ADDIS ABABA UNIVERSITY,

ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)

SCHOOL OF CHEMICAL AND BIO ENGINEERING

PRODUCTION AND OPTIMIZATION OF BIO-ETHANOL FROM AVOCADO SEED

BY:

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JUNE 06, 2018

ADDIS ABABA, ETHIOPIA

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Date

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TABLE OF CONTENTS

CONTENTS **PAGE NO**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFEX	Ammonia Fiber Explosion
ANOVA	Analysis of Variance
BAP	Biomass Action Plan
C.V	Coefficient of Variation
CCD	Central Composite Design
DF	Degree of Freedom
DOE	Department of Energy
E-85	85% ethanol and 15% gasoline
EISA	Energy Independence and Security Act
EtOH	Ethanol
EU	European Union
FTIR	Fouries Transform Infrared Spectroscopy
GHG	Greenhouse Gas
HMF	Hydroxyl Methyl Furfural
LHW	Liquid Hot Water
OECD	Organization for Economic Cooperation and Development
PH	Power of Hydrogen
Rpm	Rotation per minute
US	United State

ABSTRACT

This study employs powdered avocado seed wastes as a raw material for the production of Bioethanol. It was used dilute acid pretreatment, dilute acid hydrolysis, fermentation and distillation processes. The experiment was designed by Central Composite Design (CCD) with three factors (acid concentration, temperature and time) and triplicate run, the acid concentration varied from 1.5 to 3.5%, hydrolysis temperature varied from 125 to 145℃ and hydrolysis time was varied from 30 to 90 minutes. The different parameters of hydrolysis conditions were optimized. The effect of hydrolysis temperature, hydrolysis time and acid concentration on glucose yield was studied. The maximum yield of glucose was achieved at 60 minutes hydrolysis time, acid concentration of 1.5% and hydrolysis temperature of 135℃ with maximum yield of 49.60% under optimized conditions. Acid concentration and hydrolysis time have a statistically significant effect on the yield with p-value of 0.0016 and 0.0320 respectively. Higher acid concentration as well as increased hydrolysis temperature causes a decline in the glucose yield. Chemical characterization of the bio-ethanol produced was performed by FTIR. The result shows that, the ethanol produced contains OH, CO, CH_2, and CH_3 functional groups which indicate the presence of ethanol in the product.

1. INTRODUCTION

1.1. Background of the study

Society has been searching for better sources of sustainable energy for many years. Because of limited access to petroleum based fuels, rising of fossil fuel costs and its consequences on emission of greenhouse gases, the struggle to maintain a green planet has become more challenging over time particularly for developing countries. One of the potential options to solve the environmental and energetic problems is the use of bio-ethanol. This is a renewable fuel, which avoids the negative environmental impacts generated by petroleum-based fuels *(Reddy et al, 2011).* Bio-ethanol can be produced by using different technologies. One of the most important technology, fermentation, produces bio-ethanol by means of biological transformation of natural starch and sugars resources such as energy rich crops, (first-generation biofuels) and lignocellulosic biomass (second generation biofuels). The use of renewable biomass resources to produce liquid biofuels such as bioethanol offers attractive solutions to reducing greenhouse gas (GHG) emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies, and increasing sustainability of the world transportations system. Apart from bio-fuels, many other valuable products for chemical and pharmaceutical industry can be produced from organic byproducts through microbial fermentation. Bioethanol feedstocks can be divided into three major groups: (i) sugar containing feedstocks (e.g. sugar cane, sugar beet, sweet sorghum and fruits), (ii) starchy materials (e.g. corn, milo, wheat, rice, potatoes, cassava, sweet potatoes and barley), and (iii) lignocellulosic biomass (e.g. wood, straw, and grasses). Most current bioethanol production processes (1st generation) utilize more easily degradable biomass feedstocks such as cereals crops and sugarcane juice. However, the utilization of these agricultural crops exclusively for energy production is heavily conflicting with food and feed production *(Braide et al, 2016).* Great effort is enforced on advancing a cellulosic bioethanol concept (2nd generation) that utilizes lignocellulosic biomass.

Lignocelluloses wastes refer to plant biomass wastes that are composed of cellulose, hemicellulose and lignin. They may be grouped into different categories such as wood residues

(including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non food seeds, bagasse, domestic wastes (lignocellulose garbage and sewage), food industry residues, municipal solid wastes and the like *(Rodríguez et al., 2008 ; Hirschnitz et al , 201).* Currently, the second generation bio-products such as bioethanol, biodiesel and methane from lignocellulose biomass are increasingly been produced from wastes rather than from energy crops (jatropha, switchgrass, hybrid poplar and willow) because the latter competes for land and water with food crops that are already in high demand.

The production and use of bioethanol is increasing worldwide due to various driving factors. The production in 1990 was only 4 billion gallons (15.14 billion litres) and it took 15 years for the quantity to double to around 8 billion gallons (30.28 billion litres) in 2005. But in recent years it only took three years to double again to around 17 billion gallons (64.35 billion litres) in 2008 and today around 23 billion gallons (87 billion liters) of ethanol is produced annually worldwide *(F.O.Licht, 2007-2011).* Moreover, Predictions indicate that the production would reach 34 and 48 billion gallons (128.7 and 181.7 billion litres) by the year 2020 and 2030 respectively. With this the global future demand of bioethanol is expected to surpass the supply which signifies that there will be market opportunities for low cost producer developing countries, especially for tropical countries with low labour and land costs *(Dufey, 2007; Walter, o.a., 2007).*

In this study, the hydrolyzates of the reduced sugars obtained from hydrolysis of avocado seed wastes were the major raw materials for the production of bio-ethanol in an environmentally friendly and cost effective way. The researchers believe that the seed wastes does not disturb the human food chain and it is a massive potential source of waste in anywhere as a residue in cities and towns, causing in environmental pollution and unpleasant odor.

1.2. Statement of the problem

Inadequate agricultural solid waste collection and disposal creates a range of environmental problems in our country. In recently, due to the environmental concerns about air pollution caused by the combustion of fossil fuels, thus an alternative energy sources need to be renewable, sustainable, efficient, cost effective, convenient and safe. The use of food crops for biofuels production may cause inflation of cost of these crops leading to food insecurity. To reduce such problems, alternative and non food agricultural products must be investigated. Avocado seed is an important byproduct of avocado for every 1 million pounds of avocado fruit, with about 25% seed, 250,000 pounds avocado seeds are produced which would be a large product to be thrown away as one of no value *(Mahawana et al., 2015).* However, avocado seed is still discarded in not very productive ways usually burnt which is dangerous to environment, directly tied to air pollution. While such residues may contain valuable materials such as 38-50% cellulose, 23-32% hemicelluloses and 15-25% lignin *(Zych, 2008).* Bioethanol fermentation from edible, cellulosic feedstocks using enzymatic hydrolysis has been carried out with success; very little research has been done on fermenting bioethanol from non-edible, lignocellulosic material using acid pretreatment with acid hydrolysis on avocado seed. Due to the tough crystalline structure, the enzymes currently available require several days to achieve good results. Since long process times tie up reactor vessels for long periods, these vessels have to either be quite large or many of them must be used. Either option is expensive. Currently the cost of enzymes is also too high. Acid is low cost, non-volatileness, easily available, and productive. However, bioethanol fermentation from lignocellulosic material can only be achieved by adequately pre-treating the lignocellulosic material. Pre-treatment would remove the lignin and make the cellulose and hemicellulose accessible for conversion to sugars. Both cellulose and pentose converts in to fermentable sugar. However, the primary industrial yeast used in bioethanol production, *Saccharomyces cerevisiae* converts only hexose sugars such as glucose and is not able to co-ferment glucose and xylose *(Chang SF, 1989*). Thus, avocado seed can be used for the production of second generation biofuel. In addition to environment benefit, ethanol production from avocado seed can stimulate community based jobs and economic growth. Therefore, the aim of this work was to investigate the possibility of using and transforming avocado seed waste to something valuable product, namely ethanol.

1.3. Objectives of the research

1.3.1. General objective

The main aim of this thesis was to produce bio-ethanol from avocado seed.

1.3.2. Specific objectives

The specific objectives of the present work were:-

- To characterize avocado seed using proximate analysis (moisture content, volatile constitutes and ash content) and to find the chemical composition (extractives, hemicellulose, lignin and cellulose).
- To investigate the effects of process variables (hydrolysis time, concentration of H_2SO_4 & temperature) on the yield of glucose.
- To determine the optimal operating conditions in the hydrolysis process.
- To characterize the final product through FTIR analysis.

1.4. Significance of the study

This study has great significance in terms of assuring the production of an alternative form of energy from avocado seed wastes; which is locally available, abundant and no economic value. Producing Energy is one of the most fundamental parts of our universe. We use energy to do the work. Energy lights our cities, powers our vehicles, trains, planes and rockets, warms our homes, and cooks our food, powers machinery in factories and tractors on a farm. Everything we do is connected to energy in one form or another. This study also highly contributes in the substitute fossil fuel by bio-fuel. Fossil fuels are quickly being depleted due to extensive and continuing over-utilization. If consumption goes at this rate the fossil fuel reserve will be depleted completely within a short period of time. In addition to this, continuous burning of fossil fuel increases emissions of greenhouse gasses to the atmosphere and causes global warming. Reducing use of fossil fuels would considerably reduce the amount of CO_2 produced, as well as reduce the levels of pollutants.

2. LITERATURE REVIEW

2.1. Brief history of ethanol production

Ethanol is an alcohol made through the fermentation of plant sugars from agricultural crops and biomass resources *(NEVC, 1998).* With the rapid depletion of the world reserves of petroleum, ethanol in recent years has emerged as one of the alternative liquid fuel and has generated immense activities of research in the production of ethanol and its environmental impact.

Production of alcoholic beverages is in fact as old as human civilization. The production of pure ethanol apparently begins in the 12-14th century, along with improvement of distillation. During the middle ages, alcohol was used mainly for production of medical drugs but also for the manufacture of paint pigments. The knowledge of using starchy materials for ethanol production was first employed in the 12th century in typical beer countries like Ireland. Ethanol was one of the most popular lamp illuminates used in 1850s and approximately 90 million gallon ethanol was produced in the United States. But due to the tax imposition on ethanol to assist in financing the civil war and the cheaper price of kerosene, it quickly replaced ethanol as the premier illuminated in 1861 *(Onuki et al,. 2008).* It was only in the 19th century that this trade became an industry with enormous production figures due to the economic improvements of the distilling process. It was at the beginning of the 20th century that it had become known that alcohol might be used as fuel for various combustion engines, especially for automobiles. In the 1970s, the interest in fuel ethanol was renewed due to the oil crisis. Nearly 25 federal agencies administered various ethanol programs and the National Alcohol Fuels Commission was established to study the potential for alcohol based fuels *(Lansing, 1983).*

Bio-fuels: The term bio-fuels generally refer alcohols, ethers, esters and other chemicals made from cellulose based biomass, which include pure plant oil (PPO), bio-ethanol and biodiesel.
This includes variety of annual plants (sunflower, groundnut, soybean and rapeseed), perennial plant species (oil palms, coconut palms, physical nut and Chinese tallow tree), herbaceous , woody plant, agricultural and forestry residues and a large portion of municipal and industrial waste material *(Teixeira et al,. 2005)* (as cited in Wondale 2012).
Bio-fuels are renewable since they are produced from biomass, organic matter, such as plants. They generate about the same amount of carbon dioxide (a greenhouse gas) from the tailpipe as fossil fuels, but the plants that are grown to produce the bio-fuels actually remove carbon dioxide from the atmosphere. Therefore, the net emission of carbon dioxide will be close to zero*(Karp et al,. 2013).* The bio-fuels industry has evolved from using first generation feedstock (typically food crops) to using second and third generation feedstocks, for both ethanol and biodiesel. While the term bio-fuels denote any fuel made from biological sources, for most practical uses, the term refers to either ethanol or biodiesel. The last few years have seen tremendous growth in bio-fuels *(Badger, 2002).* Bio-ethanol and biodiesel have been emerged as bio-fuels in the above context. Global production of bio-fuels consists primarily of ethanol then biodiesel comes second.

Current Status and future trends of bio-ethanol: The production and use of bio-ethanol is rising worldwide as a result of various driving factors as described in fig. 2.1 below. The production in 1990 was only 4 billion gallons (15.14 billion liters) and it took 15 years for the quantity to double to around 8 billion gallons (30.28 billion liters) in 2005. But in recent years it only took three years to double again to around 17 billion gallons (64.35 billion liters) in 2008 and today around 23 billion gallons (87 billion liters) of ethanol is produced annually worldwide *(F.O.Licht, 2007-2011).* Moreover, Predictions indicate that the production would reach 34 and 48 billion gallons (128.7 and 181.7 billion liters) by the year 2020 and 2030 respectively as shown in figure 2.1 below. With this the global future demand of bio-ethanol is expected to surpass the supply which signifies that there will be market opportunities for low cost producer developing countries, especially for tropical countries with low labour and land costs *(Dufey, 2007; Walter, o.a., 2007).*

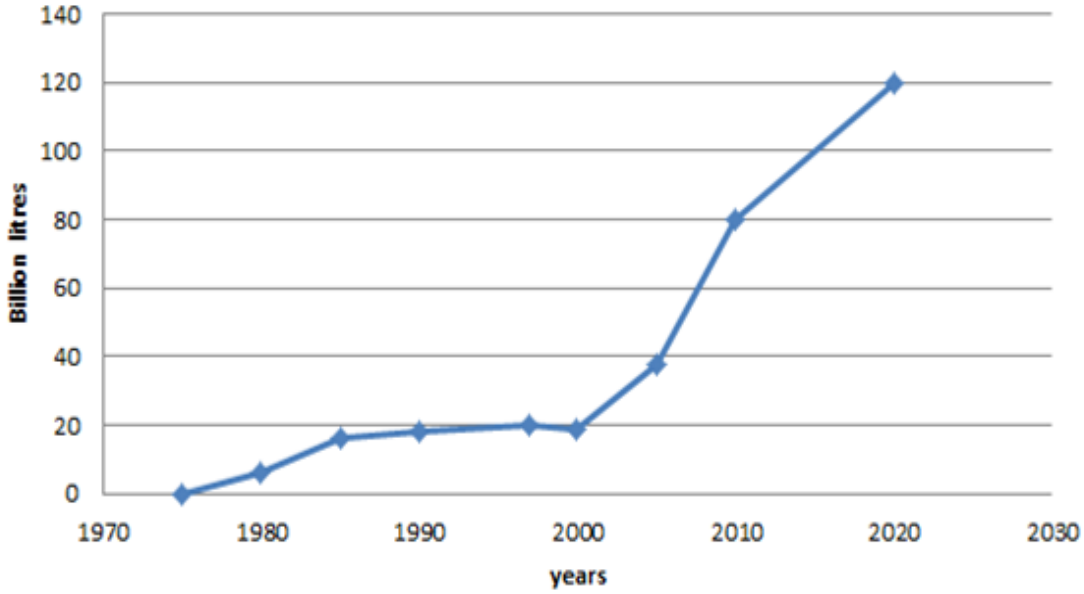


Figure 2.1 Current Status and future trend of bioethanol production

Source: Adapted from World watch Institute, 2006 and Bio-fuel market, 2007

The future supply of bio-ethanol is expected to exceed the demand which implies that there will be opportunities for low-cost producer developing countries, especially for tropical countries with low labor and land costs *(Dufey, 2007).*

2.2. Ethanol and its characteristics

Bio-ethanol or fuel alcohol refers to ethyl alcohol produced by microbial fermentation (as opposed to petrochemically-derived alcohol) that is used as a transportation bio-fuel. It is produced through distillation of the ethanolic wash emanating from fermentation of biomass derived sugars and can be utilized as a liquid fuel in internal combustion engines, either neat or in petrol blends *(Walker, 2011).* Table 2.1 summarizes some of the important characteristics of ethanol as a fuel source.

Table 2.1 Physico-chemical characteristics of ethanol as a liquid fuel

Parameter	**Characteristic properties**
Molecular formula	C_2H_5OH
Molecular mass	46.07 g/mol
Appearance	Colorless liquid
Water solubility	(Between –117°C and 78°C)
Density	0.789 kg/l
Boiling temperature	78.5°C (173°F)
Freezing point	–117°C
Flash point	12.8°C (Lowest temperature of ignition)
Ignition temperature	425°C
Explosion limits	The lowest 3.5% (v/v) Upper 19% (v/v)
Vapor pressure at 38°C	50 mm Hg
Higher heating value (at 20°C)	29,800 KJ/kg
Lower heating value (at 20°C)	21,090 KJ/kg
Specific heat	Kcal/Kg 60°C
Acidity (p*K*a)	15.9
Viscosity	1.200 maps. s (20°C)
Octane number	99

Source: (Walker, 2011)

The high octane number of ethanol makes it blend achieve the same octane boosting or anti-knock effect as petroleum derived aromatics like benzene. Aside high octane number ethanol has a high evaporation heat and high flammability temperature that influences the engine performance positively and increases the compression ratio. The blend E85 consisting of 15% unleaded gasoline and 85% ethanol has a prevalent usage as an alternative fuel because of its advantage over pure ethanol which has a high risk of cold starting problems.

2.3. Status of bio-ethanol production

2.3.1. Worldwide status of bio-ethanol production

Bio-ethanol production worldwide has increased considerably since the oil crisis in 1970*(Campbell and Laherrere, 1998).*Its market grew from less than a billion liters in 1975 to more than 65 billion liters in 2008 *(Biofuels Platform, 2010)* and is expected to reach 100billion liters in 2015 *(Licht, 2006).* According to IEA (2008b) the total worldwide demand for oil is projected to rise by 1% per year, mostly due to increasing demand in the energy market of developing countries, especially India (3.9%/year) and China (3.5%/year).

