07	4.54	
A-temperature	0.16	1	0.069	4.190E- 003	0.31	1.00
B-acid con...	0.16	1	0.069	9.315 E- 003	0.32	1.00
C-time	0.083	1	0.069	-0.071	0.24	1.00
AB	-0.017	1	0.090	-0.22	0.18	1.00
AC	7.500E-003	1	0.090	-0.19	0.21	1.00
BC	7.500E-003	1	0.090	-0.21	0.19	1.00
A^2	-0.38	1	0.067	- 0.53	-0.24	1.02
B^2	-0.25	1	0.067	-0.40	-0.100	1.02
C^2	-0.16	1	0.067	-0.31	-0.015	1.02

Before the conclusions from the analysis of variance adopted, the adequacy of the underlying model should be checked. And the normality assumption can be checked by constructing a normal probability plot of the residuals. A normal probability plot of the raw data is used to check the assumption of normality. In the analysis of variance, it is usually more effective and straight forward to do this with the residuals. If the underlying error distribution is normal, this plot will resemble a straight line. In visualizing the straight line, place more emphasis on the central values of the plot than on the extremes. In addition, the normal probability plot indicates the residuals following a normal distribution. In the case of this experiment the points in the plots shows fit to a straight line, this shows that the quadratic polynomial model satisfies the assumptions of analysis of variance (ANOVA) i.e. the error distribution is approximately normal. The points are coded by color to the level of response and they represent going from cool blue for lowest values to hot red for the highest.

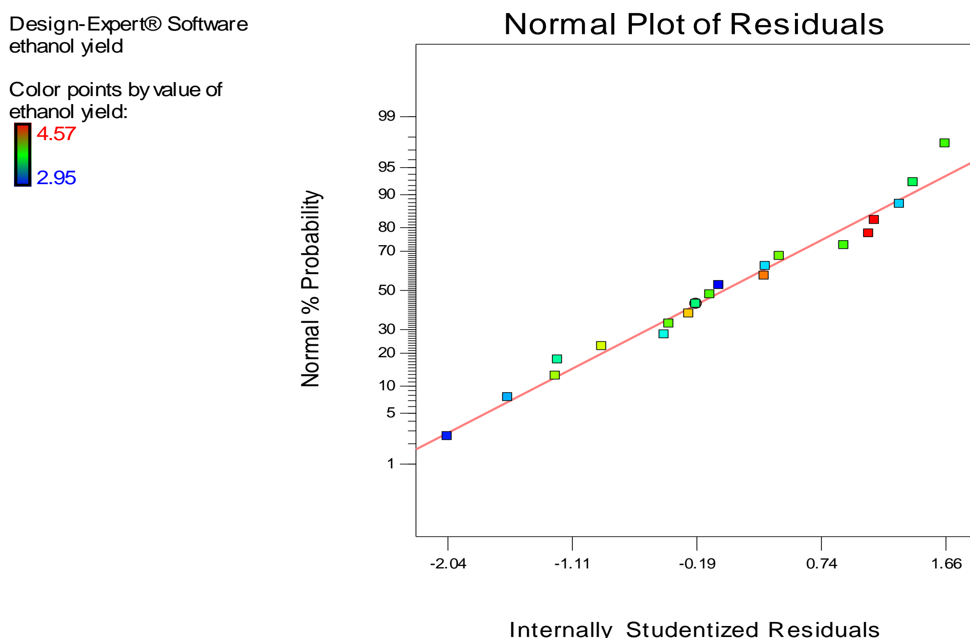


Figure 4.3 Normal plots of residuals for ethanol yield of duckweed

From the below figure assumptions of the analysis of variance (ANOVA) should be checked using the plots of residuals versus predicted. This could be useful to know how much the model is acceptable. When the model is correct and the assumptions are satisfied, the residuals is structure less; in particular, they are unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted, predicted values. This plot

should not reveal any obvious pattern. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. The plot shows random scatter which justifying no need for an alteration to minimize personal error. More scatter in the residuals for a particular block could indicate the block is not homogenous.

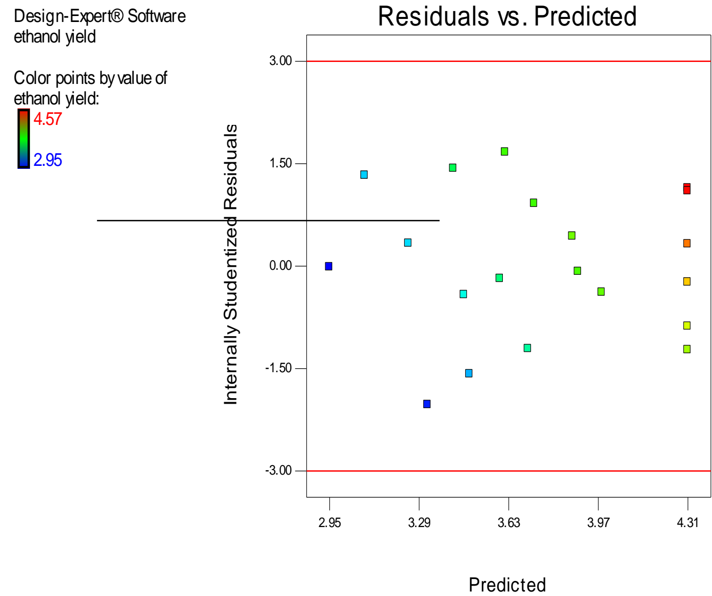


Figure 4.4 Residual versus predicted values

From the figure 4.5 below predicted versus actual value of ethanol yield was plotted. The plot shows how precisely the model modeled. The points show how the predicted value and actual values of each run approach the straight line. The straight line shows how the predicted and actual values are closer to each other. When the point is above the straight line predicted value is greater than the actual value and the reverse is also true.

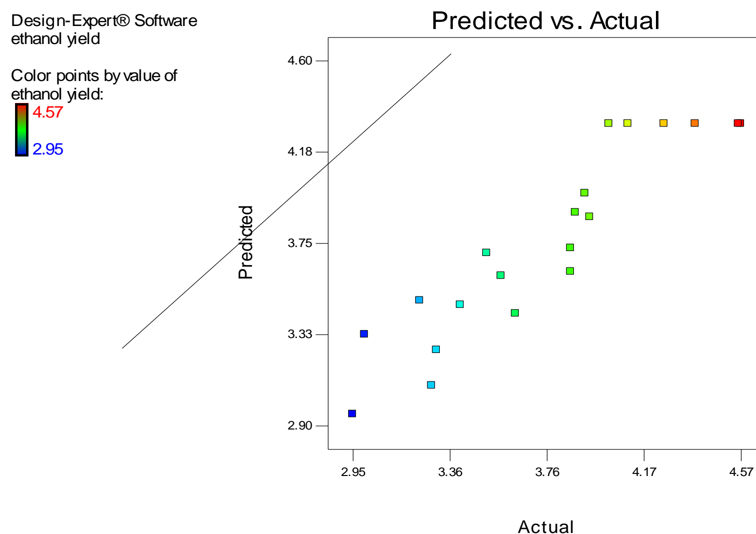


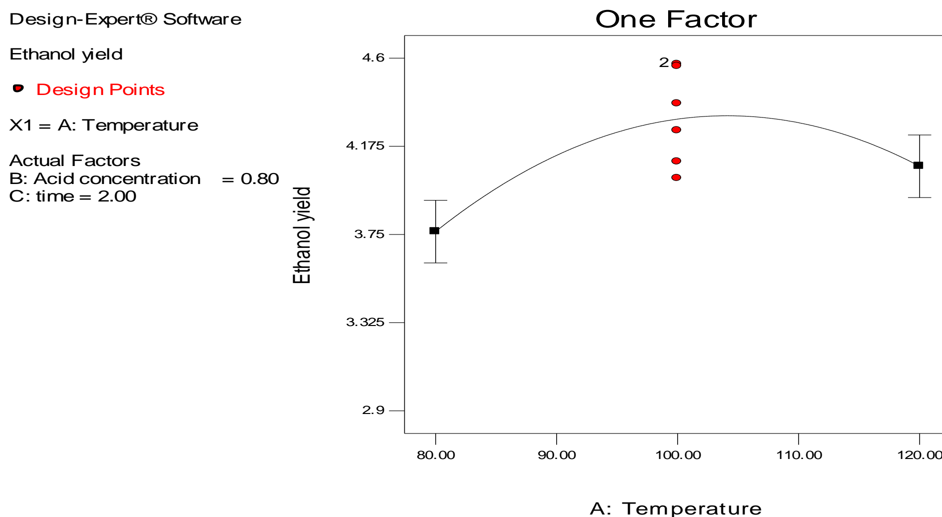
Figure 4.5 Predicted versus actual values of ethanol yield duckweed

4.5.3 Individual effect of experimental variables on the yield of ethanol

The Effect of Temperature

Figure 4.6 represents the effect of hydrolysis temperature on the yield of ethanol at constant time and acid concentration at the center point. As shown from the plot yield of Ethanol is very sensible to the hydrolysis temperature. Yield of ethanol was highly increased as temperature increase from 80°C to 105°C. Optimum yield of ethanol was obtained around 105°C hydrolysis temperature. Beyond 105°C hydrolysis temperature the yield of ethanol is slightly decrease.