Global production of bio-ethanol increased from 17.25 billion liters in 2000 *(Balat, 2007)* to over 46 billion liters in 2007 *(REN21, 2008).* Bio-ethanol production in 2007 represented about 4% of the 1300billion liters of gasoline consumed globally *(REN21, 2008).* The United States, Brazil and several EU member states have the largest programs promoting bio-fuels in the world. National bio-fuels policies tend to vary according to available feedstock for fuel production and national agricultural policies. With all of the new government programs in America, Asiaand Europe in place, total global fuel bio-ethanol demand could grow to exceed 125 billion liters by 2020 *(Demirbas, 2007).*

Bio-energy ranks second (to hydropower) in renewable U.S. primary energy production and accounts for 3% of the U.S. primary energy production *(James and Barry,2007).*The United States is the world's largest producer of bio-ethanol fuel, accounting for nearly 47% of global bio-ethanol production in 2005 and 2006 *(Balat and Balat, 2009).* The" Bio-fuels Initiative" in the US Department of Energy *(US DOE, 2004),* strives to make cellulosic ethanol cost-competitive by 2012 and supposedly to correspond and account for one third of the US fuel

consumption by 2030. In 2007, the US president signed the Energy Independence and Security Act of 2007 *(EISA, 2007),* which requires 34 billion liters of bio-fuels (mainly bio-ethanol) in 2008 increasing steadily to 57.5 billion liters in 2012 and to136 billion liters in 2022. Similar to Brazil, the US is also a big investor in bio-ethanol research *(Solomon et al., 2007)* and has increased the ethanol production from 6.16 billion liters or 1.63 billion gallons in 2000 to 39.3 billion liters or 10.4 billion gallons in 2009,representing a 24 times increase *(Petrova and Ivanova, 2010*). Currently over 95% of ethanol production in the United States comes from corn, while the rest is made from wheat, barley, cheese whey and beverage residues *(Solomon et al., 2007).* However, it is expected that about 1.53 billion liters or 405 million gallons of cellulosic ethanol will be produced by the end of 2012 *(Cristina et al,. 2017).*

The EU has also adopted a Biomass Action Plan that sets out measures to increase the development of biomass energy from wood, wastes and agricultural crops by creating market based incentives and removing barriers to the development of markets. Implementation of the plan will help the EU to cut its dependence on fossil fuels, reduce greenhouse gas emissions and stimulate economic activity in rural areas. In 2003, the European Union adopted two bio-fuels directives. These directives set targets for the share of renewable fuels in the transport fuel market (2% by the end of 2005 and 5.75% by the end of 2010) *(EC Directive,2003).* The 2005 target was not achieved, but the industry is growing rapidly and it is expected that the 2010 target will be achieved. On 23 January 2008, the European Commission proposed a binding minimum target of 10% for the share of bio-fuels in transport that envisages a 20% share of all renewable energy sources in total energy consumption by 2020 *(EC, 2008).*

The bio-ethanol sectors in many EU member states have responded to policy initiatives and have started growing rapidly. Bio-ethanol production increased by 71% and consumption reached 2.44 billion liters in 2007 *(Tokgoz, 2008).* The potential demand for bio-ethanol as a transportation fuel in the EU is estimated at about 12.6 billion liters in 2010*(Zarzyycki and Polska, 2007).*

Brazil is the world's largest exporter of bio-ethanol and second largest producer after the United States. With regard to bio-ethanol, the share of the US in the global production is50% and Brazil provides 39% of the total global supply, while the share of OECD-Europe is 5% *(Gnansounou, 2010).* Since Brazil is one of the most developed nations in ethanol production, almost all the

Brazilian vehicles use either pure ethanol or the blend of gasoline and ethanol *(Mussatto et al., 2010; RFA, 2010*).

The highest percentage in which ethanol is added to gasoline in Brazil is also an effort on the part of the government to reduce the imports of oil *(Prasad et al., 2007).* As a result of these efforts, ethanol production in Brazil has substantially risen from 555 million liters (1975/76) to 16 billion liters (2005/06) *(Orellana and Bonalume Neto, 2006; Souza, 2006).* Production has been expected to rise from 15.4 billion liters in 2004 to 26.0 billion liters by 2010. Ethanol from sugarcaneprovides 40% of automobile fuel in Brazil and approximately 20% is exported to the US, EU and other markets *(Greenergy, 2007).*

There are more than 10 ethanol bio-fuel facilities either in operation or under construction in Canada and 130 plants in the United States as of 2006 *(Allan et al., 2006; Parcell and Westhoff, 2006).* In eastern Canada and the US, corn is used as the feedstock while in western Canada wheat is used. Brazil produces a large amount of ethanol from sugarcane and many vehicles in that country has been built to run directly on ethanol fuel. In Europe, ethanol is produced in Sweden, Denmark, Germany, the United Kingdom, France, Italy and Spain. Many Asian countries such as China, India, Japan and Indonesia are also developing ethanol production capacity *(Allan et al., 2006; Worldwatch Institute, 2006).*

2.3.2. Ethiopia's status of bio-ethanol production

Ethanol production in Ethiopia is linked with sugar factories. The total identified irrigable land for sugar cane plantation in the country is about 700, 000 hectares, estimated at a potential to produce one billion liters of ethanol *(Yacob, 2013).* At present, the main supply line in the domestic market is dominated by two sugar factories (Fincha and Metehara) with the combination of their annual production capacity at around 11.1 million liters.

In order to transform this potential into reality, the government developed a strategic plan in 2007 considering jatropha as a principal feedstock for biodiesel production and sugarcane as a principal feedstock for bio-ethanol production. Among other things the strategy focused on establishing bio-fuel program, encouraging feedstock development, motivating customer demand, improving environmental sustainability, awareness conception and promotion of bio-

fuels, and renewing energy policy to incorporate bio-energy in detail. As a continuation of this endeavor, there have been repeated efforts to initiate using bio-ethanol for domestic use and particularly, blending 5% of ethanol with gasoline in the year 2008 followed by 10% in the year 2011 and to increase the percentage in the years to come was the plan set out. With these of course the plan includes expansion of sugar factories and building new ones though delayed during implementation *(Sn, 2008).*

Table 2.2 Ethiopia ethanol production in liters

Year		Ethanol produced (liters)		
		Fincha sugar factory	Metehara sugar factory	Total
1991	1998/99	1,907,000		1,907,000
1992	1999/00	720,000		720,000
1993	2000/01	1,790,571		1,790,571
1994	2001/02	209,444		209,444
1995	2002/03	894,624		894,624
1996	2003/04	911,431		911,431
1997	2004/05	1,636,047		1,636,047
1998	2005/06	6,847,816		6,847,816
1999	2006/07	6,066,860		6,066,860

2000	2007/08	5,330,337		5,330,337
2001	2008/09	5,878,516		5,878,516
2002	2009/10	7,116,585		7,116,585
2003	2010/11	7,127,895	6,373,775	13,501,670
2004	2011/12	6,794,000	7,658,000	14,452,000
2005	2012/13	7,620,500.00	7,063,000.00	14,683,500.00
2006	2013/14	11,678,000.00	7,767,000.00	19,445,000.00
2007	2014/15	10,999,000.00	8,806,000.00	19,805,000.00

Source: (Ethiopia Sugar Corporation, 2013)

2.4. Feedstocks for bio-ethanol production

There is a growing interest worldwide to find out new and cheap carbohydrate sources for production of bio-ethanol *(Mohanty et al., 2009).* For a given production line, the comparison of the feedstocks includes the following several issues: chemical composition of the biomass, cultivation practices, availability of land and land use practices, use of resources, energy balance, emission of greenhouse gases, acidifying gases and ozone depletion gases, absorption of minerals to water and soil, injection of pesticides, soil erosion, contribution to biodiversity and landscape value losses, farm-gate price of the biomass , logistic cost (transport and storage of the biomass , direct economic value of the feedstocks taking into account the co-products, creation or maintenance of employment , water requirements and water availability).

Bioethanol feedstocks can be divided into three main types: sugars, starches and cellulose materials *(Kudirat, 2012).*

2.4.1. Sugars

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethanol and other byproducts. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose are the easiest to convert to ethanol. However, since sugar materials are in the human food chain, these materials are usually too expensive to use for ethanol production. One example of a sugar feedstock is sugarcane. Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*Saccharomyces cerevisiae* also known as Baker's yeast, since it is commonly used in the baking industry) is frequently used to ferment glucose to ethanol. Theoretically, 100 grams of glucose will produce 51.4 g of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100%. Other biomass feedstocks rich in sugars include sugar beet, sweet sorghum and various fruits. However, these materials are all in the human food chain and, except for some processing residues are generally too expensive to use for fuel ethanol production *(Badger, 2002).*

2.4.2. Starchy materials

Another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials can also be fermented after breaking starch into simple glucose molecules. Examples of starchy materials commonly used around the world for ethanol production include cereal grains, potato, sweet potato and cassava. Cereal grains commonly used in the US for ethanol production include maize and wheat. Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Typically, hydrolysis is performed by mixing the starch with water to form slurry which is then stirred and heated to rupture the cell walls. Specific enzymes that will break the chemical bonds are added at various times during the heating cycle *(Chaves & Borges, 2016).*

2.4.3. Lignocellulosic biomass

Agricultural residues are a great source of lignocellulosic biomass, which is renewable, chiefly unexploited and inexpensive. Such resources include: leaves, stems, and stalks from sources like corn cobs, corn Stover, sugarcane bagasse, rice hulls, woody crops, and forest residues. Also, other multiple sources of lignocellulosic waste from industrial and agricultural processes include citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste, and paper mill sludge. In addition, dedicated energy crops for biofuels could include perennial grasses such as switchgrass and other forage feedstocks such as Miscanthus giganteus, Bermuda grass, elephant grass, poplar etc *(Kudirat, 2012).*

Cellulose materials represent the most abundant global source of biomass and have been largely unutilized. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about 200×10^9 tons per year, where about 8 to 20×10^9tons of the primary biomass remains potentially accessible *(Kudirat, 2012).* Cellulose is not used for food and the bio-fuels industries that use lignocellulosic materials do not compete for raw materials.

Cellulosic biomass such as switch grass and agricultural wastes are cheaper to produce and requires fewer inputs in form of energy. Cultivation of such plants improves soil fertility and is accompanied by less soil erosion. Moreover, the process also represents a means of effective and efficient waste management as a large proportion of agricultural and municipal wastes are lignocellulosic. Another benefit of cellulosic ethanol is the reduction in greenhouse gas emission. Compared to gasoline, ethanol burns cleaner, with greater efficiency, thereby releasing up to 85% less carbon dioxide. Lignocelluloses are complex substrates composed of a mixture of carbohydrate polymers, i.e. cellulose and hemicelluloses tightly bound to lignin, a complex aromatic polymer, mainly by hydrogen bonds but also by some covalent bonds. Lignin interferes with cellulose hydrolysis because it acts as a physical barrier that prevents the contact of cellulase to cellulose. The first step in bio-fuels production from lignocelluloses therefore is delignification to liberate cellulose and hemicelluloses from their complex with lignin. It is a very crucial, rate limiting and difficult task *(Kudirat, 2012).*

Generally the various forms of lignocellulosic feedstocks can be grouped into six main categories (Table 2.3) below:

Table 2.3 Lignocellulosic biomass categories

Biomass Category	**Common Examples**
Industrial cellulosic waste	Saw mail and Paper mill waste, Furniture Industry discards
Municipal solid waste	Newsprint and office waste paper
Agricultural residues	Wheat straw, Corn Stover, Rice hulls, avocado seed waste,Sugarcane bagasse
Dedicated herbaceous	Biomass Alfalfa hays, Switch grass, Bermuda grass, Reed canary grass, Timothy grass
Hardwoods	Aspen, Poplar
Softwoods	Pine, Spruce

Source: (Sun and Cheng, 2002; Lin and Tanaka, 2006)

Cellulose from wood, agricultural residues, etc. must be converted to sugars, either by acid or alkali hydrolysis, or by action of cellulase enzymes and the sugar can be fermented to ethanol.

The chemical compositions of lignocellulosic biomass (cellulose, hemicellulose and lignin content) of various feedstocks for bio-ethanol production (see Table2.4 below) show that, on average, agricultural wastes with the high cellulose content are corn cob, softwood stems, cotton seed hairs and saw dust. Forest residues comprise about 80% of the world's biomass *(Demirbas, 2005)* and in the United States alone 33.5-44.6 million metric tons of corn cob are available for harvest each year *(Zych, 2008).*

2.5. Compositions of lignocellulosic materials

Lignocellulosic plant biomass is an important renewable carbon resource for the bio refinery industry and is thus considered a sustainable and environment friendly alternative to the current petroleum platform *(Wongwilaiwalina et al., 2010).* Lignocellulosic biomass such as agricultural residues and herbaceous energy crops, consists mainly of three different types of polymers, i.e. cellulose, hemicellulose, lignin and pectins *(Ayeni et al,. 2015)* (table 2.4).Theses polymers need to be hydrolyzed by means of either enzymes or acids to fermentable sugars.

Table 2.4 polymer composition of lignocellulosic biomass

Polymers	Content lignocelluloses (%)	Major monomers
Cellulose	33-51	Glucose
Hemicellulose	19-34	Xylose, Glucose, Mannose, Arabinose, Rhamnose, Galactose
Lignin	20-30	Aromatic alcohols
Pectins (when present)	2-20	Galacturonic acid and Rhamnose

Source: (van Maris et al., 2006)

No matter what plant it comes from, lignocellulosic biomass is composed of a complex mixture of cellulose, hemicellulose and lignin. The composition of these constituents may vary from one plant species to another.

2.5.1. Cellulose

Cellulose was first described by Anselme Payen in 1938 as a resistant fibrous solid that remains behind after treatment of plant tissues with acid and ammonia *(Brown, 2007).* Cross and Bevan in early 1900s designated plant materials which do not dissolve in concentrated sodium hydroxide solution as alpha cellulose, meaning true cellulose; and the soluble materials were designated beta and gamma cellulose. The beta and gamma cellulose were later shown to be simple sugars and other carbohydrates, but not cellulose.

Cellulose is a complex carbohydrate consisting of 1000 – 3000 or more glucose units in a linear chain structure that can pack into the fibers of great tensile strength. Cellulose is the most abundant renewable bio-resource produced in the biosphere. It is a major component of the cell wall of plants; about 25 – 30 percent of mosses and seaweeds and 40 – 50 percent of the trees. Cotton fibers consist of about 90 – 98 percent of cellulose. It has a high degree of polymerization (DP) from 100-20,000 which is water insoluble and recalcitrant to hydrolysis into its individual glucose subunit because of tightly packed, highly crystalline structure with straight, stable supra-molecular fibers of great tensile strength and low accessibility in its polymer form *(Damon et al., 2005).*

About 33% of all plant matter is composed of cellulose. Cellulose does not melt with temperature, but its decomposition starts at 135^0C. There are several types of cellulose in wood, crystalline and non-crystalline and accessible and non-accessible. Most wood-derived cellulose is highly crystalline and may contain as much as 65% crystalline regions. The remaining portion has a lower packing density and is referred to as amorphous cellulose. Accessible and non accessible refer to the availability of the cellulose to water, microorganisms, etc. The surfaces of crystalline cellulose, are accessible, but the rest of the crystalline cellulose is non-accessible, whereas most of the non-crystalline cellulose is accessible, but part of the non-crystalline cellulose is so covered with both hemicelluloses and lignin that it becomes non-accessible *(Rowell et al., 2005; Kuhad et al., 2011a).*

2.5.2. Hemicellulose

Hemicellulose is a heterogeneous polymer, which varies in composition from plant to plant and within different parts of the same plant. It is a mixture of polymers comprising pentoses, hexoses hexuronic acids and deoxy-hexoses. Hemicelluloses differ from celluloses in the composition of various sugar units and by much shorter and branched molecular chains. In contrast to cellulose, which is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. Therefore, it is easily hydrolyzed by dilute acid or base, as well as hemicellulase enzymes *(Talebnia, 2008).*Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders it soluble at elevated temperatures. The presence of acid highly improves the solubility of hemicellulose in water.