The reason that ethanol yield is low at low level of temperature might be due the temperature is not sufficient enough to convert starch to glucose on the hydrolysis stage. The other case is the amount of ethanol yield is low at high temperature and this is might be due to the reason that starch was converted other byproducts rather than to glucose (Balat and Balat 2009).



Figur4.6 Effect of hydrolysis temperature on ethanol yield of duckweed

Effect of acid concentration

Figure 4.7 shows the effect of acid concentration on the yield of ethanol at constant temperature and time in the center point. As shown in figure below the yield of ethanol was affected slightly by acid concentration, as the concentration of acid increase from 0.6M to 0.85M the yield slightly increase, beyond 0.85M the yield of ethanol slightly decreases, because high concentrations of acid may decompose the sugar and producing inhibitor molecules for the fermentation process (Taherzadeh and Karimi 2007).

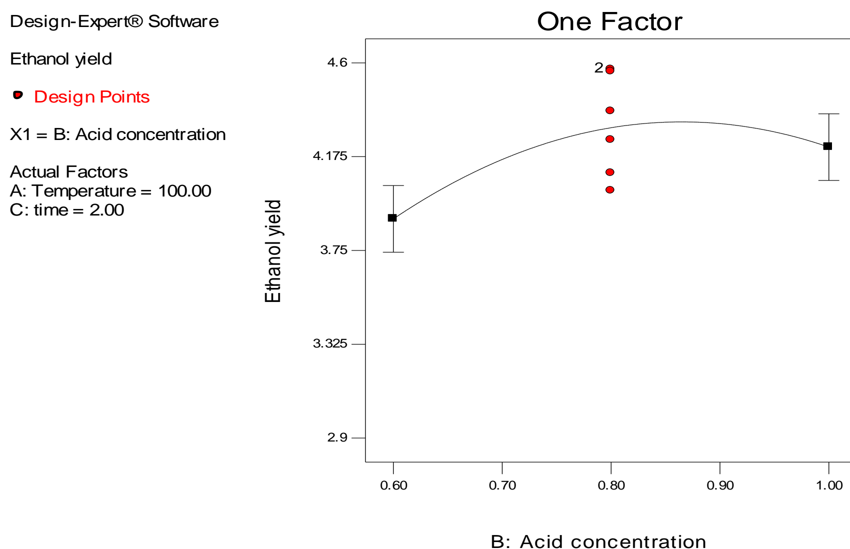


Figure 4.7 Effect of hydrolysis acid concentration on ethanol yield of duckweed

The Effect of Time

Figure 4.8 shows the one factor plot of time versus ethanol yield. The graph shows how ethanol changes when time changes from lower level to higher level. The plot shows the slightly increasing pattern of ethanol yield and then reaching maximum and then it decreases. The maximum ethanol yield is shown in the range between 1 and 2.5 hours. Beyond 2.5hr the ethanol yield was starts to slightly decrease.

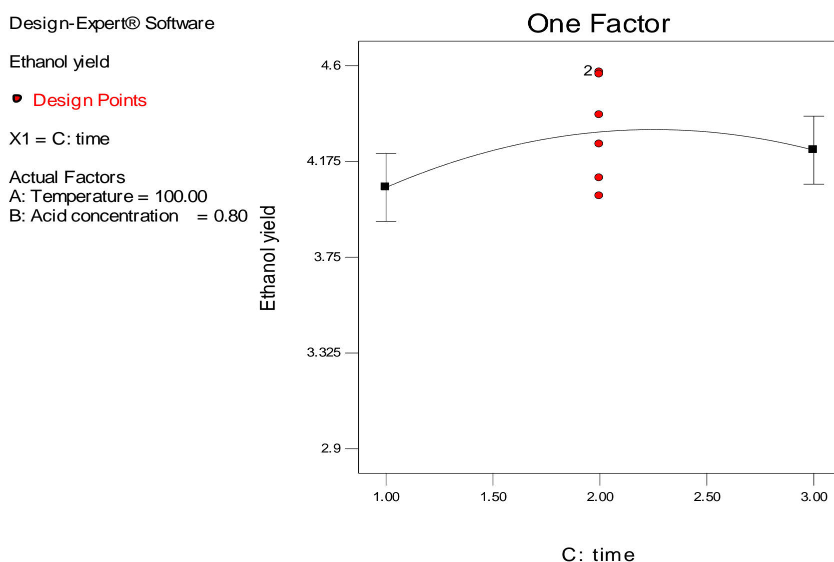


Figure 4.8 Effect of hydrolysis time on ethanol yield of duckweed

4.5.4 Response Surface and Contour plot on the Experimental Variables

In order to analyze the regression equation of the model, three-dimensional surface and 2D contour plots were obtained by plotting the response on the Z axis against any two variables while keeping the other variable at center level. These plots are created to analyze the change in the response surface. Conical shape response surface plot indicates optimum operating conditions. The response optimized value for the production of bioethanol was based on the two process variables described on the response surface plot. The effect of the independent variables and their mutual interaction on the yield of ethanol can be seen in Figures below.

The Effect of Temperature and acid concentration

The figure 4.9 shows of temperature and acid concentration on the ethanol yield when time was at the center point. As it observed from figure the temperature and acid concentration have no

interaction on the ethanol yield and it has maximum effect on the yield of ethanol at higher acid concentration until the temperature reach 105^0c. At higher level of acid concentration the amount of ethanol yield is high compared to lower acid concentration. There are two cases, the first case is when acid concentration is at higher level, the amount of ethanol yield is low at low temperature level and this might be due to the temperature is insufficient for hydrolysis of starch and the amount of ethanol yield is low. At high hydrolysis temperature, the amount of ethanol yield is low compared to the middle maximum point. This might be due to the reason that starch was converted to other by products.

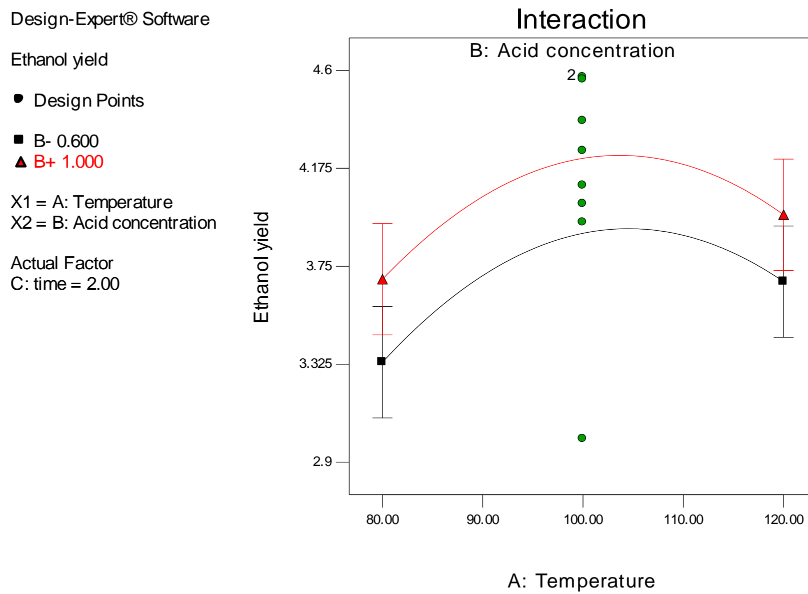


Figure 4.9 Effect and Interaction of temperature and acid concentration (fixed) on the yield of ethanol at center of time

Response Surface and Contour Plot for the Effect of Temperature and Acid concentration

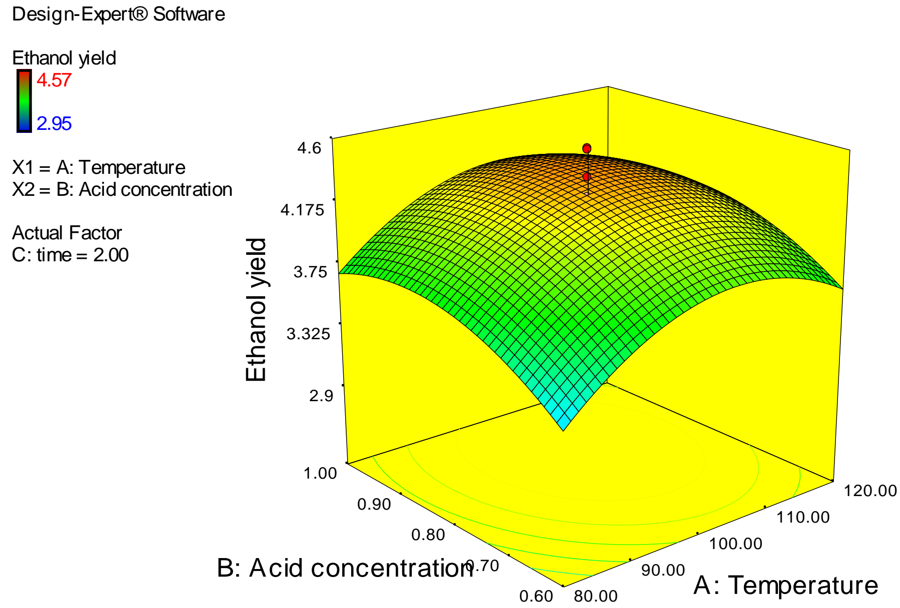


Figure 4.10 Response surface plot of the effect of temperature and acid concentration at constant time in the center

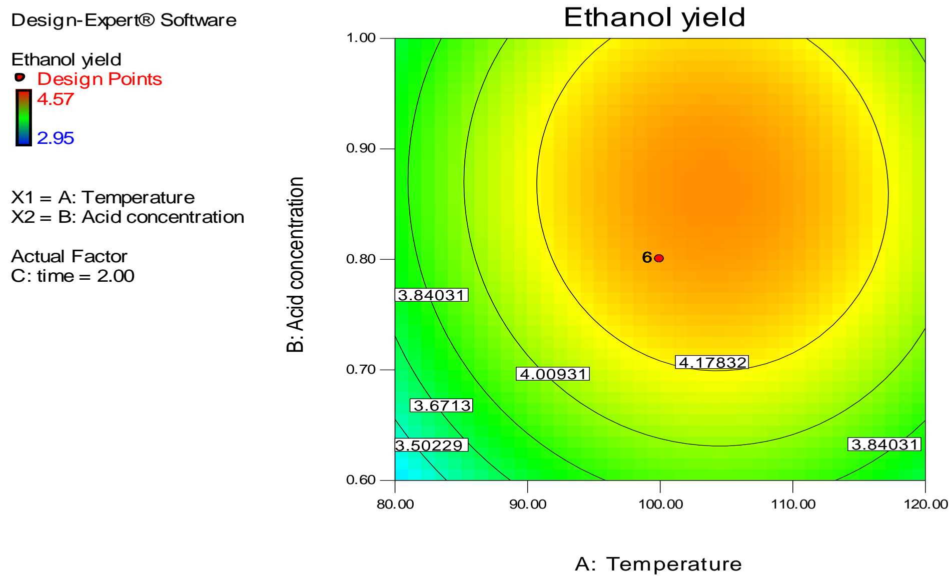


Figure 4.11 Contour plot of the effect of temperature and acid concentration at constant time in the center

Figure 4.10 and 4.11 represents the response surface and contour plots developed as a function of temperature and acid concentration respectively, while time was kept constant at 2hr. the yield of ethanol increased slightly with hydrolysis temperature from 80 to 105°C and acid concentration from 0.6M to 0.8M. However, upon increasing the hydrolysis temperature beyond 105°C, and acid concentration beyond 0.8M there was a gradual decline in the yield, because sugar degrade might be occurred.

Figure 4.10 shows response surface plot of ethanol yield with respect to temperature and acid concentration. The response surface plot shown in obtained from hydrolysis temperature and acid concentration. As shown from the Figure 4.11 contour plot the maximum yield of ethanol was occur in the region of 105°C temperature and 0.8M acid concentration. The contour plot in terms of factors temperature and acid concentration with time at the center point is shown in Figure 4.11. There is color change on the graph and the response variable is increasing from green color to red. The graph suggests operating at the center point where the response variable shows maximum amount. Operating in the red region is good to have high amount of ethanol yield.

The Effects of acid concentration and Time

The graph shown in Figure 4.12 Shows the effect of acid concentration and fixed time on ethanol yield but the temperature kept 100°C. The graph shows effect acid concentration on ethanol yield at fixed time when the temperature held at center point. The red and black color shows the high and low level of time. When the acid concentration increases for both high level and low level time the amount of ethanol yield increases and reaching the maximum it starts to decrease.

As it observed from figure below the acid concentration and time have no interaction on the ethanol yield and it has maximum effect on the yield of ethanol at higher time until the acid concentration reach 0.85M. But, beyond 0.85 M acid concentration at fixed time the yield of ethanol slightly decreases due to the fact that starch material is decomposed in to unwanted product. This could be due to the reason that the amount of starch is low at low level of the acid concentration and time. At high level of acid concentration and time the amount of ethanol yield is low compared to the middle point.

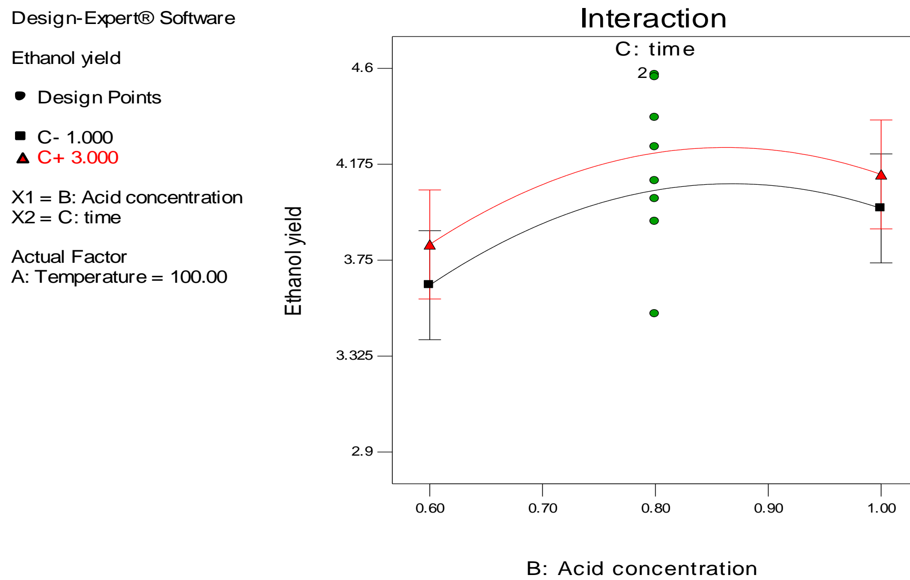


Figure 4.12 Effect of acid concentration and time (fixed) on the yield of ethanol at center of temperature