2.5.3. Lignin

Lignin is the third component of lignocellulosic biomass, its main function is to surround and protect the cellulose and hemicellulose from degradation of microorganisms in plants. It constitutes around 20-30 percent of plant and wood biomass. It is a complicated structure of various compounds. The alcohols p-coumaryl-, coniferyl- and sinapyl-alcohol are the precursors of the most common building blocks in lignin. Softwood lignins are predominantly polymers of coniferyl alcohol. Hardwood lignins are made from coniferyl and sinapyl alcohol and lignin in grass contain all the three. It is one of the most complicated natural polymers with respect to its structure and heterogeneity, which make it extremely resistant to chemical and biological degradation *(Talebnia, 2008).*

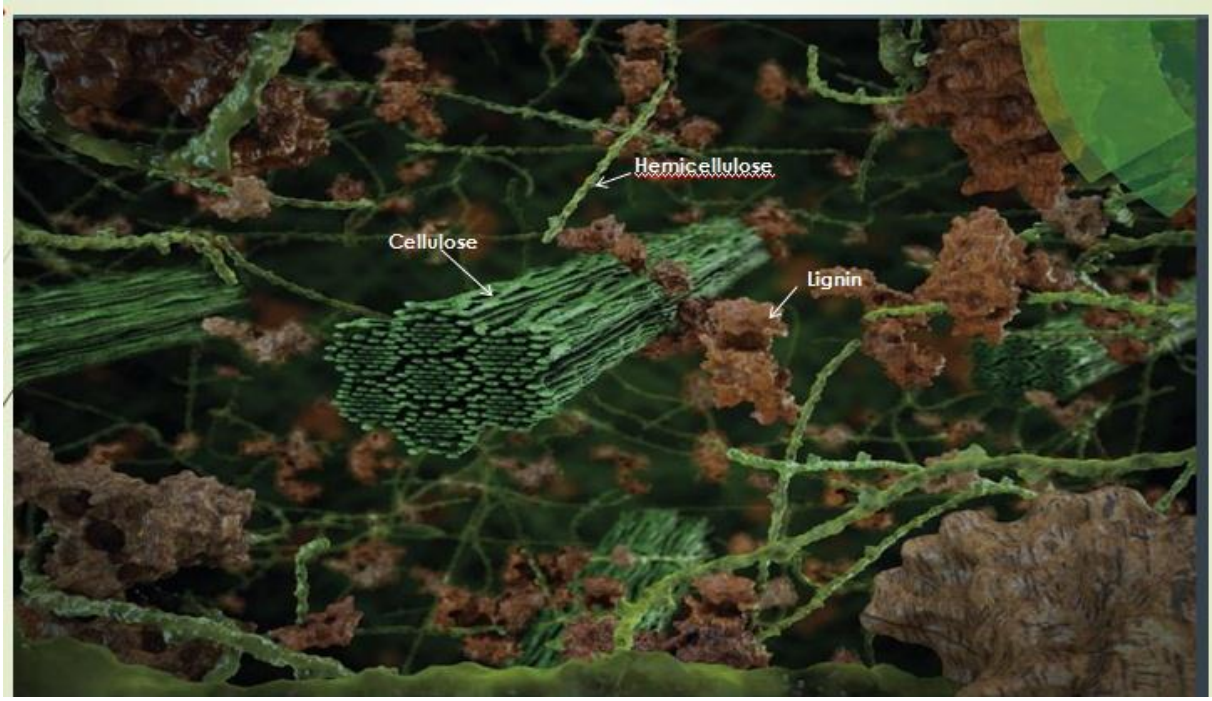


Figure 2.2 Ultra-structural view of lignocellulosic

2.6. Avocado seed as a feedstock of ethanol

Avocado seeds are abundantly found and are wastes, therefore if utilized for ethanol, making as feedstock, will be of great importance. Utilization of seeds will greatly reduce space and money for waste disposal and management.

Avocado seeds have a devastating effect, especially when they pile up, because they act like environmental eye sore. Making ethanol from them will solve a multifaceted problem environmentally and scientifically.

Avocado seed is often being viewed as one of the most disposable parts of an avocado. The seeds themselves can be pressed for oil, but even then the husks are thrown away. If you have a million pounds of cull fruit, with about 25% seed, you would have 250,000 pounds of seed which would be a large product to be thrown away as of no value *(Mahawan, Tenorio, & Gomez, 2015)*. The first thing to do in determining the possible uses of seed is to ascertain its composition. The avocado seed contains the following different compositions *(Nairobi et al., 2006).*

Table 2.5 Compositions of avocado seed

Component	Wet basis (%)	Dry basis (%)
Ash	1.3	2.7
Protein	2.5	5
Reduced sugars	1.6	3.2
Common sugar	0.6	1.2
Starch	**29.6**	**60**
Pentosans	1.6	3.3
Arabinose	2	4.1
Ether extract	1	2
Fiber	3.7	7.2
Water	50.4	

Source: California Avocado association 1934, 132-134

All the sugars present in the seed i.e. common sugar, reducing, pentosans and arabiose are dissolved in the water and eliminated. Soluble proteins are removed with water while the rest is removed by centrifugation through density difference with water and starch. As a renewable raw material, the avocado seed is a potential feedstock for the production of bio-ethanol, biogas and biodiesel to fulfill the increasing demand for bio-fuels *(Weatherby, 1934).*Before its use as a substrate for fermentation processes, the raw material has to be pretreated. Pretreatment is one of the many steps in the cellulose-to-ethanol process, but represents a currently critical step for hydrolysis. An effective pretreatment is performed at conditions that avoid degradation of pentose from hemicellulose, or glucose from cellulose, and limit formation of degradation products that inhibit the growth of fermentative microorganisms.

The lignocellulosic structure is destroyed by treatment with high temperature and saturated steam in a reactor followed by a sudden pressure decrease *(Limayem & Ricke, 2012).* Avocado seed is a lignocellulosic material composed of cellulose, hemicellulose and lignin. These polymeric fibers consist of monomeric molecules. Cellulose is built of C_6 sugars; hemicellulose mainly of the C_5 sugars xylose and arabinose. Lignin consists of phenolic macromolecules. According to study of (Feurete, 2007) was reported that the chemical compositions of avocado seed contains 51.34 % cellulose, 5.75% hemicellulose and 28.5% lignin *(Mahawan et al., 2015).*

2.7. Overview of bio-ethanol process

Ethanol can be produced in two different ways. Either chemically, by hydration of ethylene, which is derived from crude oil or natural gas, or by fermentation of sugar containing feeds, starchy feed materials or lignocellulosic materials. About 5% - 10% of the ethanol produced in the world is a petroleum product. The Petroleum ethanol product is made by the catalytic hydration of ethylene with sulfuric acid as the catalyst. It can also be obtained via ethylene or acetylene, from calcium carbide, coal, oil gas, and other sources. The two primary ways of producing fuel ethanol from cellulosic feedstock are: Biochemical conversion process and thermo chemical conversion process *(Otulugbu, 2012)* (as cited in Wodale, 2012).

The technology of ethanol production from biomass feedstocks consists of several steps, and varies depending on the type of raw materials used. It becomes more sophisticated as the raw

materials turn from sugars to starches and cellulosic materials. Unlike starch, the specific structure of cellulose favors the ordering of the polymer chains into tightly packed, highly crystalline structures those are water-insoluble and resistant to depolymerization. For production of ethanol from cellulosic feedstocks, four major steps are required: pretreatment, hydrolysis, fermentation and distillation of the product mixture to separate ethanol *(Braide et al., 2016).*

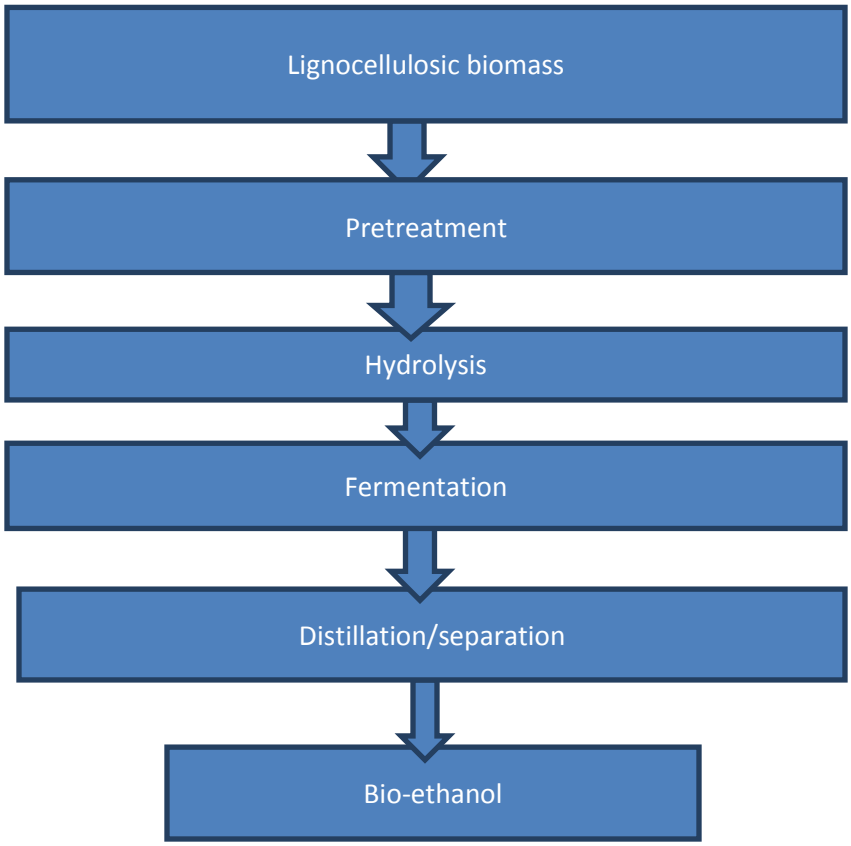


Figure 2.3 General outlines of the lignocelluloses to bio-ethanol production process

2.7.1. Pretreatment of biomass to ethanol

Pretreatment is a vital step in production of ethanol from lignocellulosic materials. It required in order to change the structures of cellulosic materials and to make cellulose more accessible to the enzymes or dilute acids, which convert the carbohydrate polymers in to fermentable sugars *(Ververis, 2007).*

It is technically challenging and forms a large part of the process cost and therefore will need to be optimized prior to commercialization. Due to the different types of carbohydrates contained in biomass, a package of enzymes/microbes will be required for hydrolysis and fermentation; this package is a significant process cost and requires optimization *(Tekaligne et al., 2015).*The aim of the pretreatment step is to destroy lignin shell protecting cellulose and hemicellulose within the plant materials.

Pretreatment of lignocellulose is required to alter its complex structure, thereby increasing its surface area which facilitates rapid and efficient hydrolysis of the polymer to fermentable sugars (Pretreatment, Lignocellulose, Of, In, & To, 2010).It is normally applied to separate the mixed polymers of lignin, hemicellulose and cellulose to provide the sugars needed for the hydrolysis and the fermentation processes *("Potential for Bioethanol Production from Wet Coffee Pulp Waste in," 2014).*

A successful pretreatment must meet the following requirements: (i) Improve the formation of sugars or the ability to subsequently form sugars by acidic or enzymatic hydrolysis; (ii)avoid the degradation or loss of carbohydrate; (iii) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes and (iv) be cost effective. There is not a general agreement on which pretreatment can be considered as the best one *(Pretreatment et al., 2010).*The categories of available pretreatment options are: physical, physico-chemical, chemical and biological pretreatment. Since different lignocellulosic materials have different physico-chemical characteristics, it is necessary to adopt suitable pretreatments technologies based on the lignocellulosic biomass properties of each raw material*(Fungi and bacteria) (Badger, 2002).*

a) Physical pretreatment

The purpose of physical pretreatment is to reduce biomass particle size mechanically, cellulose crystallinity and degree of polymerization as well as increasing specific surface area.

Physical approaches on biomass to ethanol involves mechanical size reduction, internal delamination and surface fibrillation such as chipping, grinding, milling, extrusion, refining, pyrolysis etc *(Karp et al., 2013).*

Generally, chemicals are not required when doing physical pretreatment. However, most methods are high energy demanding with little lignin removal. Hence, physical pretreatment like refining is combined with chemical pretreatments (usually as a post-treatment) to lower the overall energy and chemical demand, while still achieving desirable conversions without too much sugar degradation *(Aysu & Durak, 2015).*

b) Chemical pretreatment

Acid pretreatment: Inorganic acids such as H_2SO_4, HCl and HNO_3 and organic acids such as fumaric and maleic acid are used to treat lignocellulosic materials. Early studies show that concentrated acid (30-70 percent) at low temperature (e.g. 40°C) is effective in terms of breaking down lignocellulosic structure and achieving high sugar conversion *(Limayem & Ricke, 2012).*However, interest has been reduced in this process due to corrosive conditions, high cost on non metallic or alloy equipment and the challenge of acid recovery.

Dilute acid pretreatment using H_2SO_4 appears to be more favorable for practical application and have been studied for pretreating a wide range of lignocellulosic biomass materials. This is typically conducted using low acid concentration and can be performed at lower temperatures (less than 160°C) for longer periods of time (30-60 min); or at higher temperature (e.g. 160-240°C) for shorter periods of time (e.g. 10 min)*(Otulugbu, 2012).* The mechanism behind this process is the solubilization of hemicellulose, and also converts solubilized hemicellulose to fermentable sugars. It is not effective in lignin removal, but can disrupt lignin to a certain extent to increase enzyme accessibility to cellulose *(Harmsen & Huijgen, 2010).*

High hydrolysis yields have been reported when pretreating lignocellulosic biomass with diluted H_2SO_4. However, several draw-backs are present in the dilute acid pretreatment method. First,

the pH of the process stream following pretreatment must be neutralized to the optimum range of enzymes for hydrolysis and further adjustments are necessary for fermentation. This process also suffers from low yields because of sugar decomposition. Sugar degradation in a single stage dilute acid pretreatment process tend to form furfural, HMF, carboxylic acids, furans and phenolic compounds, which are potentially inhibitory to fermentation. In order to reduce sugar degradation, studies have been done using two or more stages of dilute acid hydrolysis with mild conditions in the first stage and more severe conditions in the subsequent stage(s) *(onuki et al., 2010).*

Alkaline pretreatment: Alkali pretreatment refers to the utilization of base chemicals in the pretreatment process. A wide range of alkaline chemicals have been studied for pretreatment use such as: NaOH, Ca $(OH)_2$, Na_2CO_3, $NaHCO_3$, ammonia and furthermore, SO_2 in alkaline conditions have also been applied *(Danmaliki, Muhammad, Shamsuddeen, & Usman, 2016).*
The mechanism behind the alkali pretreatment process is to swell lignocellulosic materials, causing disruption in lignin structures and linkages between lignin and carbohydrates. An increase of internal surface area and decrease in cellulose crystallinity and degree of polymerization was observed. Alkali pretreatment has been shown to cause less sugar degradation than acid pretreatment and it was reported to be more effective on non-woody (agricultural residues) than on woody biomass *(Tekaligne et al., 2015).*
There is also a consensus that dilute alkaline pretreatment is more effective on hardwood than softwood *(Sun & Cheng, 2002; Wu et al., 2010).*

To achieve higher lignin removal, alkali pretreatment can be combined with ammonia, O2 or H_2O_2 at temperature of 100-220°C *(Onuki et al., 2008).* High sugar yields in enzymatic hydrolysis with effective lignin removal (40-85%) has been reported in these alkali based wet oxidation techniques *(Harmsen & Huijgen, 2010).*

Ozonolysis: Ozone is used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw, and poplar sawdust. The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. Ozonolysis pretreatment has the following advantages: it effectively removes lignin; it does not produce toxic residues for the downstream processes; and

the reactions are carried out at room temperature and pressure. However, a large amount of ozone is required, making the process expensive *(Onuki et al., 2008).*

Oxidative delignification: Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2. The pretreatment of cellulosic biomass with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% lignin and most hemicellulose were solubilized by 2% H_2O_2at 30 °C within 8 h, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45 °C for 24 h *(Eriksson, 2013).*

Organosolv process: In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, and triethylene glycol and tetrahydrofurfuryl alcohol. Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process. At high temperatures, the addition of catalyst was unnecessary for satisfactory delignification. Usually, a high yield of xylose can be obtained with the addition of acid. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system is necessary because the solvents may be inhibitory to the growth of organisms, hydrolysis, and fermentation*(Hossain & Fazliny, 2010).*

c) Physico-Chemical Pretreatment

Steam explosion (auto hydrolysis): Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials. In this method, chipped biomass is treated with high-pressure saturated steam and then pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 90 - 160 ºC for several minutes to a few hours the material is exposed to atmospheric pressure.

The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis and steam treatment with dilute acid of 0.5 to

1%, temperature of 90 to 140^{o}C and residence time of 10 to 40 minutes gives highest yield of ethanol *(Produkcji & Na, 2010).*

The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The environmental mechanical methods require 70 % more energy than steam explosion to achieve the same size reduction. Steam explosion is recognized as one of the most cost effective pretreatment process for hardwoods and agricultural residues, but it is less effective for softwoods. Limitations of steam explosion include destruction of a portion xylan fraction, incomplete disruption of the lignin-carbohydrate matrix and generation of compounds that may be inhibitory to microorganisms used in downstream processes. Because of the formation of degradation products that are inhibitory to microbial growth, enzyme hydrolysis and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water soluble hemicelluloses *(Parameters, Kernel, Application, Advisor, & Yimam, 2015).*

Ammonia fiber explosion (AFEX): AFEX is physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time and then pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1-2 kg ammonia /kg dry biomass, temperature 90ºC, and residence time 30 minutes. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials, including alfalfa, wheat straw, wheat chaff, barley straw, corn Stover, rice straw municipal solid waste, softwood newspaper, coastal Bermuda grass, switch grass, aspen chips and bagasses. The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment and acid-catalyzed steam explosion. The composition of materials after AFEX pretreatment was essentially the same as the original materials. Over 90 % hydrolysis of cellulose and hemicellulose has been obtained after AFEX pretreatment of Bermuda grass and bagasses. To reduce the cost and protect to the environment, ammonia must be recycled after the pretreatment. The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash is not necessary. AFEX pretreatment does not require small particle size for better efficiency *(Hong, 2013).*

CO_2 explosion: Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for the pretreatment of ligocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. Dale and Moreira (1982) used this method to pretreat alfalfa (4 kg CO_2/ kg fiber at the pressure of 5.62 MPa) and obtained 75 % of the theoretical glucose released during 24 h of enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper and found that CO_2explosion was more cost effective than ammonia explosion and did not cause the formation of inhibitory compounds and could occur in steam explosion*(Amanda et al., 2015).*

d) Biological Pretreatment

Among all the methods, fungal pretreatment provides the mildest environmental condition with low capital and energy cost, no chemical requirements and unit operation simplicity. Microorganisms such as brown, white and soft-rot fungi have been used. Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin *(Sun & Cheng, 2002).* Lignin degradation by white-rot fungi is the most effective for biological pretreatment of lignocellulosic materials. It degrades lignin and hemicellulose with major cellulose preservation *(Shi et al., 2008*). The major drawbacks for moving this biological pretreatment forward is the low hydrolysis rate in the processes *(Sun & Cheng, 2002).* Thus, biological study of more basidiomycetes fungi or even genetic modification can enable more efficient fungi delignification.The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low *(Nirbhay Gupta, 2008).*