Response Surface and Contour Plots of the Effects of acid concentration and Time

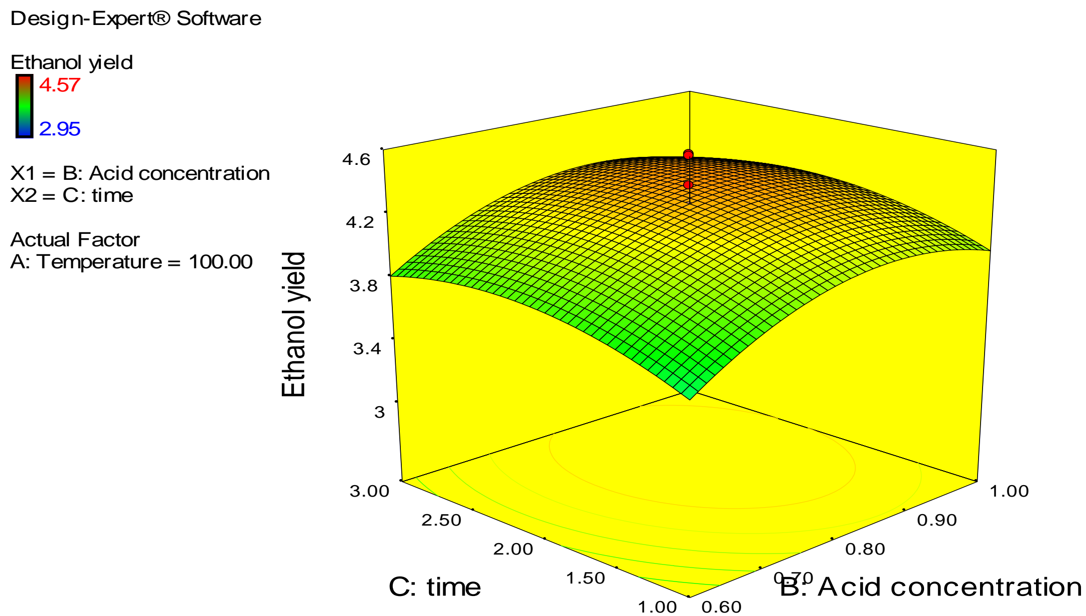


Figure 4.13 Response surface plot of the effect of acid concentration and time on the yield of ethanol at constant temperature

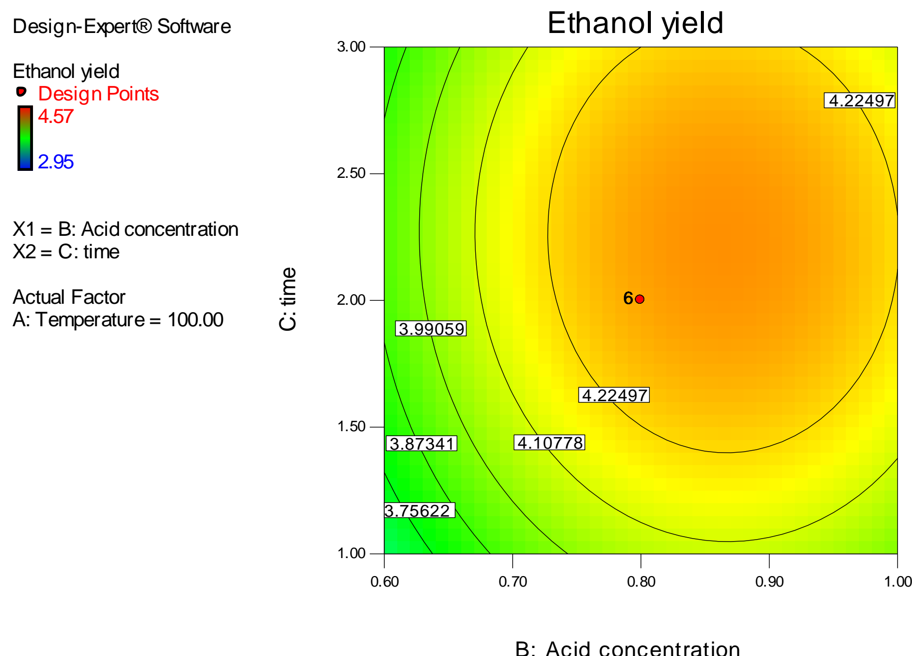


Figure 4.14 Contour plot of the effect of acid concentration and time on the yield of ethanol at constant temperature

Figure 4.13 and 4.14 shows the response surface and contour plots developed as a function of acid concentration and time respectively, while the temperature was kept constant at 100°C. Upon increasing the acid concentration 0.6 to 0.85M with an increase of hydrolysis time from 1 to 2hr, the yield of ethanol increased in small extent. Beyond this time the yield of ethanol was gradually decreased. The highest yield was obtained at 2hr hydrolysis time and 0.85M acid concentration. The decrement of ethanol yield with increasing of acid concentration from 0.85-1M is due to the decomposition of sugar and the formation of some inhibitor.

The Effects of Temperature and Time

As it observed from the temperature and time have no interaction on the ethanol yield and it has maximum effect on the yield of ethanol at higher time until the temperature reach 105^{0}C. Beyond this Temperature 105^{0}c the yield of ethanol was gradually decreased. The amount of ethanol yield at high temperature is higher than at lower temperature. The maximum amount is found in between the high and low temperature. At fixed hydrolysis time at low level of temperature the amount of ethanol yield is low. This might be due to the reason that the starch was not converted

to glucose; therefore the ethanol yield is low. At high level of hydrolysis temperature, the amount of ethanol yield is low compared to the middle maximum point. This might be due to the reason that starch was converted to other by products.

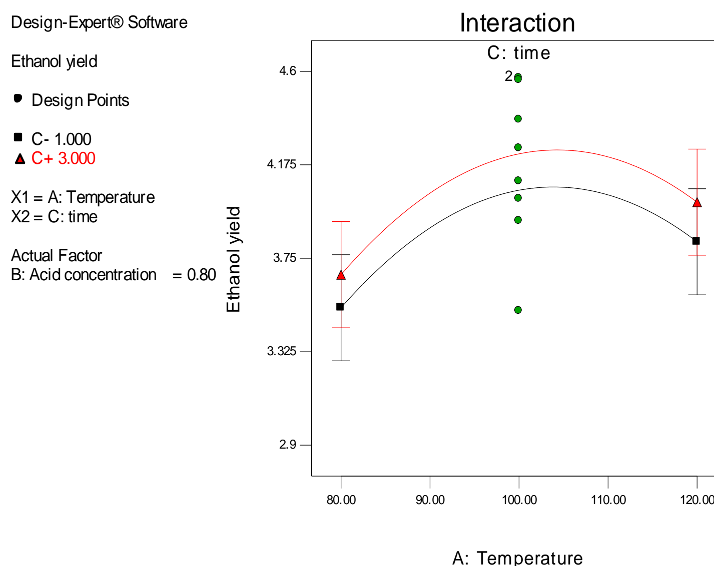


Figure 4.15 Effect and interaction of temperature and time (fixed) on the yield of ethanol at center of acid concentration

Response Surface and Contour Plots of the Effects of temperature and Time

Figure 4.16 and 4.17 shows the response surface and contour plots respectively developed as a function of temperature and time, while the acid concentration was kept constant at 0.8M. It was observed that the yield of ethanol was more sensitive change, when the temperature change from 80-105°C and time changes from 1 to 2hr the yield of ethanol reaches at the peak and beyond 105°C and time beyond 2hr the yield slightly decreased. The reason for this observation is due to the fact that when the starch exposes to high temperature and longer time, the sugar which obtained from starch degraded into not fermentable product and gives low yield of ethanol.

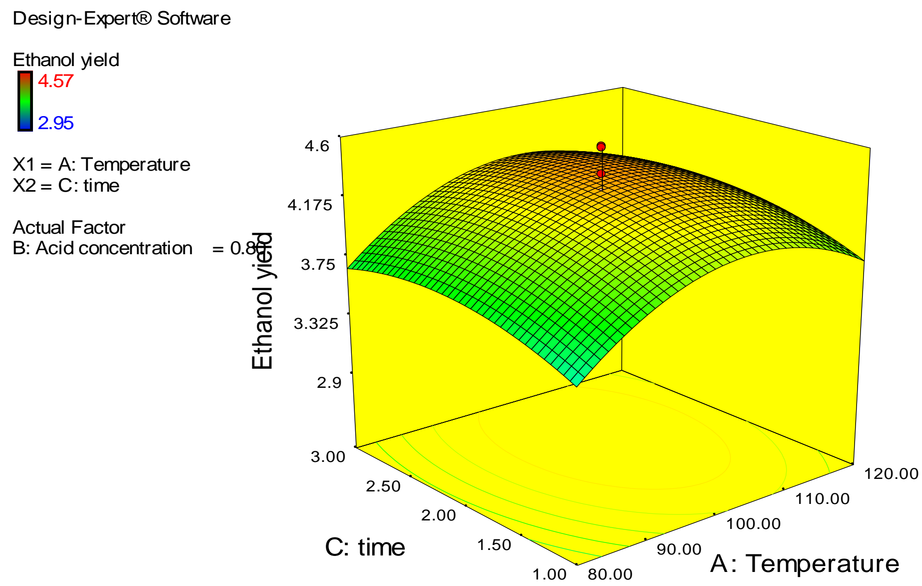


Figure 4.16 Response surface plots of the effect of temperature and time on the yield of Ethanol at Constant acid concentration.

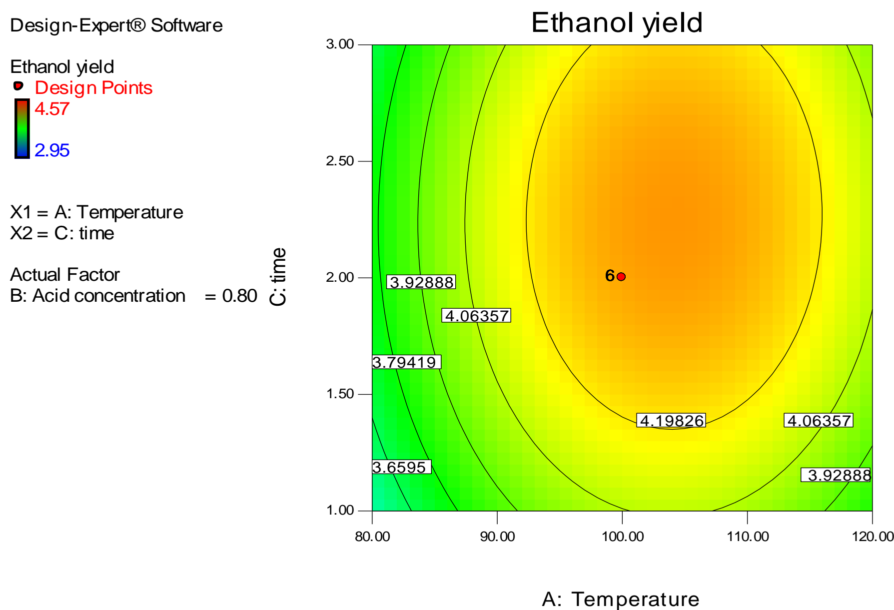


Figure 4.17 Contour plots of the effect of temperature and time on the yield of Ethanol at Constant acid concentration

4.6 Optimization of operating process variables in hydrolysis process using RSM

One of the primary objectives of the present study was to find the optimum process parameters for maximizing the quantity of ethanol yield, glucose concentration. The process variables such temperature, acid concentration and time have been optimized using central composite experimental design and their output values are executed using design-expert software 7.0. In optimizing process, the temperature, acid concentration and time are a set of process parameters held to be "in range" while the ethanol yield and glucose concentration, set of responses that need to be "maximized". Table 4.9 shows the summary of factors responses and goals and the corresponding set of specific objectives that will optimize the process condition. The table below exhibits the desired combinations of process parameters that would provide the highest responses by using Numerical optimization. Numerical optimization was used to optimize any combination of one or more goals. The goals may be apply either factors or responses. The model capable of predicting the maximum ethanol yield, and glucose concentration value showed that the optimum values of the process variables were the operating process variables are putting on the range.

Table 4.9 Constraints applied for optimization

Name	Goal	Lowe Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	is in rang	80	120	1	1	3
Acid con..	is in range	0.6	1	1	1	3
Time	is in range	1	3	1	1	3
Ethanol yield	Maximize	2.95	4.57	1	1	3
glucose con..	Maximize	32.27	44.39	1	1	5

By using the Numerical optimization criteria in Table 4.9, the design expert solution was obtained. The possible solution for this model with the given factors that change the amount of the produced ethanol yield and glucose concentration is shown in Table 4.10. The optimum possible solutions in hydrolysis of duckweed for yield of maximum of concentration glucose

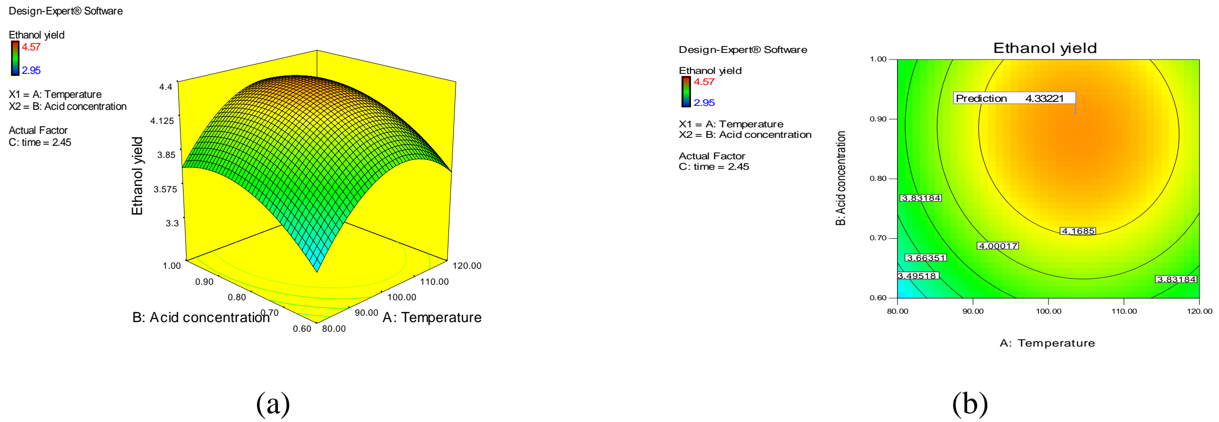
and bioethanol are presented in table 4.10 and in Figure 4.18-4.20 in the form of response surface plot and the contour plot.

Table 4.10 Optimum possible solutions

No	Temperatue	Acid con….	Time	Ethanol yield	Glucose con..	Desirability	
1	103.59	0.91	2.45	4.3322	44.2471	0.935	Selected
2	103.59	0.91	2.45	4.3326	44.2462	0.935	
3	103.61	0.91	2.45	4.3321	44.246	0.935	
4	103.60	0.91	2.45	4.3321	44.2465	0.935	
5	103.62	0.91	2.45	4.3327	44.2472	0.935	
6	103.60	0.91	2.45	4.3323	44.2459	0.935	
7	103.58	0.91	2.45	4.3324	44.2469	0.935	
8	103.61	0.91	2.45	4.3323	44.2459	0.935	
8	103.58	0.91	2.45	4.3326	44.2473	0.935	
9	103.61	0.91	2.45	4.3325	44.2468	0.935	
10	103.62	0.91	2.45	4.3325	44.2463	0.935	
11	103.63	0.91	2.45	4.3328	44.2467	0.935	
12	103.59	0.91	2.45	4.3326	44.2457	0.935	
13	103.58	0.91	2.45	4.3325	44.2458	0.935	
14	103.62	0.91	2.45	4.3325	44.2458	0.935	
15	103.64	0.91	2.45	4.3326	44.2462	0.935	
16	103.60	0.91	2.45	4.3324	44.2453	0.935	
17	103.62	0.91	2.45	4.3322	44.2454	0.935	
18	103.62	0.91	2.46	4.3325	44.2478	0.935	
19	103.59	0.91	2.45	4.3324	44.2458	0.935	
20	103.59	0.91	2.45	4.3322	44.2471	0.935	

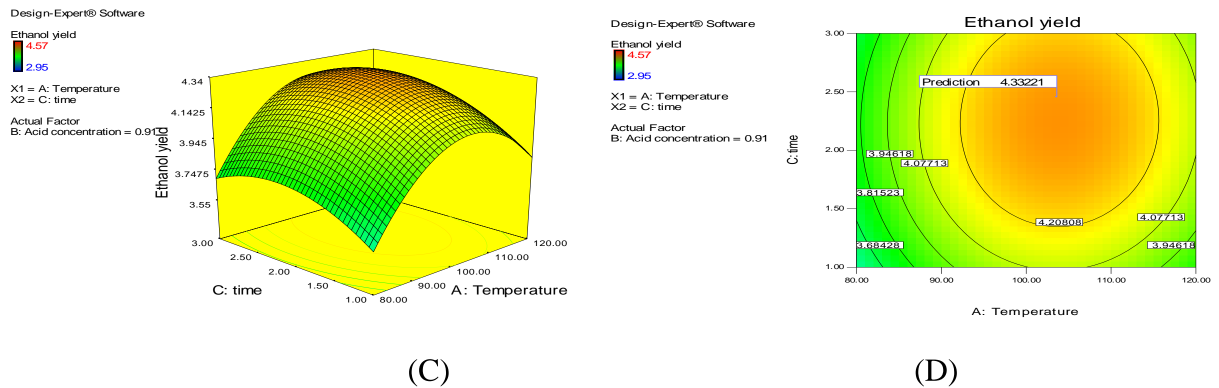
The result in Table 4.10 has shown that the three process optimal value of the variables namely, temperature, acid concentration and time that give maximum ethanol yield glucose

concentration. Therefore, the next step is validation of the model result using the response surface and couture plots of optimum possible solutions.