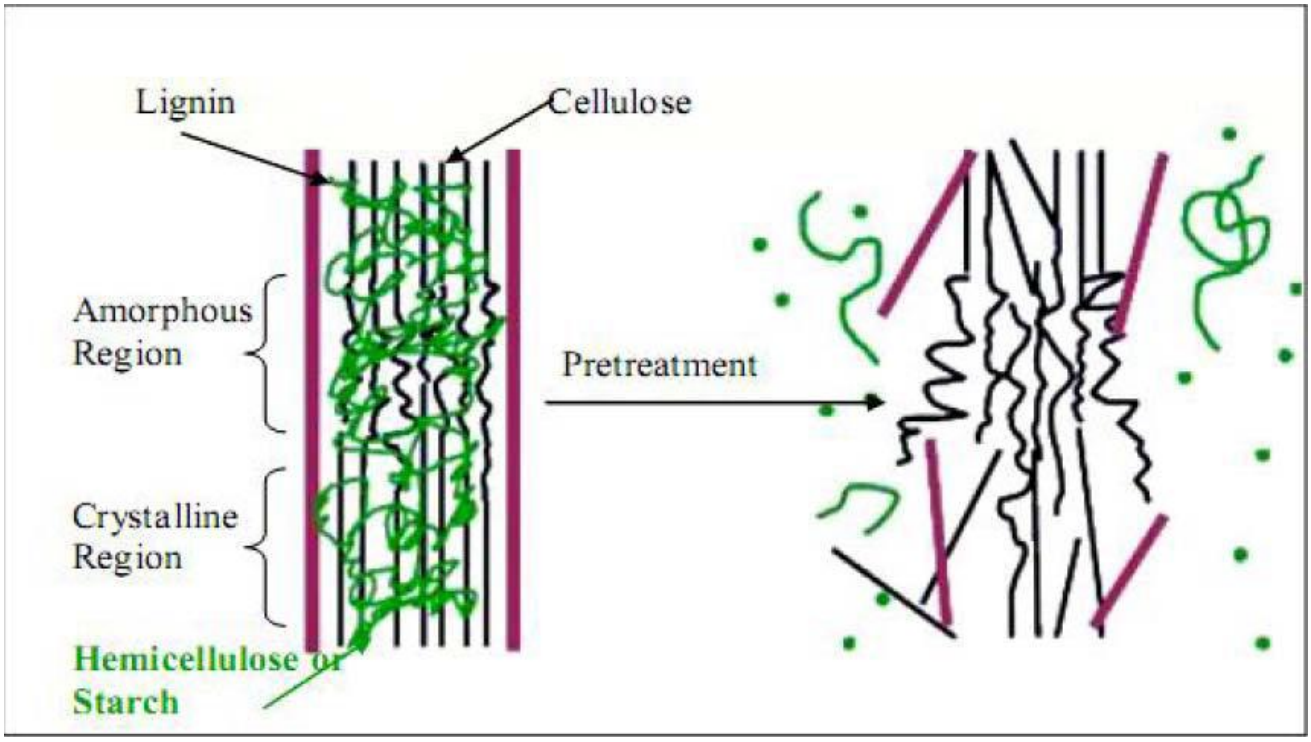


Figure 2.4 Schematic representation on biomass pretreatment (Mosier et al, 2004)

Table 2.6 Summary of various processes used for the pretreatment of lignocellulosic materials (Adapted from Parveen Kumar et al., 2009)

Pretreatment process	Advantage	Disadvantage
Mechanical comminution	Reduce cellulose crystallinity	Consumes higher power than inherent biomass energy
Acid pretreatment	Hydrolyslzes hemicelluloses to xylose and alter lignin structures	High cost, equipment corrosion and formation of toxic substances
Alkaline pretreatment	Remove hemicelluloses and lignin; increase accessible surface area	Long residence times required and irrecoverable salts formed
Ozonolysis	Reduce lignin content; does not produce toxic residues	Large amount of ozone required; makes the process expensive
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to drained from reactor, evaporated, condensed and recycled; high cost
Steam explosion	Cause hemicellulose degradation and lignin transformation; cost effective	Destruction of a portion of xylan fraction; incomplete disruption of the lignin carbohydrate matrix; generation of inhibitory compounds such as furfural
AFEX	Increase accessible surface area, remove hemicelluloses and lignin to an extent; doesn't produce inhibitors for downstream process	Not efficient for biomass with high lignin content
Biological	Degrades hemicelluloses and lignin; low energy requirements	Rate of hydrolysis is very low

2.7.2. Hydrolysis of lignocellulosic materials

After pretreatment of lignocellulosic biomass, the cellulose is prepared for hydrolysis, which means, that a molecule is cleaved by addition of a water molecule*(Reddy et al,. 2011).* After the pretreatment process, there are two types of processes to hydrolyze the feedstocks for fermentation into ethanol, most commonly used are acid (dilute and concentrated) and enzymatic hydrolysis of cellulosic materials includes the processing steps that convert the carbohydrate polymers e.g. cellulose and hemicellulose into monomeric sugars. Cleavage of these polymers can be catalyzed enzymatically by cellulases or chemically by acids such as sulfuric acid*(Woldu & Tsigie, 2015).*

The cellulose molecules are composed of long chains of glucose molecules. In the hydrolysis process, these chains are broken down to free the sugar, before it is fermented for alcohol production. There are two major hydrolysis processes: a chemical reaction using acids, or an enzymatic reaction using enzymes.

i) Acid hydrolysis

Acid hydrolysis is a possible process for treating lignocellulosic materials such as avocado seed waste , wood chips ((Journal, 2011), rice straw *(Almeida 1991),* sugar beet pulp and wheat straw. According to Parisi (1989), the mineral acids act simply and rapidly as reaction catalyzers of polysaccharide fractions. Sugarcane bagasse can be hydrolyzed using dilute acid to obtain a mixture of sugars with xylose as the major component. However, in the hydrolyzate some byproducts generated in the hydrolysis, such as acetic acid, furfural, phenolic compounds, or lignin degradation products, can be present. These are potential inhibitors of a microbiological utilization of this hydrolyzate.

Processes such as two-stage acid hydrolysis can be employed to produce xylose and glucose *(Beck, 1986).* Treatment with dilute sulphuric acid at moderate temperatures (the first stage of acid hydrolysis) has proven to be an efficient means of producing xylose from hemicellulose.

In general, acid treatment is effective in solubilizing the hemicellulosic component of biomass. Proper combinations of pH, temperature, and reaction time can result in high yields of sugars, primarily xylose from hemicellulose *(Kam, Hii, Sim, Gaik, & Ong, 2016).*

ii) **Enzymatic hydrolysis of cellulose**

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes, which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose.

Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50°C) and does not have a corrosion problem. Both bacteria and fungi can produce cellulase for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora, and Streptomycescan produce cellulases. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as Clostridium thermocellumand and Bacteroides cellulosolvens produce cellulases with high specific activity, they do not produce high enzyme titres. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi.Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase which hydrolyzes cellobiose to produce glucose. During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented.

2.7.3. Fermentation

The fermenting of the biomass is conducted under standard fermenting conditions and will utilize all the major biomass. Yeast is the most commonly used microorganism in fermentation processes. Yeasts are minute, often unicellular fungi. The yeasts used are typically brewer's yeasts. Examples of yeast capable of fermenting the decaying biomass include, but are not limited to, *Saccharomyces cerevisiae* and *Saccharomyces uvarum*. Non-Sacharomyces yeasts, also known as non-conventional yeasts, are also used to make a number of commercial products. Some examples of non-conventional yeasts include *Kuyberomyces lactis, Yarrowia lipolytica, Hansenula polymorpha and Pichia pastoris (Nichols et al., 2008).*

Microorganisms other than yeast can also be useful in making fermentation products. For example, cellulosic ethanol production also utilizes fungi and bacteria. Examples of these cellulolytic fungi include *Trichoderma reesei* and *Trichoderma viride*. One example of a bacteria used in cellulosic ethanol production is *Clostridium Ijungdahlii*. Mid- to long-term technology under development are expected to improve the fermentation efficiency of the organism, producing higher yields in less time, and an organism requiring less detoxification of the hydrolyzate *(Cellulosic Ethanol, 2010).*

$$3C_5H_{10}O_5 \xrightarrow{Yeast} 5C_2H_5OH \quad + 5CO_2 \Delta H = \textit{-74.986 Kj/mol}$$

$$3H_5H_{12}O_6 \xrightarrow{Yeast} 2C_2H_5OH + 2CO_2 \Delta H = \textit{-84.394Kj/mol}$$

2.7.4. Distillation

Distillation is one of the steps of the purifications. Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. This is because all materials have intermolecular interactions with each other, and two materials will co-distill during distillation. *(Onuki, 2005* as cited in Wondale, 2012).

Whatever method of preparation is used, the ethanol is initially obtained in a mixture with water. The ethanol is then extracted from this solution by fractional distillation. Although the boiling point of ethanol, 78.3℃, is significantly lower than the boiling point of water, 100℃, these materials cannot be separated completely by distillation. Instead, an azeotrope mixture (i.e. a mixture of 95% ethanol and 5% water) is obtained, and the boiling point of the azeotrope is 78.15℃. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill from the distillation flask, and this material is the azeotrope of 95% ethanol which has the lowest boiling point. If an efficient fractionating column is used, 95% alcohol could be obtained first and then a small intermediate fraction of lower concentration, and then water. But no matter how efficient the fractionating column used, 95% alcohol cannot be further concentrated by distillation because the vapor has exactly the same composition as the

liquid; towards distillation, then, 95% alcohol behaves exactly like a pure compound *(Jackman, 1987* as cited in Wondale, 2012).

2.7.5. Dehydration

After distillation, some amount 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% due to the formation of a low boiling water-ethanol azeotrope. For blending with gasoline, purity of 99.5 to 99.9% is required, depending on temperature, to avoid separation. Currently, the most widely used purification method is a physical absorption process using molecular sieves. Another method is azeotropic distillation *(Onuki, 2005* as cited in Wondale, 2012).

Molecular sieves: There is a lower bound on the fraction of ethanol entering the molecular sieve (0.8). Adsorption takes place at 95 °C. Heat exchanger heats the inlet stream from the mixer up to 95 °C. The molecular sieve is a bed of zeolite that operates in semi-continuous mode. The bed is saturated with water after a period of time and is then regenerated. Hence, there are usually two sieves being operated in parallel – one being saturated with water while the other is being regenerated (or dehydrated) using air under vacuum. Heat exchanger heats air with an assumed relative humidity of 70% at 20 °C to 95 °C. The air at the outlet of the dehydrating molecular sieve is cooled down to 25 °C in heat exchanger and this stream leaves this exchanger saturated with water at 25 °C (Mariano Martín and Ignacio E. Grossmann, Carnegie Mellon University).

3. MATERIAL AND METHODS

The experiments of production of ethanol from avocado seed were carried out in the laboratory of school of chemical and bio engineering at the Institute of Technology and Arat kilo college of natural and computational science department of chemistry, Addis Ababa University.

3.1. Materials use for the experiments

The materials used to run all experiments were listed below:

Equipments: Plastic bags to collect and transport samples to the laboratory, knife for cutting the avocado seed wastes in to pieces, oven loading (model 100 -800) to dry the sample, crushers to crush the dried sample, sieves (mesh size of 2.0 mm, Sortmks-3332, PFEUFFR, Germany) to sieve the crushed sample to the particle size of 2mm, Digital balance**s (**model-Sartorius with 0.01mg sensitivity, and model EP214C) to weigh samples, pH- Meter to measure the pH of the hydrolyzates before fermentation, vessels to hold samples and additives for hydrolysis, fermentation and distillation set up , centrifuge to separate the soluble liquid from non soluble part, graduated cylinders of different volumes for volume measurement, vertical autoclave for sterilization and hydrolysis, shaker incubator to shake sample and its additives after hydrolysis, Vacuum Filter (model-BN 3 STAATLICH, Berlin) and Fourier Transform Infrared spectroscopy (FTIR).

Chemicals: Sulfuric Acid (H_2SO_4, (98%, England)), used as a pretreatment and hydrolysis of avocado seed, sodium Hydroxide (NaOH, min. assay 98% BDH Chemicals Ltd pool England cellulose),) used to adjust the pH of soluble cellulose and hemicelluloses before fermentation, Benedict's solution used to determine reduced sugars, yeast extracts (Agar) ,urea, dextrose sugar, Mg SO_4.7 H_2, and yeast (*Saccharomyces cerevisiae*) used as media preparation (manufactured in france by S.I. Lesaffre with the strain"safinstant").

3.1.1. Characterization of avocado seed

3.1.1.1. Proximate analysis

The proximate analysis gives moisture content, volatile matter content, the fixed carbon content, the ash content (the inorganic residue remaining after combustion of the sample).

Determination of moisture content

2 g of the ground avocado seed sample was weighed out into the crucible, after the crucible has been heated and weighed. The moisture content was determined by oven drying at 105^{o}C until constant weight was obtained *(Pearson, 1980)* for an hour. This was removed and cooled in a desicator and then weighed using sensitive balance. This was repeated until constant weight was obtained. The percent moisture content was determined using the following formula.

$$\text{Moisture content (\%)} = \frac{W1-W2}{W1} * 100 \qquad (3.1)$$

Where; $W1$= weight of the sample before drying

$W2$ = weight of the sample after drying

Determination of ash content

A crucible was weighed empty, and then 3 g of ground avocado seed sample was put in it and placed in a temperature controlled furnace at 550^{o}C for about 2 hours for proper ashing. The crucible was removed from the furnace and placed in a desicator to cool, then was reweighed. The process was repeated until constant weight was obtained. The percent ash content was determined using the formula:

$$\text{Ash content (\%)} = \frac{W1-W2}{W1} * 100 \qquad (3.2)$$

Where; $W1$= original weight of the sample

$W2$ = weight of the sample after cooling

Volatile matter content

Measuring of moisture content is important know the combustibility of bio-ethanol produced from avocado seed waste as feedstock. A crucible was weighed empty, and then 1.5 g sample was put in it. The sample and the crucible were placed in a muffle furnace for 30 min at 600^{o}C *(Pearson, 1980)*. The crucible was removed from the furnace and placed in a desiccators to cool, then was reweighed. The process was repeated until constant weight was obtained. The percent volatile matter content was determined using the formula:

$$\text{Volatile content (\%)} = \frac{W1-W2}{W1} * 100 \quad (3.3)$$

Where; $W1$= original weight of the sample

$W2$ = weight of the sample after cooling

Fixed Carbon Content

This is the residue left after the moisture, volatile and ash is given up. It is deduced by subtracting from 100, the percentage of moisture, volatile matter and ash content. The fixed carbon content (FC) is given as:

FC = 100 – (%moisture + %volatile matter + % ash) (3.4)

3.1.1.2. Chemical composition

Determination of extractives: 3 g of oven dried at 60 ^{o}C for 72 hours of avocado seed powder was loaded in to the Soxhlet extraction tube by stabling inside the millimeter paper, 150 ml of acetone was used as solvent for extraction. Residence times for the boiling and rising stages was carefully adjusted to 70 ^{o}C and 25 min respectively on the heating mantle for a 4 h run period. After extraction, the sample was air dried at room temperature for a few minutes. The constant weight of the extracted material was achieved in a convection oven at 105^{o}C. The % (w/w) of the extractives content was evaluated as the difference in weight between the raw extractive and extractive free sample *(Blasi et al, 1999).*

Figure 3.1 Soxhlet extraction unit

The % (w/w) of the extractives content was evaluated using the formula:

$$\text{Extractive } (\%) = \frac{W1-W2}{W1} * 100 \qquad (3.5)$$

Where; $W1$= oven dry sample

$W2$ = extracted residue

Determination of hemicellulose: 1 g of extractive free sample was transferred into a 250 ml of flask. Add 150 ml of 0.5 mol/m3 NaOH was added. The mixture was boiled for 3.5 h with distilled water. It was filtered after cooling through vacuum filtration and washed until neutral pH. The residue was dried at 105 ^{o}C in a convection oven. The difference between the sample weights before and after this treatment is the hemicellulose content (%w/w).

$$\text{Hemicellulose (\%)} = \frac{W1-W2}{W1} * 100 \quad (3.6)$$

Where; $W1$= oven dry sample, $W2$ = extracted residue

Determination of lignin: 0.5 g extractive free sample was placed in a flask and 3 ml of 72% H_2SO_4 was added. The sample was kept at room temperature for 2 h with carefully shaking at 30 min intervals to allow for complete hydrolysis. After the initial hydrolysis, 84 ml of distilled water was added. The second step of hydrolysis was made to occur in an autoclave for 1 h at 121 °C. The slurry was then cooled at room temperature. Hydrolyzates were filtered by vaccum filtration. The acid insoluble lignin was determined by drying the residues at 105 ^{o}C and accounting for ash by incinerating the hydrolyzed samples at 575 ^{o}C in a muffle furnace. The acid soluble lignin fraction was determined by measuring the absorbance of the acid hydrolyzed samples at 320 nm. The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin *(Sluite et al, 2008).*

$$\text{Lignin (\%)} = \frac{W1-W2}{W1} * 100 \quad (3.7)$$

Where; $W1$= oven dry sample

$W2$ = extracted residue

Determination of Cellulose: The cellulose content (%w/w) was calculated by difference, assuming that extractives, hemicellulose, and lignin are the only components of the entire biomass *(Ayeni,2013).*

$$WC + WH + WE + WL = 100 \quad (3.8)$$

$$WC = 100 - WH - WE - WL$$

Where: WC, WH, WE, WL are cellulose content, hemicellulose, extractive and lignin content respectively.

3.2. Methods

3.2.1. Sample collection

The main aim of the research was ethanol production from avocado seed. First 3kg of raw material was collected from the juice houses processing found in the Addis Ababa city, Ethiopia. The seeds were washed with tap water and the outer covering of the seeds were manually removed while washing in order to remove the dirt particles from the cover, and cracked to obtain the kernel. Then, the kernel was cut using a knife into pieces of about 3-5 cm length to reduce the size of the sample and allowed to dry in an oven at 60° C for 72 hours to obtain easily crushable material.