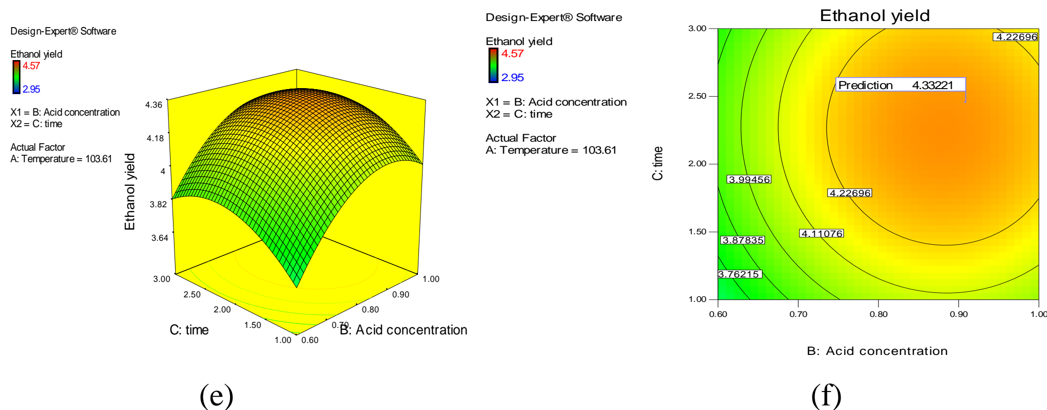
(a) (b)

Figure 4.18 (a) Response surfaces plot of predicted ethanol yield a constant time (b) Contour plot of predicted ethanol yield at constant time



(C) (D)

Figure 4.19 (c) Response surfaces plot of predicted ethanol yield a constant acid concentration (d) Contour plot of predicted ethanol yield at constant acid concentration



(e) (f)

Figure 4.20 (e) Response surfaces plot of predicted ethanol yield a constant temperature (f) Contour plot of predicted ethanol yield at constant temperature

4.7 Model validation

According to the response surface and counter plot method result using Design-Expert 7.0 software, an experiment with hydrolysis temperature, acid concentration and time were conducted in order to study the outcome or effect of the central composite. The experiment was carried out at the optimized conditions. As it can be seen from figure 4.18 - 4.20 above ethanol yield of average predicted value was 4.33221/20g sample (0.17g/g). As a result, the model was considered to be accurate and reliable for predicting the yield of ethanol from duckweed using dilute acid hydrolysis. The optimal values of variables were calculated at a temperature 103.59 ^{0}c ,acid concentration 0.91M, and time 2.45 hr.

4.8 Bioethanol Characterizations by FTIR

Alcohols have characteristic IR absorptions associated with the O-H, C-O and the C-H stretching vibrations. When run as a liquid film the region 3500-3200 cm^{-1} with a very intense and broad band indicated the O-H stretch of alcohols, while the region 1260-1050 cm^{-1} confirms the C–O stretch. The bands at around 2880 and 2930 cm^{-1} were assigned as the symmetric stretching modes of the –CH_2 and –CH_3 groups, respectively (Coates and Meyers, 2000). This assures that the product obtained from duckweed is exactly ethanol due to the confirmation of these regions as show.

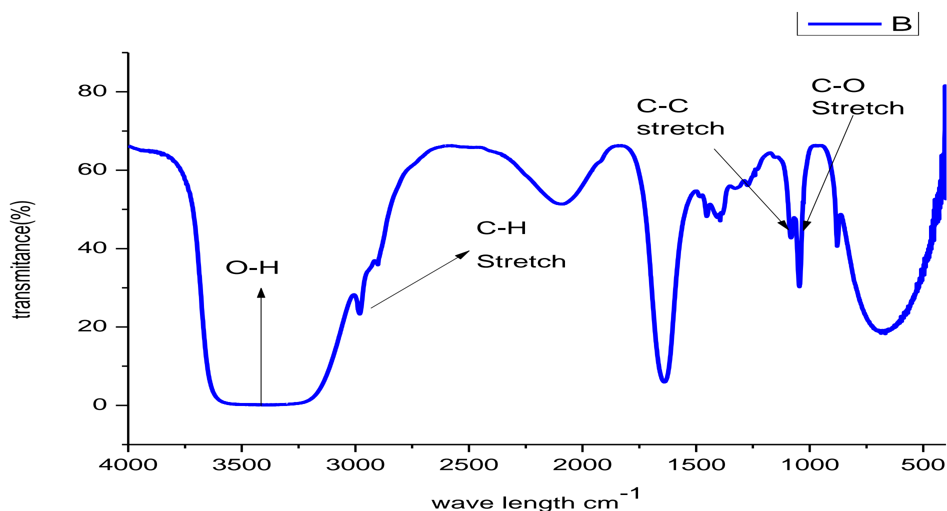


Figure 4.21 Fourier transforms Infrared spectra of the produced bioethanol from duckweed

5. Conclusion and Recommendations

5.1 Conclusion

Production of ethanol (bioethanol) from biomass is one way to reduce both consumption of crude oil and environmental pollution. Bioethanol is appropriate for the mixed fuel in the gasoline engine because of its high octane number, and its octane number and high heat of vaporization impede self-ignition in the diesel engine. So, ignition improver, glow-plug, surface ignition, and pilot injection are applied to promote self-ignition by using diesel-bioethanol-blended fuel, in this research ethanol was produced from duckweed. Starch hydrolysis with dilute acid was carried out. The effect of temperature, acid concentration and time on hydrolysis of duckweed investigated. Hydrolysis with process parameters temperature, acid concentration and time, results the maximum and minimum ethanol yield 4.57ml/20g (0.18g/g) and 2.95ml/g (0.12g/g) value respectively. Based on analysis of variance (ANOVA) hydrolysis temperature, acid concentration has significant effect on the yield of ethanol. Samples containing high amount of glucose concentration produced high amount of ethanol as one might expect. As the result of, RSM optimization 4.33221/20g sample (0.17g/g) yield of ethanol was found at 103.59^0c, acid concentration 0.91M, and time 2.45hr. Generally, the ethanol synthesis from duckweed may replenish the fuel availability and it may lead to the sustained development.

5.2 Recommendation

This research would like to suggest the following recommendation

- Further researches have to be carried out to increase the yield of bioethanol from duckweed by use other microorganisms which are capable of converting starch into ethanol.
- The government should involve on bioethanol production from aquatic plant because most the aquatic plants have generally, low in lignin but with a high proportion of carbohydrate, do not compete with food crops for arable land and they commonly have higher productivity because of their fast proliferation, many types of aquatic plants could be cultured in waste water system for decontamination.

- Further researches have to be done on hydrolysis of starch so as to get high amount of glucose concentration which gives high amount of ethanol because from the research the amount of glucose concentration and ethanol has direct relationship.
- Producing ethanol from renewable resources is becoming an important issue for the whole world. Therefore, the work needs to be continued for scaling up of ethanol production from duckweed.

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Appendices

Appendix A: Laboratory equipment and sample photos

Pretreated duckweed

Sample ready for Hydrolysis

Autoclave (reactor)

Sample after hydrolysis

Vacuum filtration machine

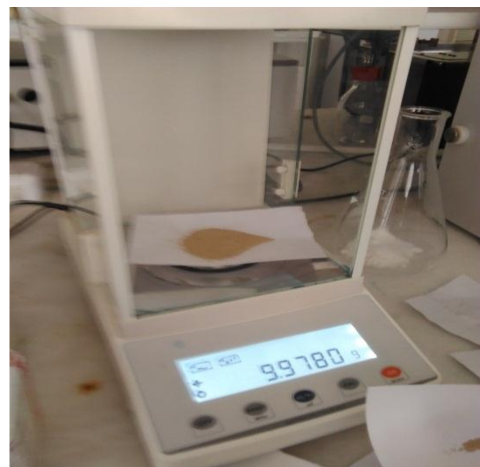
Filtered sample

waste after hydrolysis

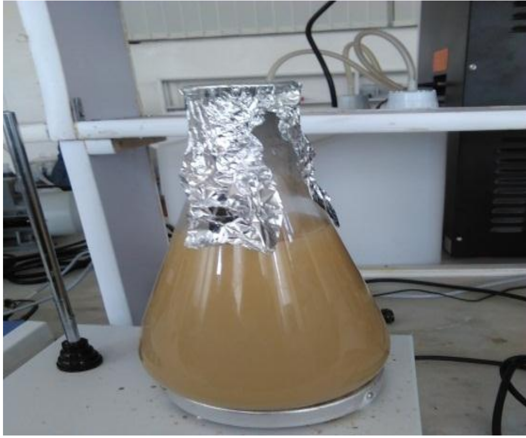
PH meter

Filtered sample after neutralization

Ingredient for culture preparation

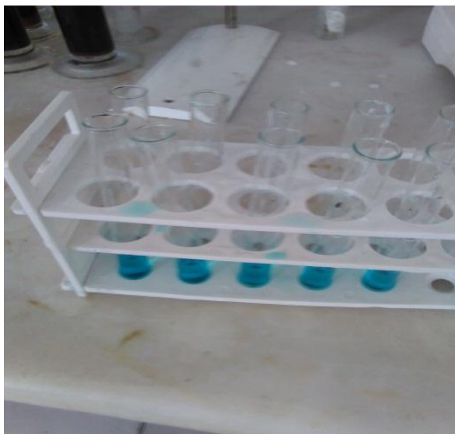


Weight balance



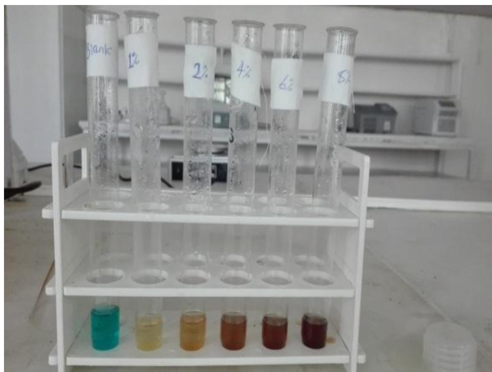
Cultured media

sterilizer



Benedict solution

water bath



Standard glucose after reaction with benedict solution

Samples ready for analysis

Digital spectrometer

Distillation Apparatus

Density meter

sample products

Appendix B: Properties of Ethanol

Molecular formula	C_2H_5OH
Molar mass	46.07g/mol^{-1}
Appearance	Colorless clear liquid
Density	0.879g/cm^3
Melting point	−114.3 °C, 159 K, -174 °F
Boiling point	78.4 °C, 352 K, 173 °F
Solubility in water	Fully miscible
Acidity	15.9
Viscosity	1.200 mPa·s (cP) at 20.0 °C
Dipole moment	5.64 fC·fm (1.69 D) (gas)

Appendix C: Central composites design for hydrolysis

Std	Run	Block 1	Temperature	Factor 3 acid con.	Factor 2 time (hr)
15	1	Block 1	100.00	0.80	2.00
4	2	Block 1	80.00	1.00	1.00
2	3	Block 1	100.00	0.80	2.00
16	4	Block 1	120.00	0.60	1.00
8	5	Block 1	80.00	1.00	3.00
17	6	Block 1	100.00	0.46	2.00
5	7	Block 1	100.00	0.80	2.00
13	8	Block 1	80.00	0.60	3.00
14	9	Block 1	100.00	1.14	2.00
19	10	Block 1	100.00	0.80	0.32
6	11	Block 1	100.00	0.80	2.00
9	12	Block 1	100.00	0.80	2.00
10	13	Block 1	120.00	1.00	3.00
18	14	Block 1	66.36	0.80	2.00
12	15	Block 1	80.00	0.60	1.00
20	16	Block 1	120.00	1.00	1.00
7	17	Block 1	120.00	0.60	3.00
3	18	Block 1	100.00	0.80	3.68
11	19	Block 1	133.64	0.80	2.00
1	20	Block 1	100.00	0.80	2.00

Appendix D. Diagnostics Case Statistics of ethanol

Run Order	Actual Value	Predicted Value	Residual	Leverag e	Internally Studentized Residual	Externally Studentized Residual	Influence on Fitted Value DFFITS	Cook's Distance
15	3.28	3.09	0.19	0.670	1.322	1.381	1.967	0.355
4	3.63	3.42	0.21	0.670	1.425	1.514	* 2.16	0.412
2	3.40	3.46	-0.062	0.670	-0.425	-0.406	-0.579	0.037
16	3.86	3.73	0.13	0.670	0.910	0.901	1.284	0.168
8	3.30	3.25	0.048	0.670	0.327	0.312	0.444	0.022
17	3.86	3.62	0.24	0.670	1.662	1.853	* 2.64	0.560
5	3.57	3.60	-0.027	0.670	-0.188	-0.179	-0.254	0.007
13	3.88	3.89	-0.012	0.670	-0.085	-0.081	-0.115	0.001
14	2.95	2.95	-0.002	0.607	-0.018	-0.017	-0.022	0.000
19	3.23	3.48	-0.25	0.607	-1.586	-1.739	* -2.16	0.389
6	3.00	3.32	-0.32	0.607	-2.035	-2.523	* -3.14	0.641
9	3.94	3.87	0.069	0.607	0.431	0.413	0.513	0.029
10	3.51	3.70	-0.19	0.607	-1.216	-1.249	-1.554	0.229
18	3.92	3.98	-0.062	0.607	-0.389	-0.371	-0.462	0.023
12	4.10	4.31	-0.21	0.166	-0.887	-0.877	-0.392	0.016
20	4.25	4.31	-0.056	0.166	-0.241	-0.230	-0.103	0.001
7	4.38	4.31	0.074	0.166	0.319	0.304	0.136	0.002
3	4.57	4.31	0.26	0.166	1.137	1.156	0.516	0.026
11	4.02	4.31	-0.29	0.166	-1.232	-1.269	-0.567	0.030
1	4.56	4.31	0.25	0.166	1.094	1.106	0.494	0.024

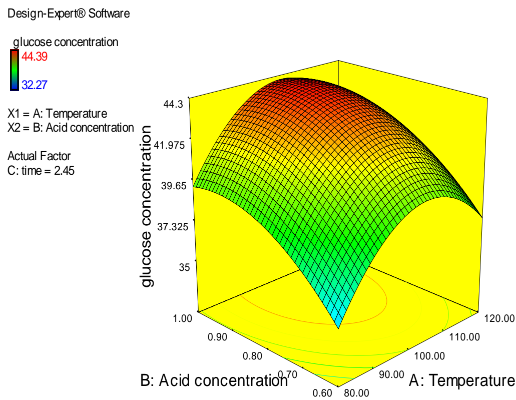
Appendix E: Densities of Mixtures of C_2H_5OH and H_2O at 20°C