The dried sample was ground with grinder machine to reduce the particle size to 2 mm and measures using sieves with a size of 2mm. The sample of larger particle size than 2 mm was ground over and over again until all particle size became 2 mm. Grinding of avocado seed in powder form increases the surface area of the sample which enhances the contact between hemicelluloses and cellulose with dilute acid to reduce cellulose crystallinity.

3.2.2. Acid pretreatment of avocado seed powder

According Zhang *et* al., 2011, dilute acid hydrolysis pretreatment for biofuel production apply from 0.05 to 2% H_2SO_4 (w/v) at between 120 and 220^{o}C) for 2 to 90 minutes (Thus, in this study dilute sulfuric 1.5% concentration and 90gram of avocado seed powder with a ratio of 1:10(w/v) sample to solution ratio was used and pretreated inside autoclave at a temperature of 125^{o}C for 60 minutes. After that it was cooled and filtered using filter vacuums. The residue was washed four times by distilled water to remove sulfuric acid from it till the pH becomes 6.78 which is on the recommended interval during pretreatment. And by putting the solid part in the oven at 60^{0}c temperature for 24 hrs then it becomes dry and kept for hydrolysis purpose.

The purpose of the pretreatment was to remove lignin, reduce cellulose crystallinity and increase the porosity of the materials. Pretreatment must meet the following requirements: improve the formation of sugar, avoid the degradation or loss of carbohydrate, avoid the formation of by-product inhibitors and must be cost effective

3.2.3. Dilute acid hydrolysis

Even though there are many types of hydrolysis types, dilute acid hydrolysis is an easy and productive process and the amount of alcohol produced in case of acid hydrolysis is more than that of alkaline hydrolysis. Research works on the dilute acid hydrolysis of different lignocellulosic materials have defined optimal process conditions: temperature 80-200^{o}C, sulfuric acid concentration 0.25–8 wt%, and reaction time 10-2000 min *(Gladyshko, 2011).*

In this study the acid hydrolysis procedure of the experiment was started by adding about 1.5% to 3.5% (v/v) dilute sulfuric to the non soluble component from pretreatment steps and the avocado seed was hydrolyzed in the reactor at three levels of temperature (125,135, and 145^{o}C), time of (30, 60 and 90 min). Next separate the solid particles from the liquid in the hydrolyzate by vacuum filtration (to remove the non fermentable lignin portion). After separating the solid part, keep the liquid part for further work (fermentation).

Figure 3.2 Hydrolysis in the autoclave

pH Adjustment : Before addition of any micro-organism to the above prepared sample, pH of the sample has to be adjusted. Otherwise the micro-organism will die in hyper acidic or basic state. A pH of around 5.0 -5.5 is maintained *(Cristina et al., 2017)* (as cited at Wondale, 2012). The hydrolyzed solution was primarily checked for pH using a digital pH meter. Since, the sample was more acidic media, and then it would maintain the pH (5.45) by adding sodium hydroxide solution by drop wise.

Sterilization: The reactor and all the equipments that were used for fermentation purposes were sterilized (autoclaved). The sterilization was carried out at a temperature of 121^{o}C for 15 minutes.

3.2.4. Measurement of reducing sugars

Benedict's solution method for determining of glucose concentration

The powdered avocado seed through hydrolysis at different acid concentration, hydrolysis time, and hydrolysis temperature on the amount of sugar produced was investigated. In this study, the total reduced sugar content through hydrolysis process was investigated by Benedict's solution method. Means I used Benedict's solution as measuring for the reducing sugar. If the sugar is present in the samples the Benedict's solution turn color (shades of yellow, orange brown). So in this case glucose concentration and its absorbance results were recorded.

Standard glucose solution preparation: This standard solution is important to determining the slop and intercept which are important in determining of the glucose concentration of the samples. I have prepared this standard by weigh accurately 0, 0.06, 0.08, 0.1 , 0.12, 0.14 grams of glucose and prepare 6 test tubs with 5ml of distill water for each test tubes and dissolve these glucose samples in 5ml distill water of test tubs for each one remains free since the glucose samples are 5 in numbers. Then shake the sample until the glucose is completely dissolve in the distil water. And prepare 6 other test tubs with 5ml of Benedict's solution for each. Then 1 ml of each of the standard solutions were pipetted out and taken into test tube which contains the Benedict's solution. The mixture was kept in water bath at a temperature of 90^{o}c for 5 minutes after rapid cooling, filtered and then the absorbance was recorded at 540 nm using UV-visible spectrophotometer and the results are presented (Table 4.3) below.

The amount of total glucose concentration present in the sample solution was calculated using the standard graph.

$$Y = mx + b \qquad (3.9)$$

Where; y = absorbance

x = glucose concentration

m = the slop and b is the intercept

3.2.5. Determination of glucose concentration from samples

The concentrations of unknown sugar samples were determined from a standard curve of glucose ($Y = 0.02751X - 0.04394$; $R^2 = 0.979$) (Figure 4.1) below. To determine the concentration of unknown samples we need the absorbance of the unknown samples then, first prepare 20 test tubs as the standard one each with 5ml of Benedict's solution. Then 1 ml of each of the 20 samples were pipetted out and taken into test tube which contains the Benedict's solution. The mixture was kept in water bath at a temperature of 90^{o}c for 5 minutes and filtered. Then the absorbance of each was read at 540 nm using UV-visible spectrophotometer and the results are presented (Table 4.4). After recorded the samples absorbance the glucose concentration and yield of unknown samples can be calculated using equation (3.10) and (3.11) in the below.

$$\text{Conc. of unknown sample (c)} = \frac{absorbance\ of\ \ unknown\ sample\ -\ y\ intercept}{slop} \tag{3.10}$$

$$\text{Yield of glucose} = \frac{gram\ of\ \ glucose\ produced}{raw\ material\ used} * 100 \tag{3.11}$$

$$Raw\ material\ used = \frac{gram\ of\ sample\ used\ during\ hydrolysis}{mililiter\ of\ solution} \tag{3.12}$$

3.2.6. Fermentation

The aim of the experiment was to measure the ethanol production by yeast (*Saccharomyces cerevisiae*) using avocado seed hydrolyzed as energy and carbon source. The clear solutions then go to fermentation. The fermentation was carried out under anaerobic condition by covering the flask which contains the sample by aluminum foil at a temperature of 30^{o}C, pH 5.45 with 200 rpm stirring conditions for 3 days. Before conducting fermentation I had prepared the media for the yeast. In order to prepare the media I should have the favorable condition for yeast growth or to supply the required amount of nutrients. Mix the following nutrients in the following proportions.

Media Preparation: For preparing 250 ml media, add 4 gm sugar (Dextrose), 0.5 gm yeast extract, 2.5 gm Urea, 2.5 gm $MgSO_4.7\ H_2O$ and 250 ml make up water. Then to the 250 ml media, 1.5ml pure yeast *Saccharomyces cerevisiae* which was obtained from Ethiopian bio diversity institute and added in a 350 ml conical flask. The conical flask was properly covered with aluminum foil. It was then placed in a shaking incubator for 24 hours, a temperature of 30^{o}C and 200rpm in order to grow the yeast.

Chamber cell counting method: I was prepared nine test tubes which contain all 9 ml of distilled water each and 1ml was pipetted out from the 350 ml growth media and taken into the first test tube then serial for the remaining test tubes and mix by vortex mixer. Then I prepared 9 agar plates to count the cells. Then pipetted out 1ml from each of the 9 test tube to these agar plates not serial but from the first test tube to the first agar plate and from the second test tube also to the second agar plate the same is true for the remaining then distributed the 1ml by loop over the agar plate. Then incubate for 3 days at 30^{o}c temperature for colony formation. I was taken 2.8g from nutrient agar and suspend this mass in 900ml of distilled water and this was autoclaved sterilized by autoclaving at 121^{o}c for 15 minutes. Then take 100ml from this to each of the nine agar plates then wait until it makes solid. Finally, the nine agar plates were placed inside the bio-incubator in order to grow.

Then counting the number of cells using cell chamber and found 400 cells in the fith (5^{th}) agar plate which has a number of cells 400. Then the total number of cells in the fermentation would be:

Total number of cells = number of cells * dilution * ml of prepared media

$$\text{Total number of cells in the fermentation} = 400 * 1 * 10^{5} / \text{ml} * 26\text{ml}$$

$$\text{Total number of cells in the fermentation} = 1.04 * 10^{9}$$

The Procedure for Fermentation: The 260 ml sample was sterilized to temperature of 121^{o}C for 15 minutes before fermentation step was started. Then the prepared media to sample were mixed in the 500ml flasks with the proportion of 1:10 (prepared media to sample ratio).Then, placed on shaking incubator at a temperature of 30^{o}C and at 200rpm for 72 hrs. And after 72 hours of fermentation, the samples were taken out and distilled.

Figure 3.3 (a) Fermentation setup (b) Sample ready for fermentation

3.2.7. Distillation

After fermentation separation of two liquids based on their boiling different boiling points was made using distillation setup at a temperature of 85^{o}C for 3 hours (*Fagal et al., 2010).* Consequently the sample ethanol and standard ethanol density was measured using density meter the results are shown below (table 3.1). Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. In this experiment separation was made by rotary evaporator at a temperature of 85 ^{o}C for 3hrs.

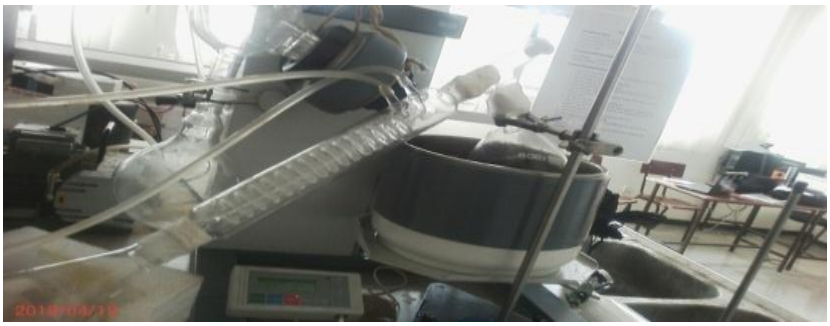


Figure 3.4 Distillations (Rotary Evaporator)

To calculate percentage of ethanol in the sample we need the following experimental designs.

Requirements:

- Chromic acid reagent (20gram of potassium chromate, H_2O and 300ml concentrated sulfuric acid) which is used for confirming test to proof that the distillate was actually ethanol.

- Ethanol sample
- Ethanol standard
- Distilled water

Procedure:

- ✓ Collect 1ml ethanol sample by distillation of fermented sample
- ✓ To 1ml of sample/solution, add 25ml of chromic acid reagent
- ✓ Place a tube in a water bath at 70^{o}c for 15 minutes
- ✓ Take out the tubes and immediately add 24ml of distilled water to it to stop the reaction
- ✓ Measure the absorbance at 600nm the recorded data is shown blow (table 3.1)

Percentage of ethanol in the sample (%) = $(\frac{Cs}{Cu})*(\frac{Au}{As})*100$ (3.13)

Where; Cs=concentration of standard ethanol

Cu = concentration of sample

Au = Absorbance of standard

As = Absorbance of sample

Table 3.1 Standard ethanol absorbance, sample ethanol absorbance, concentration of standard ethanol and sample ethanol

No	Standard EtOH absorbance@600nm	Sample EtOH absorbance@600nm	Conc.Standard ethanol(g/cm3)	Conc.sample EtOH (g/cm3)
1	0.95	0.125	1.59	0.93
2	0.95	0.130	1.59	0.92
3	0.95	0.145	1.59	0.91
4	0.95	0.21	1.59	0.95
5	0.95	0.19	1.59	0.90
6	0.95	0.20	1.59	0.74
7	0.95	0.14	1.59	0.69
8	0.95	0.17	1.59	0.89
9	0.95	0.19	1.59	0.87
10	0.95	0.24	1.59	0.96
11	0.95	0.25	1.59	0.85
12	0.95	0.15	1.59	0.79
13	0.95	0.13	1.59	0.69
14	0.95	0.18	1.59	0.87
15	0.95	0.143	1.59	0.93
16	0.95	0.23	1.59	0.94
17	0.95	0.22	1.59	0.79
18	0.95	0.27	1.59	0.69
19	0.95	0.19	1.59	0.75
20	0.95	0.3	1.59	0.87

4. RESULTS AND DISCUSSION

In this section the study discussed proximate analysis and chemical composition of the sample, effect of process variables (hydrolysis time, acid hydrolysis and temperature) on glucose yield and finally chemical composition of the product was analyzed using FTIR.

There were 20 experiments conducting by varying hydrolysis time, hydrolysis temperature and diluted sulfuric acid concentration. The amount of product obtained for each sample was measured and recorded.

4.1. Characterization of avocado seed

4.1.1. Proximate analysis

Literature (Hass,2007) reported that moisture content, volatile content, ash content and fixed carbon was 12.51, 78.76, 2.84, and 7.03 respectively. However, the results reported by (Hass, 2007) are not in accordance with my result shown below (table 4.1), probably due to the differences in species of varieties used, technology used, material used and chemicals used. Moisture content analysis used for the determination of proportionality of solid to liquid ratio in the pretreatment and hydrolysis method with increasing moisture content it affects the product quality. The analysis of total moisture is used to determine other properties such as volatile matter, ash content and fixed carbon. The sample of avocado seed with higher moisture content needs more heat for moisture vaporization. Ash is a measure of inorganic impurities in the avocado seed. In this study low ash content of avocado seed constituents, so decreasing sludge formation in the ethanol production. Finally, fixed carbon it is the carbon found in the material which is left after volatile materials are driven off this is used for the determination of carbon in the avocado seed.

Table 4.1 proximate analysis of the avocado seed sample results

Physical composition	Weight percentage (%Wt.dry basis)
Moisture	9.75
Volatile	80.52
Ash	0.48
Fixed carbon	9.25

4.1.2. Chemical composition analysis

Literature (Feurte, 2007) data for avocado seed of chemical composition analysis range from 32.84 to 54.94 % of cellulose, 28.35 % hemicellulose, 25.78% lignin and 3.15 % extractive. The results with my study shown below (table 4.2) were in a comparable range with literature values as reported by the above researcher. Therefore, the determination of cellulose and hemicellulose can be applied to quantify the theoretical production of ethanol. However, *Saccharomyces cerevisiae* only converts glucose. In this study, avocado seed contained high contents of the total cellulose of approximately 50% cellulose. The higher the cellulose content the higher glucose would obtained. And avocado seed contains a lower lignin content comparing with cellulose and hemicelluloses. The lower the lignin contents the easier hydrolysis condition, decrease formation of inhibitors and increase the contact between cellulose and hemicelluloses.

Table 4.2. The results of chemical composition of avocado seed sample

Chemical composition	Weight percentage (W/W%)
Extractive	3.23
Cellulose	50.84
Hemicelluloses	25.43
Lignin	20.5

4.2. Effect of acid hydrolysis on the glucose yield

In this study, the total reduced sugar content through hydrolysis process was investigated. The powdered avocado seed through hydrolysis at different acid concentration, hydrolysis time, and temperature on the amount of sugar produced was investigated. The results of glucose concentration and its absorbance are shown below in (table 4.3) and the result of glucose calibration curve is shown in (figure 4.1).

Table 4.3 Results of glucose concentration and its absorbance

Glucose concentration (mg/ml)	0	12	16	20	24	28
Absorbance @ 540 nm	0	0.24	0.372	0.49	0.60	0.78

The standard curve so formed is shown as below (figure 4.1)

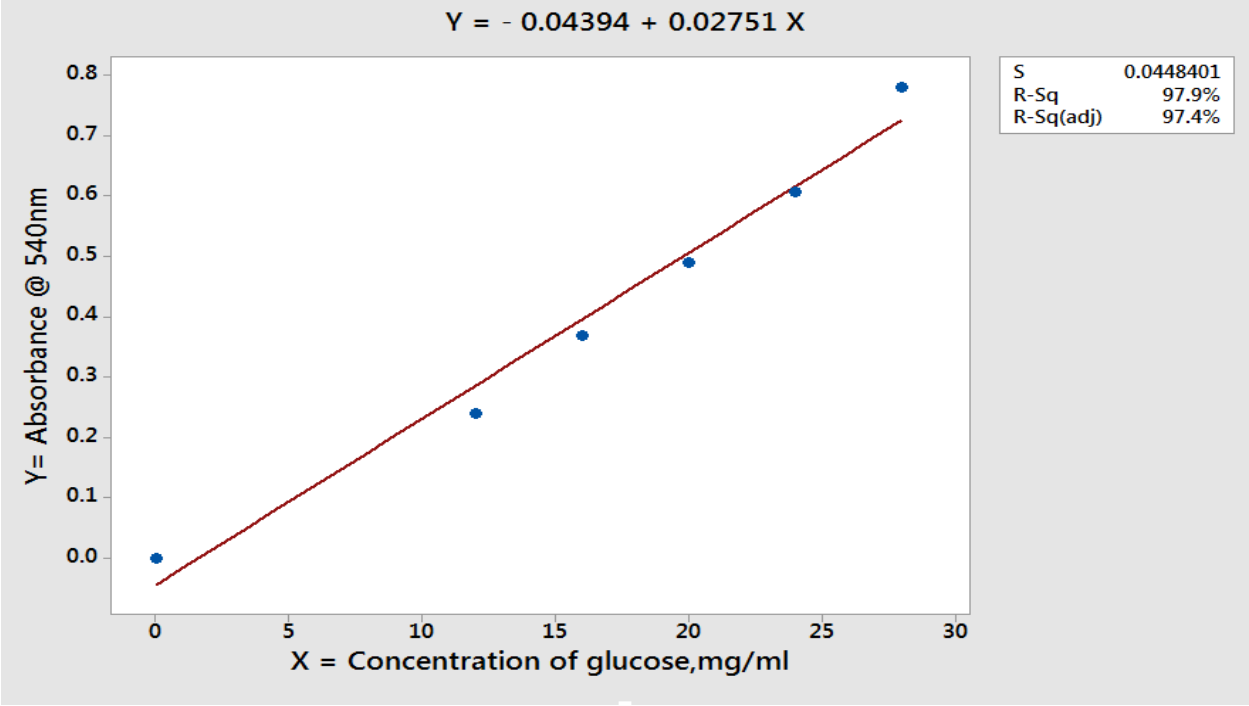


Figure 4.1 Calibration curve of glucose standard for determination of total sugar content

The concentrations of unknown sugar samples were determined from a standard curve of glucose (Y = 0.02751X – 0.04394: R^2 = 0.979) (Figure 4.1) using (equation 3.10) above and the results are summarized at (table 4.4) below.

Table 4.4 Glucose concentration (mg/ml), Glucose yield (%) and Ethanol yield (%) produced at various temperature, acid concentration and reaction time

No	A:Acid Conc. (%)	B: Temp.(°C)	C:Time (min)	Absorbance @540nm	Glucose con.(mg/ml)	Glucose yield (%)	EtOH yield (%)
1	1.5	125	30	0.561	21.50	40.2	38.5
2	3.5	125	30	0.205	21.67	40.5	38.7
3	1.5	145	30	0.450	21.94	41	39.05
4	3.5	145	30	0.230	20.65	38.6	37.25
5	1.5	125	90	0.620	23.17	43.3	42.3
6	3.5	125	90	0.210	21.08	39.4	43.6
7	1.5	145	90	0.571	22.79	42.6	41
8	3.5	145	90	0.189	21.67	40.5	39.4
9	1.5	135	60	0.691	26.54	49.6	48.15
10	3.5	135	60	0.370	23.86	44.6	43.7
11	2.5	125	60	0.390	22.90	42.8	40.3
12	2.5	145	60	0.311	23.75	44.1	38.2
13	2.5	135	30	0.470	23.11	43.2	36.5
14	2.5	135	90	0.421	24.24	45.3	42
15	2.5	135	60	0.580	25.89	48.4	46
16	2.5	135	60	0.551	25.20	47.1	44
17	2.5	135	60	0.590	24.98	46.7	45.4
18	2.5	135	60	0.601	25.91	47.3	39.9
19	2.5	135	60	0.578	25.63	47.9	46.3
20	2.5	135	60	0.569	25.31	47.3	45.9

Table 4.4 above showed us the glucose and ethanol yield decreased with acid concentration and temperature increment but increases with time increment. Based on this, the maximum yield of glucose and ethanol were noted for 1.5 % of acid concentration, at a temperature of 135°C and hydrolysis time of 60 min. For this condition the obtained glucose and ethanol yield were 49.6% and 48.15% respectively. But the glucose concentration and ethanol yield were observed to decrease at high acid concentration, and high temperature and low time. This may be due to formation of other intermediates products *(Huijegen et al,.2010).* Table 4.4 above also indicated us the varying glucose yield at different acid concentration, time and temperature. To analyze the Experimental results, Design expert® 7.0.0 software was used. From table 4.4 above, the maximum glucose yield 49.6%, and maximum ethanol yield 48.15% were obtained at an experiment number 9 at 135℃ temperature, 1.5 % acid concentration and at 60 minute of hydrolysis time. While the minimum yield glucose 38.6%, and minimum yield of ethanol 37.25% were obtained at experiment number 4 at a temperature of 145℃, 3.5% acid concentration and 30 minutes of hydrolysis time. The decrease and increase of the yield was depending on the level of factors. The resulting data, from table 4.4, were analyzed using Design expert® 7.0.0 software to determine the effect of temperature, acid concentration and time. The dependent variable used as a response parameter was the glucose yield.

Table 4.5 Design summary

Study type	Response surface
Initial point	Central composite design
Center point	6
Design Model	Quadratic polynomial
Run	20
Block	No

To determine whether or not the quadratic model is significant, it was crucial to perform analysis of variance (ANOVA), table 4.6 below. The probability values (P-values) were used to perform as a device to check the significance of each coefficient, which also showed the interaction strength of each parameter. The smaller the p- values are, the bigger the significance of the corresponding coefficient.

Table 4.6 Analysis of variance (ANOVA)

Source	Sum squares	DF	Mean square	F-value	p-value prob >F
Model	200.14	9	22.24	23.88	<0.0001 significant
A-acid conc.	17.16	1	17.16	18.43	0.0016
B-temp	0.036	1	0.036	0.039	0.8481
C-time	5.78	1	5.78	6.20	0.0320
AB	0.10	1	0.10	0.11	0.7484
AC	1.90	1	1.90	2.04	0.1835
BC	0.28	1	0.28	0.30	0.5947
A^2	3.636E-003	1	3.636E-003	3.905E-003	0.9514
B^2	37.7	1	37.7	40.13	<0.0001
C^2	22.91	1	22.91	24.60	0.0006
Residual	9.31	10	0.93		
Lack of Fit	7.48	5	1.5	4.07	0.0747 not significant
Pure error	1.83	5	0.37		
Cor Total	209.45	19			

Here the Model F-value of 23.88 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to personal error or disturbance. Probability values and/ or "Prob > F" values less than 0.0500 indicate model terms are significant. In this case A, C, 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 4.07 implies the lack of fit is not significant. There is a 7.47% chance that a "Lack of Fit F- value" this large could occur due to noise. Significant lack of fit is bad we want the model to fit.

Coefficient of Variation, the standard deviation expressed as a percentage of the mean; Predicted Residual Error Sum of Squares, which is a measure of how the model fits each point in the design; the R-Squared, measure of the amount of variation around the mean explained by the model; Adj R-Squared that is a measure of the amount of variation around the mean explained by the model, Pred R-Squared, a measure of the amount of variation in new data explained by the model, and Adequate Precision, this is a signal to disturbance ratio due to random error, presented in the table 4.7, below, are used to decide whether the model can be used or not.

Table 4.7 Model adequacy measures

Std.Dev	0.96	R-Squared	0.9555
Mean	44.02	AdjR-Squared	0.9155
C.V	2.19	Pred R-Squared	0.5285
PRESS	98.76	Adeq Precision	14.224

The "Pred R-Squared" of 0.5285 is not as close to the "Adj R-Squared" of 0.9155 as one might normally expect. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response tranformation, outliers, etc. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 14.224 indicates an adequate signal. This model can be used to navigate the design space.

By the designed experimental data from table 4.4 above, the quadratic polynomial model for ethanol production from avocado seed waste by dilute acid hydrolysis was retreated and shown as below:

Equation in terms of coded factors:

Glucose yield = +47.32 -1.31* A +0.060* B + 0.76* C - 0.11* A * B - 0.49* A * C +0.19* B * C -0.036 * A^2 -3.69* B^2 -2.89 * C^2

Final equation in terms of actual factors:

Glucose yield= -636.51483+1.36557*Acid con.+9.94981 temp +0.36643 * time -0.011250* Acid con. * temp - 0.016250* Acid con. * time +6.25000E-004* temp * time -0.036364

* Acid con.^{2} -0.036864* temp^2 -3.20707E-003 * time ^{2}

Diagnostic plot

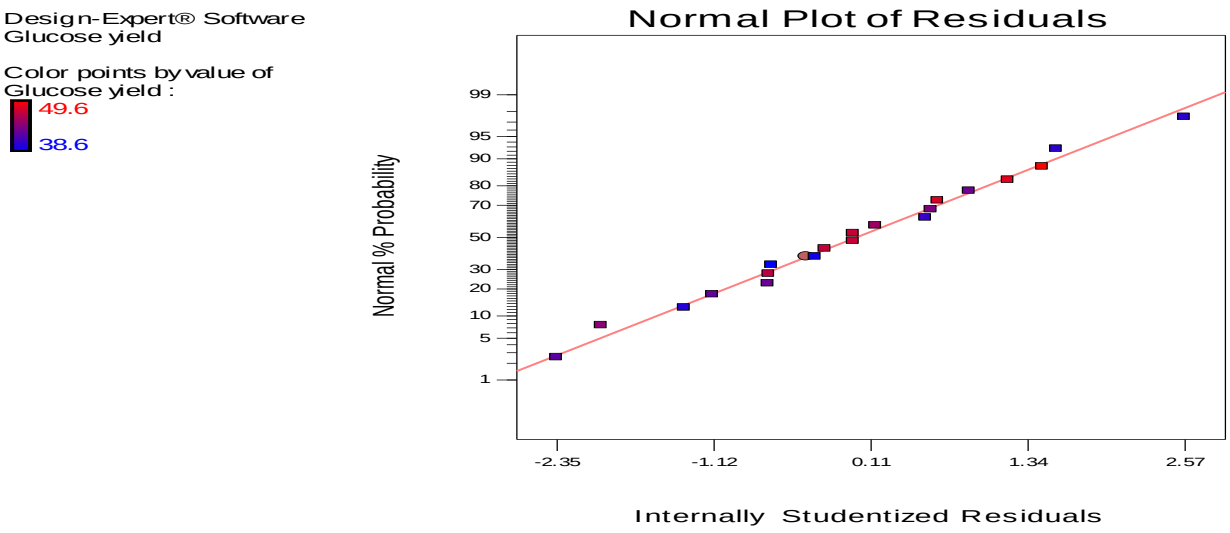


Figure 4.2 Normal plots of residuals

From the plot as shown above, 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 in the figure, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

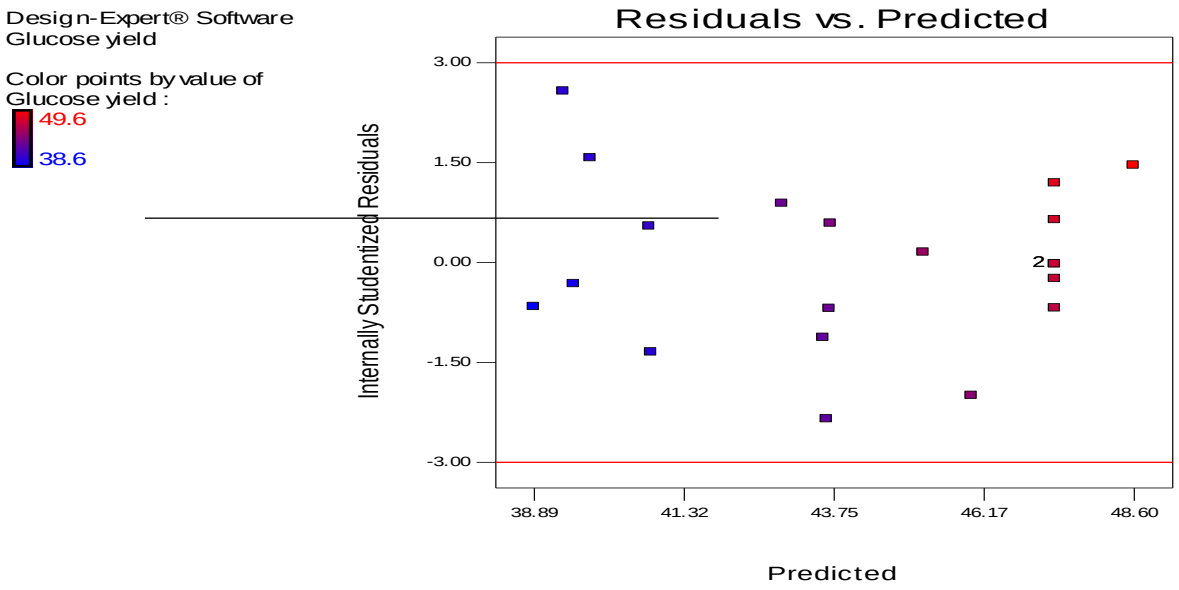


Figure 4.3 Plot of residuals versus model predicted values

If the model is correct and the assumptions are satisfied, the residuals should be structure less; in particular, they should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. 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.

4.3. Determination of the optimum operating conditions

The effects of the operating conditions on the glucose yield were investigated and the optimal values were determined in this study.

4.3.1. Effect of temperature on glucose yield

The resulting plot of temperature versus the glucose yield, when Acid concentration and hydrolysis time were actual factors, was depicted in Figure 4.4 below. From the plot as temperature increases from 125℃ to 135℃, glucose yield increased to 47.3 %. Beyond 135°C, decrease the yield was observed which is due to further conversion of other by product. Therefore, the optimum temperature was found to be 135℃ and the yield at this temperature was 47.3%.

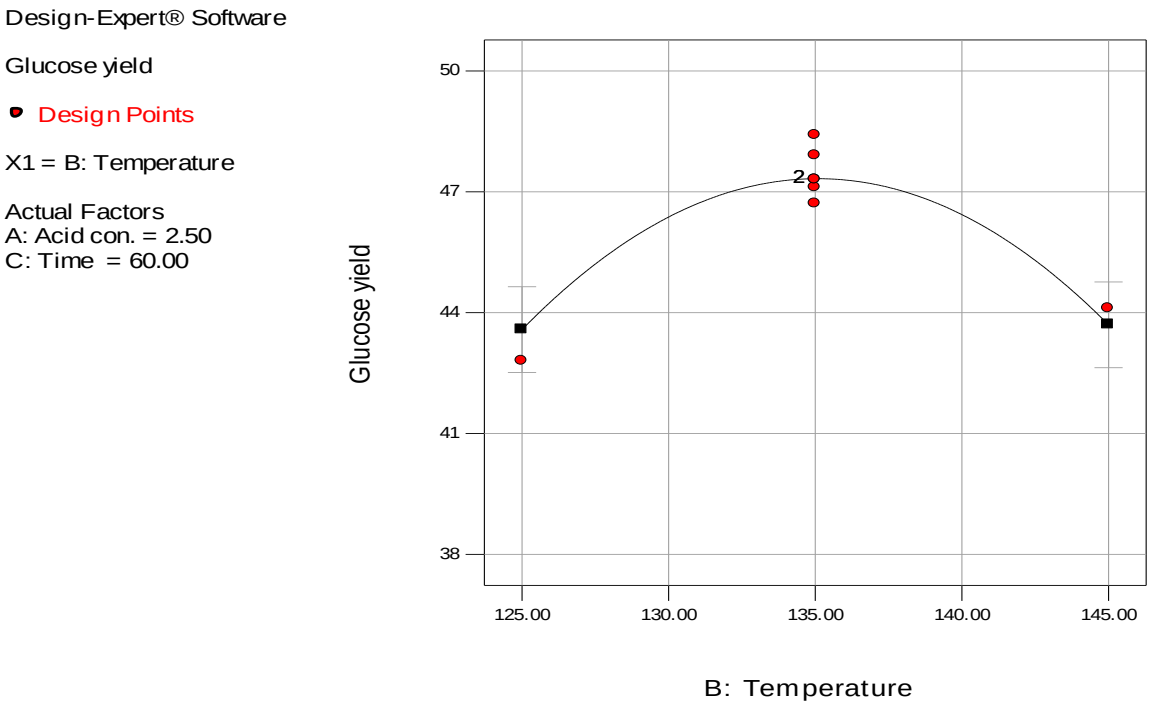


Figure 4.4 Effect of temperature on the glucose yield

4.3.2. Effect of time on glucose yield

The resulting plot of time versus the glucose yield, when Acid concentration and hydrolysis temperature were actual factors, was depicted in Figure 4.5 below. As shown from the plot

increasing time from 30 to 90 minute, glucose yield decreased the reason was due to formation of degradation product. Therefore, the optimum time was found to be 60 min and the yield at this temperature was 47.3 %. Beyond 60min, decrease the yield was observed which is due to further conversion of other by product. Therefore, the optimum time was found to be 60min and the yield at this time was 47.3%.

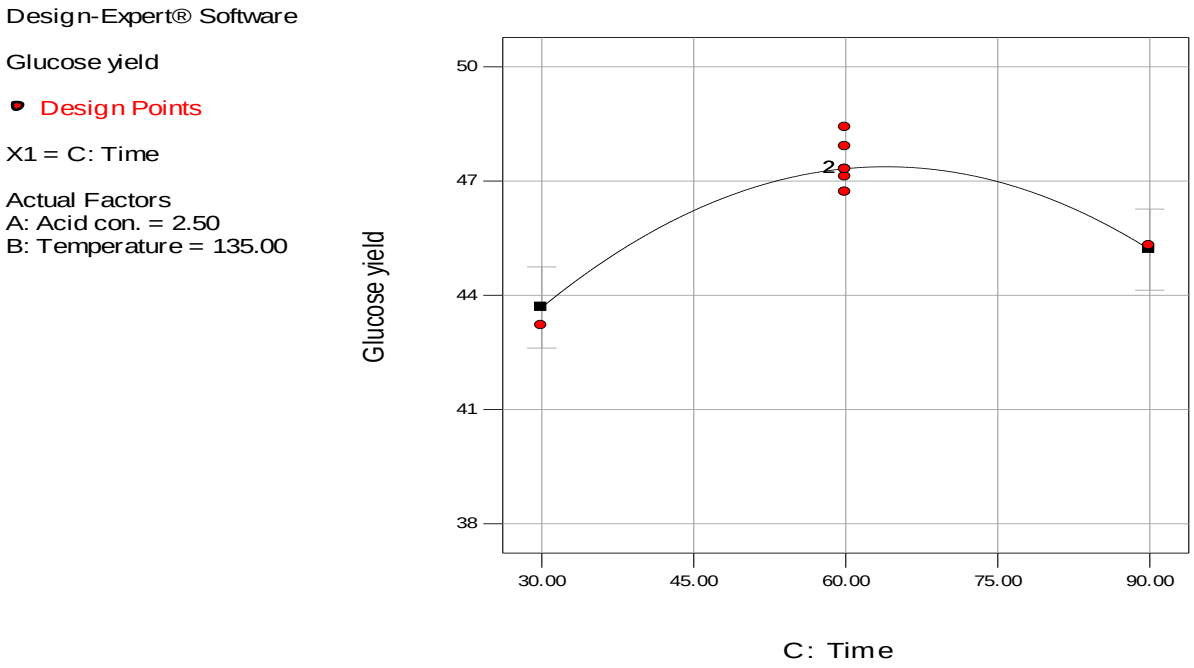


Figure 4.5 Effect of time on the glucose yield

4.3.3. Effect of acid concentration on the glucose yield

The resulting plot of acid concentration versus the glucose yield, when temperature and hydrolysis time were actual factors, was depicted in Figure 4.6 below. As shown from the plot increasing acid concentration from 1.5% to 3.5%, glucose yield decreased the reason was due to degradation of pentoses, hexoses, and the lignin present. These products can include furfural, acetic acid, 5-hydroxymethyl-2-furaldehyde (HMF), and formic acid. Therefore, the optimum acid concentration was found to be 1.5 % and the yield at this acid concentration was 48.6%.