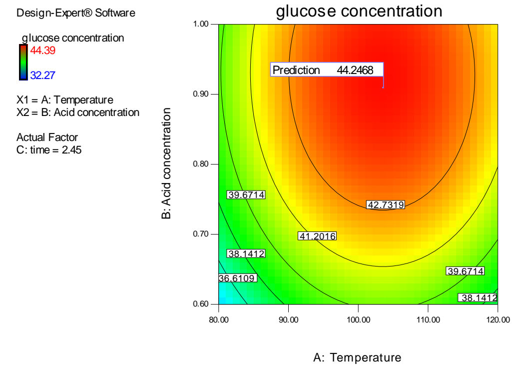
%	10°C	15°C	20°C	25°C	30°C	35°C	40°C
0	0.99973	0.99913	0.99823	0.99708	0.99568	0.99406	0.99225
1	785	725	636	520	379	217	034
2	602	542	453	336	194	031	.98846
3	426	365	275	157	014	.98849	663
4	258	195	103	.98984	.98839	672	485
5	098	032	.98938	817	670	501	311
6	.98946	.98877	780	656	507	335	142
7	801	729	627	500	347	172	.97975
8	660	584	478	346	189	009	808
9	524	442	331	193	031	.97846	641
10	393	304	187	043	.97875	685	475
11	267	171	047	.97897	723	527	312
12	145	041	.97910	753	573	371	150
13	026	.97914	775	611	424	216	.96989
14	.97911	790	643	472	278	063	829
15	800	669	514	334	133	.96911	670
16	692	552	387	199	.96990	760	512
17	583	433	259	062	844	607	352
18	473	313	129	.96923	697	452	189
19	363	191	.96997	782	547	294	023
20	252	068	864	639	395	134	.95856
21	139	.96944	729	495	242	.95973	687
22	024	818	592	348	087	809	516
23	.96907	689	453	199	.95929	643	343
24	787	558	312	048	769	476	168
25	665	424	168	.95895	607	306	.94991
26	539	287	020	738	442	133	810
27	406	144	.95867	576	272	.94955	625
28	268	.95996	710	410	098	774	438
29	125	844	548	241	.94922	590	248
30	.95977	686	382	067	741	403	055
31	823	524	212	.94890	557	214	.93860
32	665	357	038	709	370	021	662
33	502	186	.94860	525	180	.93825	461
34	334	011	679	337	.93986	626	257
35	162	.94832	494	146	790	425	051
36	.94986	650	306	.93952	591	221	.92843
37	805	464	114	756	390	016	634
38	620	273	.93919	556	186	.92808	422
39	431	079	720	353	.92979	597	208
40	238	.93882	518	148	770	385	.91992
41	042	682	314	.92940	558	170	774
42	.93842	478	107	729	344	.91952	554
43	639	271	.92897	516	128	733	332
44	433	062	685	301	.91910	513	108
45	226	.92852	472	085	692	291	.90884
46	017	640	257	.91868	472	069	660
47	.92806	426	041	649	250	.90845	434
48	593	211	.91823	429	028	621	207
49	379	.91995	604	208	.90805	396	.89979

%	10°C	15°C	20°C	25°C	30°C	35°C	40°C
50	0.92126	0.91776	0.91384	0.90985	0.90580	0.90168	0.89750
51	.91943	555	160	760	353	.89940	519
52	723	333	.90936	534	125	710	288
53	502	110	711	307	.89896	479	056
54	279	.90885	485	079	667	248	.88823
55	055	659	258	.89850	437	016	589
56	.90831	433	031	621	206	.88784	356
57	607	207	.89803	392	.88975	552	122
58	381	.89980	574	162	744	319	.87888
59	154	752	344	.88931	512	085	653
60	.89927	523	113	699	278	.87851	417
61	698	293	.88882	446	044	615	180
62	468	062	650	233	.87809	379	.86943
63	237	.88830	417	.87998	574	142	705
64	006	597	183	763	337	.86905	466
65	.88774	364	.87948	527	100	667	227
66	541	130	713	291	.86863	429	.85987
67	308	.87895	477	054	625	190	747
68	074	660	241	.86817	387	.85950	407
69	.87839	424	004	579	148	710	266
70	602	187	.86766	340	.85908	470	025
71	365	.86949	527	100	667	228	.84783
72	127	710	287	.85859	426	.84986	540
73	.86888	470	047	618	184	743	297
74	648	229	.85806	376	.84941	500	053
75	408	.85988	564	134	698	257	.83809
76	168	747	322	.84891	455	013	564
77	.85927	505	079	647	211	.83768	319
78	685	262	.84835	403	.83966	523	074
79	442	018	590	158	720	277	.82827
80	197	.84772	344	.83911	473	029	578
81	.84950	525	096	664	224	.82780	329
82	702	277	.83848	415	.82974	530	079
83	453	028	599	164	724	279	.81828
84	203	.83777	348	.82913	473	027	576
85	.83951	525	095	660	220	.81774	322
86	697	271	.82840	405	.81965	519	067
87	441	014	583	148	708	262	.80811
88	181	.82754	323	.81888	448	003	552
89	.82919	492	062	626	186	.80742	291
90	654	227	.81797	362	.80922	478	028
91	386	.81959	529	094	655	211	.79761
92	114	688	257	.80823	384	.79941	491
93	.81839	413	.80983	549	111	669	220
94	561	134	705	272	.79835	393	.78947
95	278	.80852	424	.79991	555	114	670
96	.80991	566	138	706	271	.78831	388
97	698	274	.79846	415	.78981	542	100
98	399	.79975	547	117	684	247	.77806
99	094	670	243	.78814	382	.77946	507
100	.79784	360	.78934	506	075	641	203

*For data from −78° to 78°C, see p. 2-142, Table 2N-5, ***American Institute of Physics Handbook***, McGraw-Hill, New York, 1957.

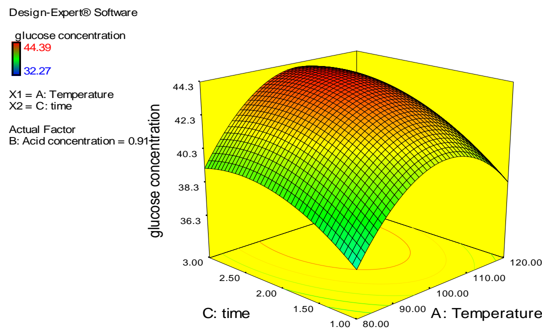
Appendix F: Response surface and Contour plot of optimized yield of concentration of sugar

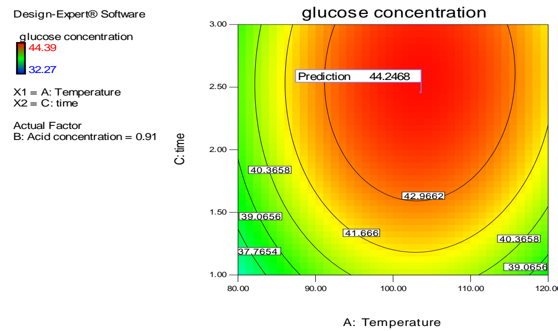




(a) (b)

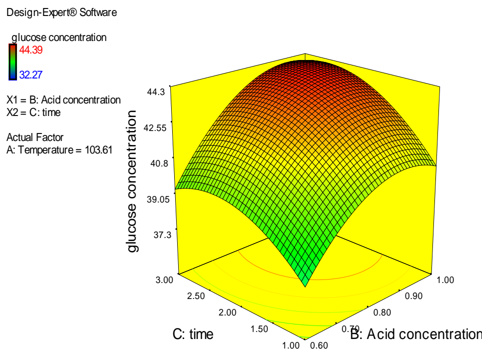
(a) Response surfaces plot of predicted glucose concentration yield a constant time (b) Contour plot of predicted glucose concentration yield at constant time

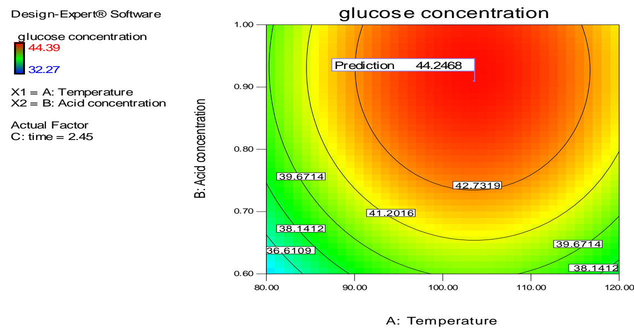




(C) (d)

(c) Response surfaces plot of predicted glucose concentration yield a constant acid concentration (d) Contour plot of predicted glucose concentration yield at constant acid concentration





(e) (f)

(e) Response surfaces plot of predicted glucose concentration yield a temperature (f) Contour plot of predicted glucose concentration yield at constant temperature

Appendix G: Ethanol standard spectrum graph