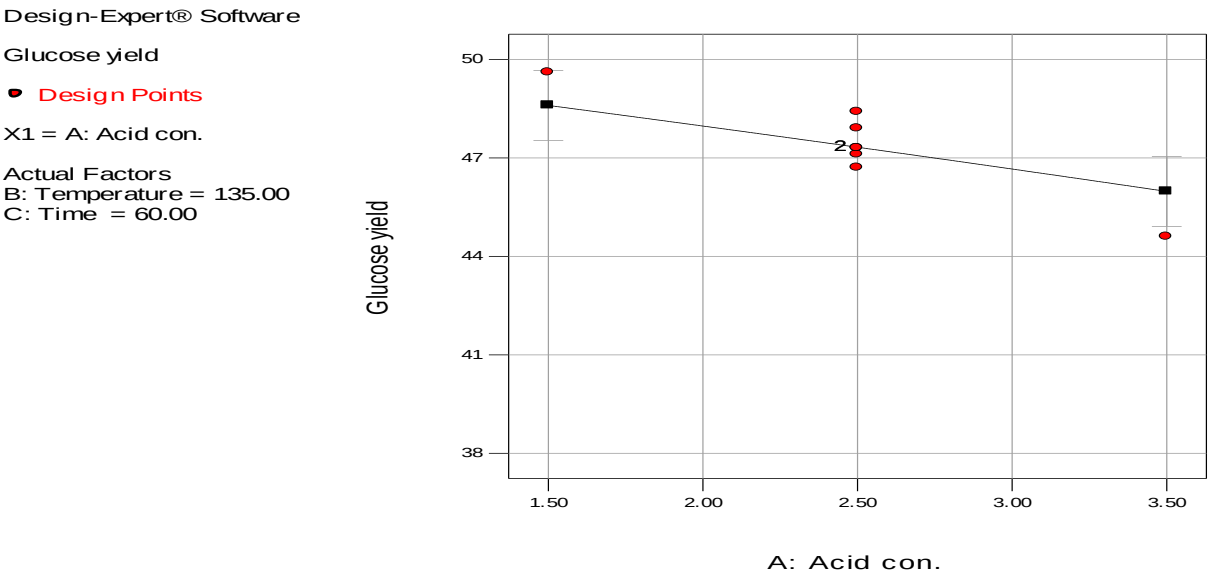


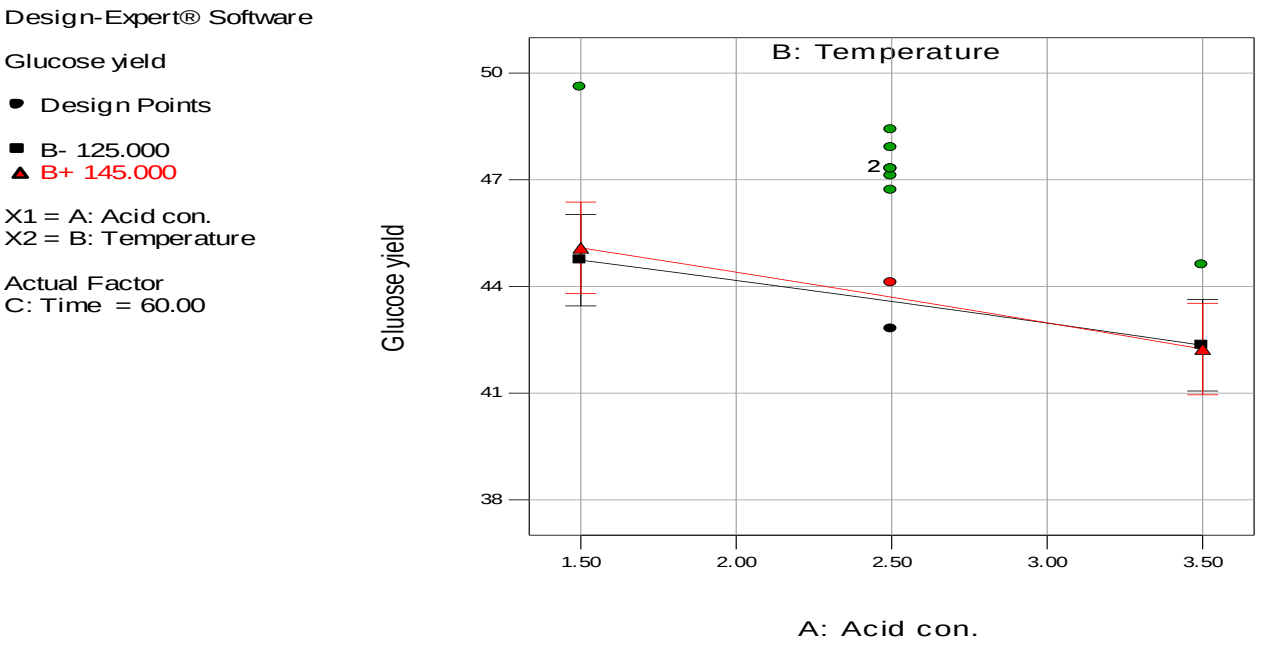
Figure 4.6 Effect of acid concentration on the glucose yield

4.4. Effects of experimental variables on acid hydrolysis

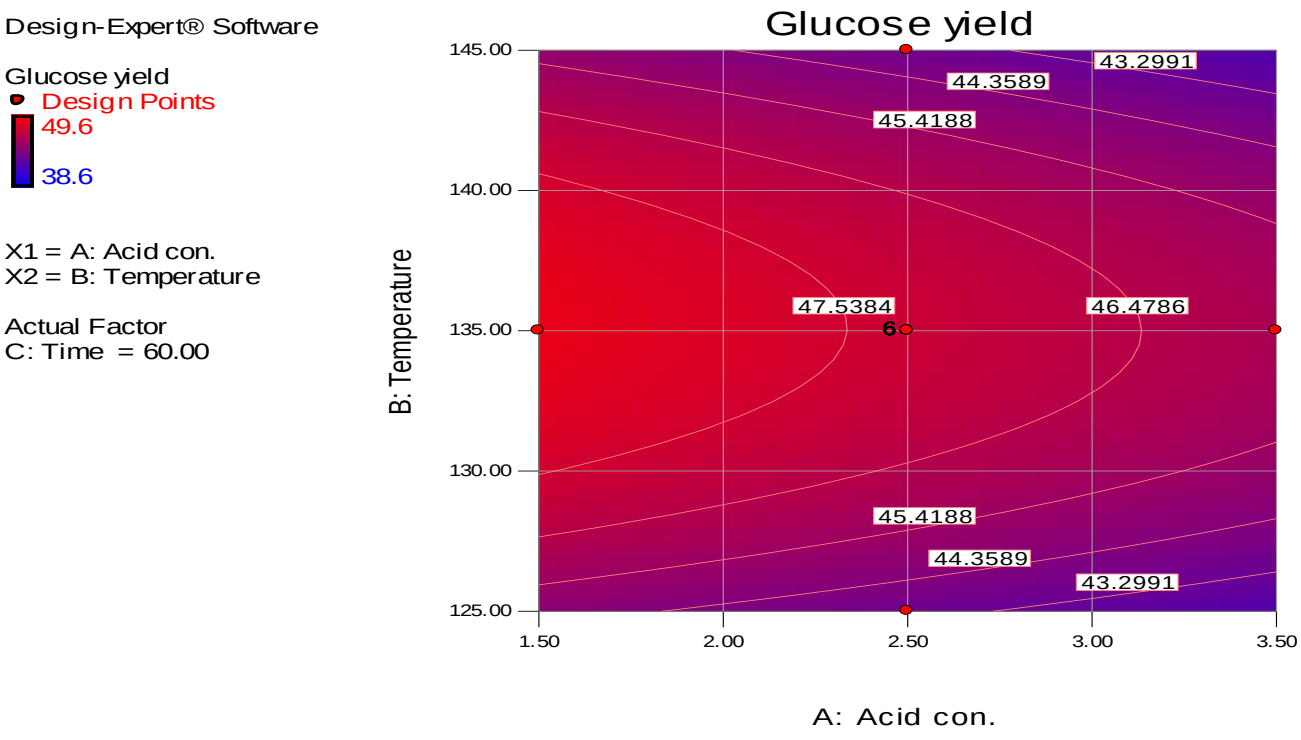
Ethanol production can be affected by many parameters starting from sample preparation to distillation, the hydrolysis steps has a complex connection with independent variables. The best way of showing the effects of this parameter for the yield of glucose are to generate response surface plots of the equation. The three dimensional i.e. interactions, contours and response surfaces effect were plotted in figures (4.7), (4.8) and (4.9) below as a function of the interactions of any two of the variables by holding the other value of the variable at center.

Interaction effects are effects that independent variable impose on one another. All controllable factors are obvious variables which affect the output of the response variable. In this research, there are three controllable factors in the acid hydrolysis step, namely: hydrolysis temperature, hydrolysis time and acid concentration. The main effects of acid concentration and hydrolysis time depended on the level of hydrolysis temperature.

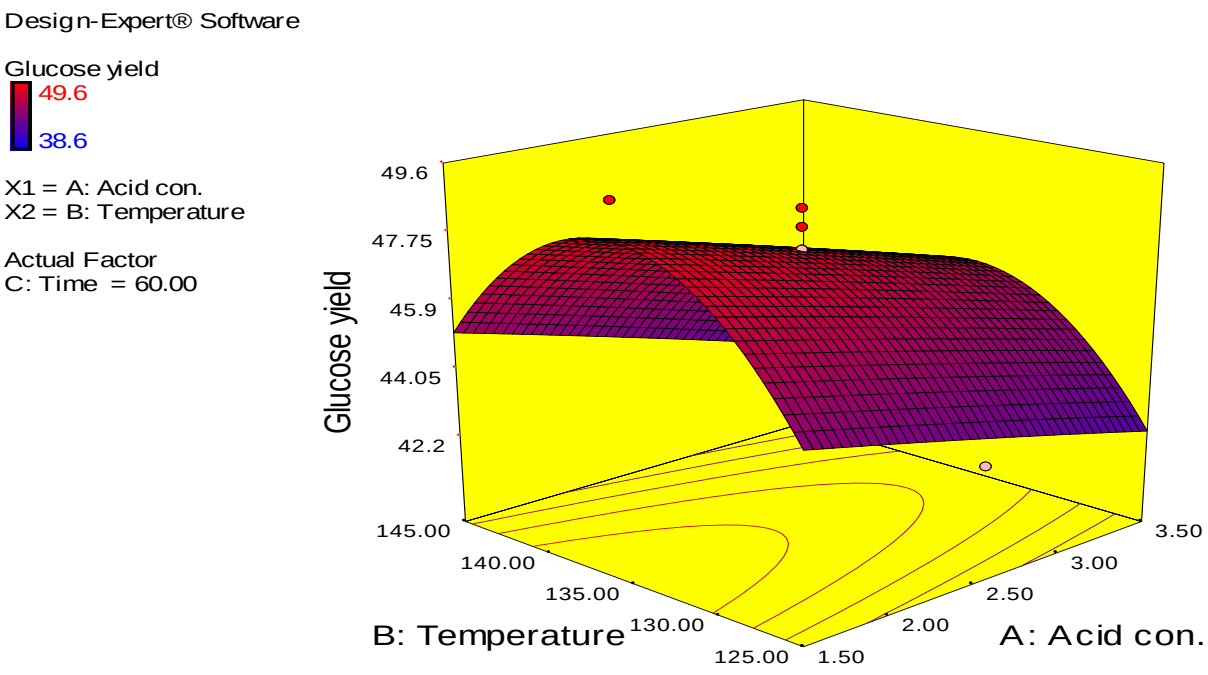
An interaction is the failure of the one factor to produce the same effect on the response at different levels of another factor. From this it is possible to conclude that, there a high interaction effect. For the interaction figures, black and red line indicates low and high level of parameters respectively. Figure 4.7(a) below shows that there is an interaction among each factor. This shows us an increment in acid concentration decrease the glucose yield.



(a) The effects of temperature and acid concentration on the yield of glucose, when the time was at the center point.



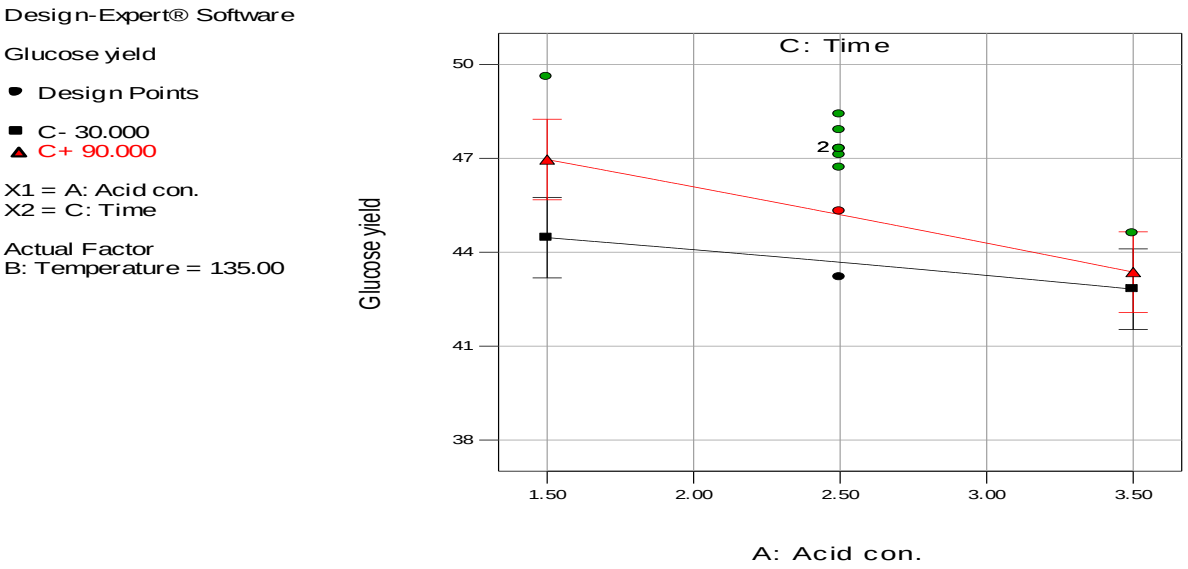
(b) Contour plot of the effects of acid concentration and temperature on the yield of glucose.



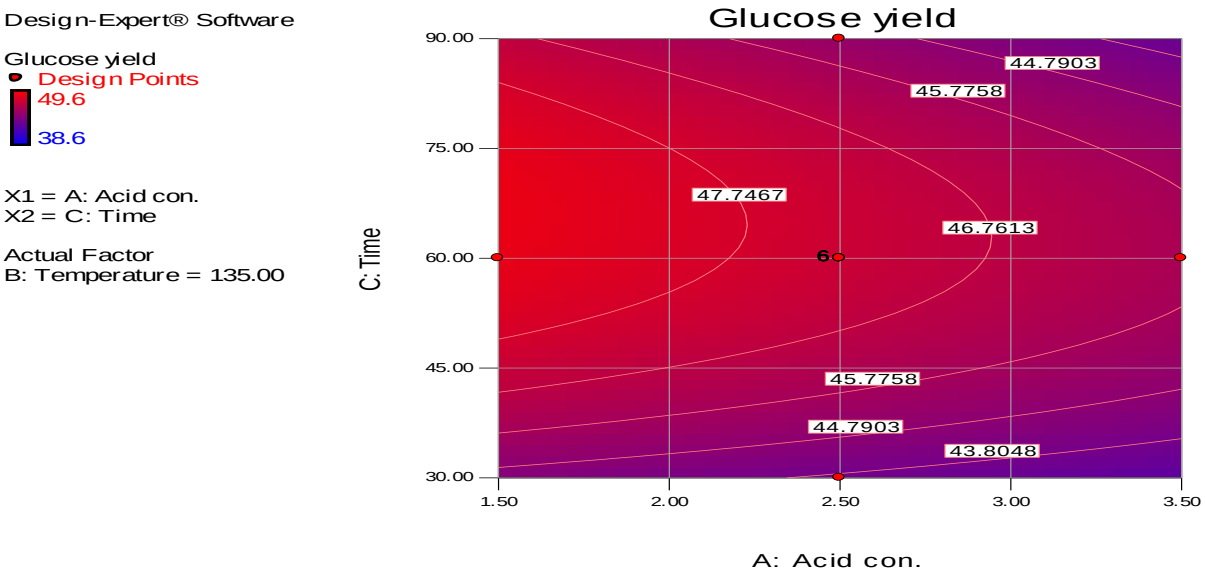
(c) Surface plot of the effects of temperature and acid concentration on the yield of glucose.

Figure 4.7 Effect of acid concentration and temperature on the yield of glucose when time was at the center point (a, b and c)

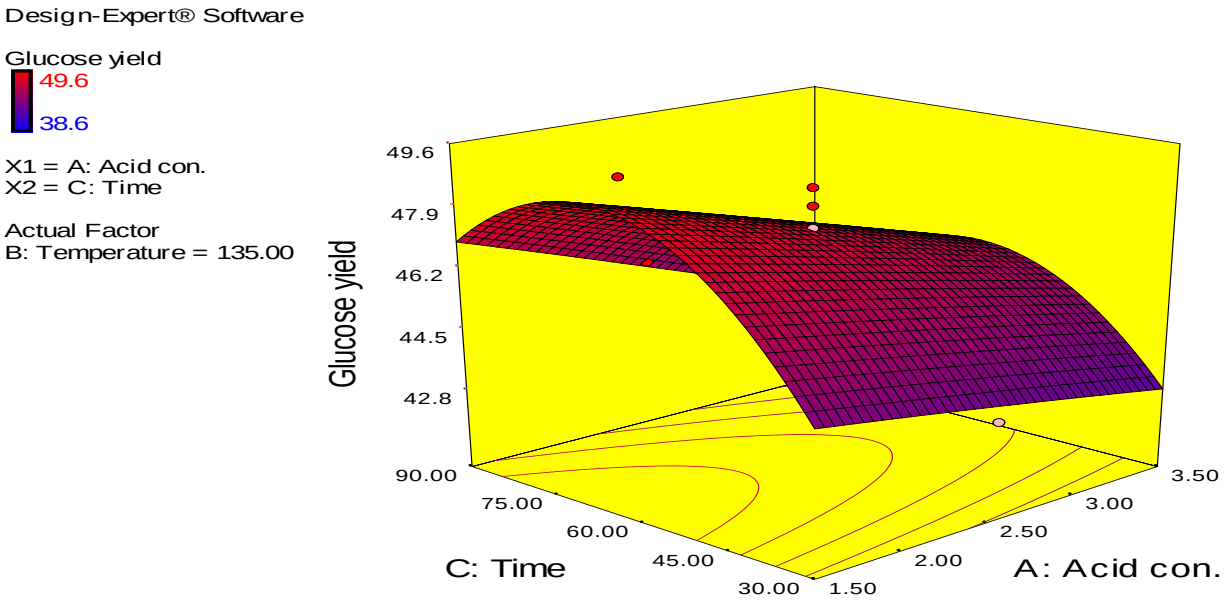
In contour and 3D surfaces graph figures 4.7 (b) and (c) above also shown that the effect of acid concentration and temperature on the glucose yield. At the lower and higher levels of temperature, the production level of glucose yield decrease. However, at lower acid concentration increases the yield. This is because it has effect of the hydrolysis treatment. At lower temperature the cellulose might not hydrolysis to simple glucose and at higher acid concentration and temperature cellulose forms other degradation products. Hence both acid concentration and temperature have strong relationship for the yield of glucose production. Similarly, low levels of acid concentration and moderate temperature had a positive effect on the glucose yield. However, at the higher levels of both temperature and acid concentration, the yield of glucose declined as a result ethanol yield also decreases. Certainly this is due to presence of the strong interaction between these two variables.



(a) The interaction effects of acid concentration and time on the yield of glucose, when the temperature was at the center point.



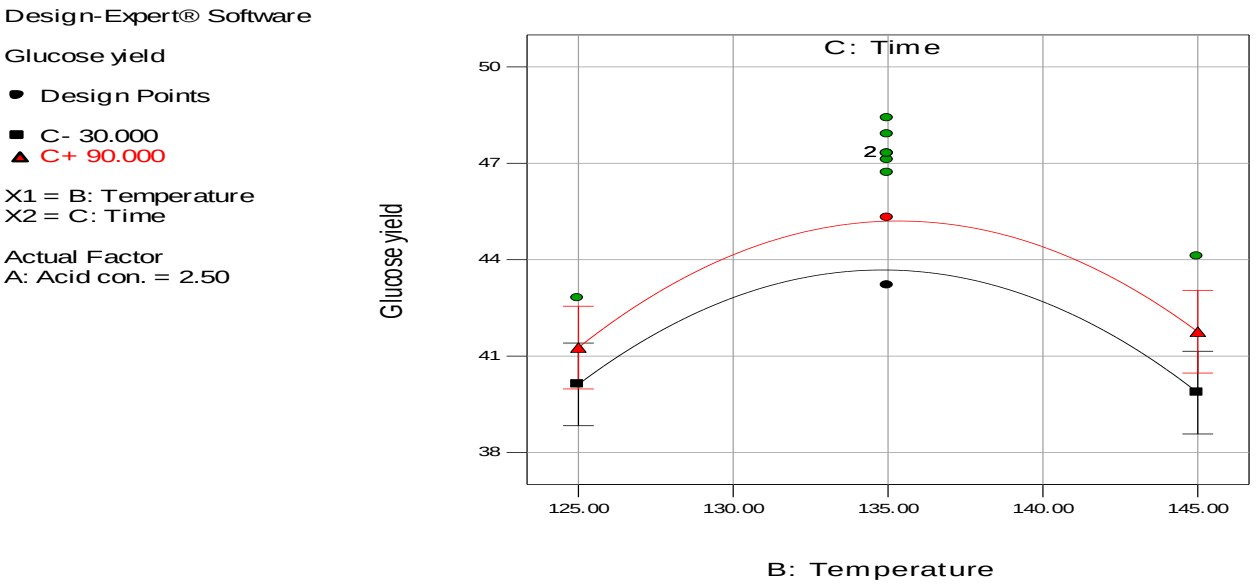
(b) Contour plot of the effects of acid concentration and time on the yield of glucose.



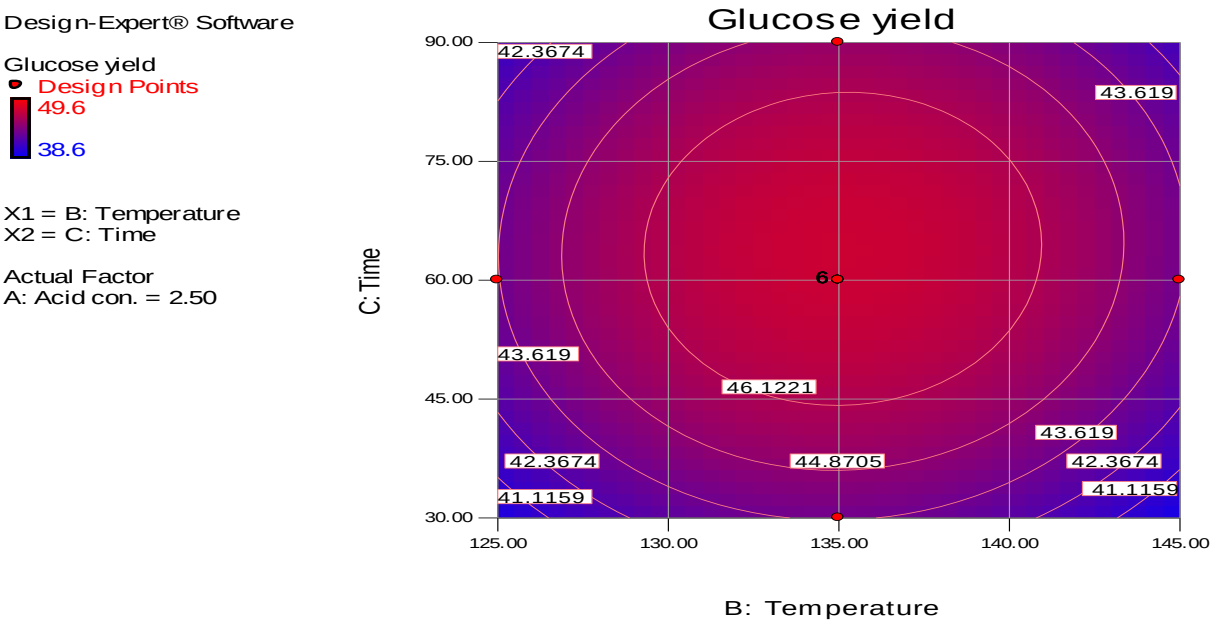
(c) Surface plot of the effects of time and acid concentration on the yield of glucose.

Figure 4.8 Effect of acid concentration and time on the yield of glucose when temperature was at the center point (a, b, and c)

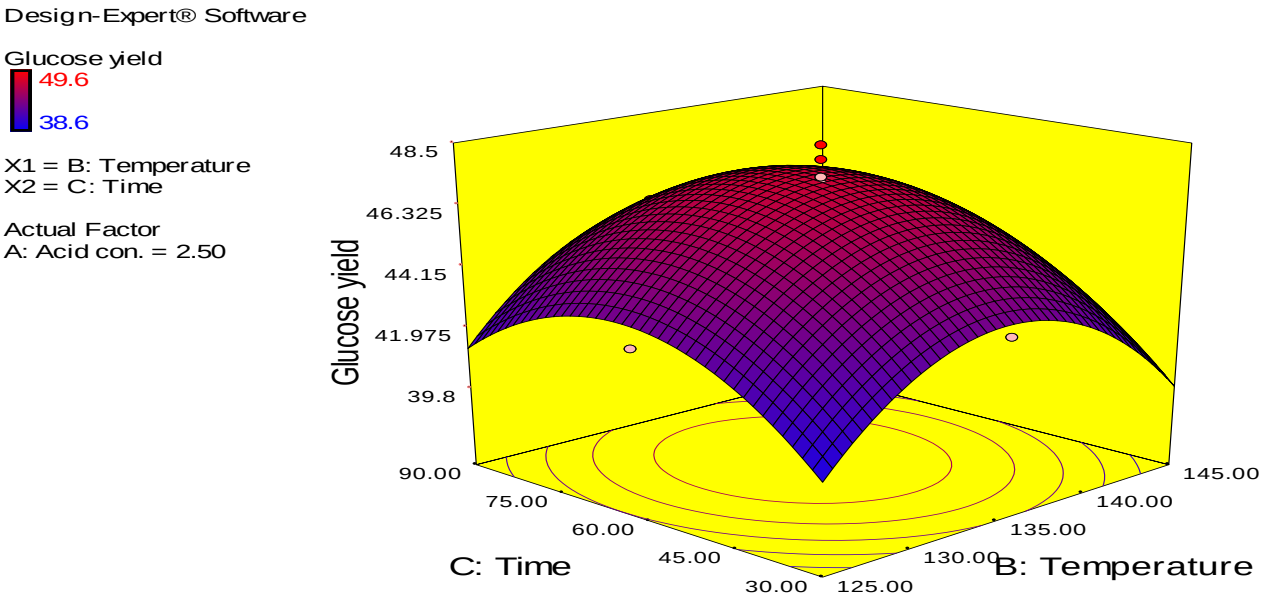
The effects of acid concentration and time on the yield of glucose, temperature was selected at the center point, are shown in figure 4.8 above. The maximum yield of glucose was observed at lower cid concentration and medium hydrolysis time. At increasing acid concentration, and time decreasing the yield of glucose became decreases since the possible formation of other molecules instead of glucose formation or the conversion glucose in to other fermentation inhibitors such as furfural.



(a) The interaction effects of temperature and time on the yield of glucose, when the acid concentration was at the center point



(b) Contour plot of effects of temperature and time on the yield of glucose



(c) Surface plot of the effects of temperature and time on the yield of glucose

Figure 4.9 Effect of temperature and time on the yield of glucose when acid concentration was at the center point (a, b and c)

The effects of acid concentration and time on the yield of glucose, acid concentration was selected at the center point, are shown in figure 4.9 above. The maximum yield of glucose was observed at medium temperature and medium hydrolysis time. At increasing/decreasing temperature, and time from the medium the yield of glucose became decreases since the possible formation of other molecules instead of glucose formation or the conversion sugars such as glucose, arabinos, galactose, frurural and xylose in to other fermentation inhibitors.

4.5. Optimizations

Optimization of glucose yield was carried out by a multiple response method called desirability (D) function to optimize different combinations of process parameters. The goal of optimization was to maximize economic benefit or increasing glucose yield by minimizing process cost.

To investigate the optimum values of glucose production from avocado seed wastes using dilute acid hydrolysis are summarized as follows:

Table 4.8 Optimization criteria for optimum yield of glucose

Name	Goal	Lower Limit	Upper Limit
Acid concentration (%)	Minimize	1.5	3.5
Temperature (^{o}C)	Minimize	125	145
Time (min)	Minimize	30	90
Glucose yield (%)	Maximize	38.6	49.6

Table 4.9 Optimum possible solutions

Run No	Acid Conc. (%)	Temp.(^{o}c)	Time (min)	Yield of glucose (%)	Desirability
1	1.5	129.11	42.78	48.3702	0.843 (selected)
2	1.5	129.21	43.00	48.4524	0.843
3	1.5	128.83	43.40	48.8315	0.842
4	1.5	129.11	41.75	48.2070	0.842
5	1.5	129.03	41.47	48.1224	0.842
6	1.5	128.66	43.47	48.2595	0.842
7	1.5	129.14	44.28	48.6129	0.842
8	1.5	129.94	39.53	48.1818	0.839
9	1.5	127.98	46.00	48.2659	0.838
10	1.5	129.91	38.75	48.0315	0.838

The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If a response falls within the unacceptable intervals, the desirability is 0, and if a response falls within the ideal intervals or the response reaches its ideal value, the desirability is 1. Based on the above analysis best local maximum for glucose yield 48.3702% was found at acid concentration 1.5%, temperature 129.11℃ and time 42.78 minutes and the value of desirability obtained was 84.3%.

4.6. Validation of the model

According to the central composite design result using Design-Expert® 7.0.0 software, an experiment with acid concentration, temperature and hydrolysis time were conducted in order to study the outcome or effect of the design. The experiment was carried out at the optimized conditions. Based on the second-order models, numerical optimization was carried out to maximize the yield of glucose, using the response optimizer in Design expert®7.0.0. The optimal values test factors were 1.5 % acid concentration, 129.11°C temperature and 42.78 minutes time (obtained from table 4.9). Glucose yield of 48.37 % (average) obtained and was in good agreement with the predicted one. Therefore the model is considered to be accurate and reliable for predicting the yield of glucose.

4.7. FTIR characterization of the produced bio-ethanol

Alcohols have characteristic infrared absorptions associated with the O-H, C-O and the C-H stretching vibrations. When the wave length range from 3500-3200 cm^{-1} indicates the O-H, around 2930 cm^{-1} indicates the -CH_3 and around 2880 cm-1 indicates the –CH_2 functional groups of the alcohols *(Coates and Meyers, 2000; Yu et al, 2007).*

This ascertains that the product obtained from powdered avocado seeds is definitely ethanol due to the confirmation of these regions as shown below (Figure 10).

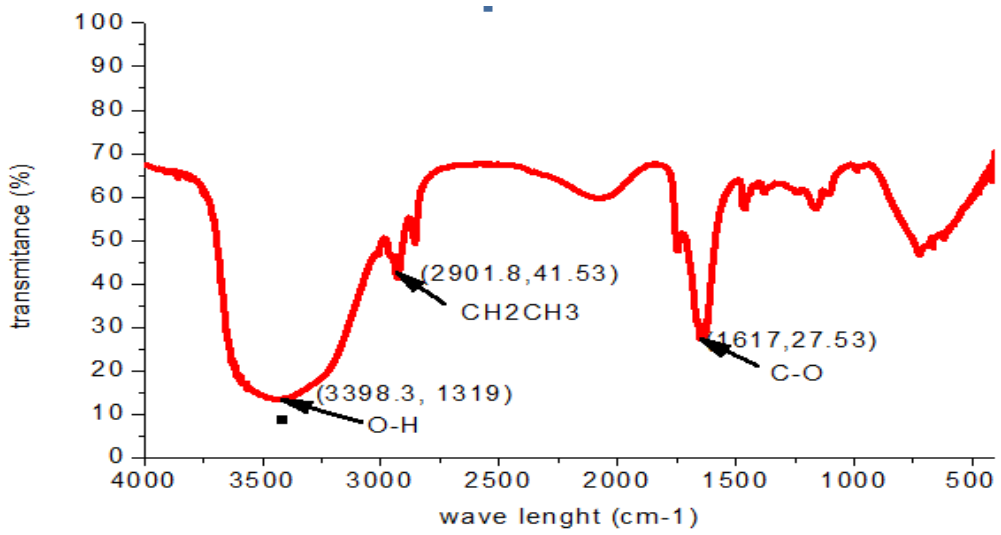


Figure 4.10 FTIR result of the ethanol yield at acid conc. of 1.5%, temp of 135℃, and for a hydrolysis of 60 min

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Proximate and chemical composition analysis was employed to characterize avocado seed. It shows a physical composition of 9.75% moisture, 80.52% volatile matter content, 0.48% ash content and 9.25% fixed carbon content and chemical composition of 3.23% extractive, 50.84% cellulose, 25.43% hemicelluloses and 20.5% lignin. From this result there is low content of ash (0.48%) and ash is a measure of inorganic impurities in the avocado seed then I can conclude that a low ash constitute of avocado seed would decreasing sludge formation in ethanol production. And also avocado seed has high content of volatile matter implies the fuel produced using this feedstock would be highly combustible. Avocado seed contained high contents of the total cellulose (50.84%). The higher the cellulose content the higher glucose would obtained. And also avocado seed has a lower level of lignin implies the easier hydrolysis condition, decrease formation of inhibitors and increase the contact between cellulose and hemicelluloses.

Bioethanol production from avocado seed wastes and optimization of different factors in the hydrolysis process were investigated. The experimental design was conducted by central composite design (CCD) to study the effects of three variables acid concentration (1.5, 2.5 and 3.5%), temperature (125, 135 and 145℃) and time (30, 60 and 90minutes). The optimized conditions for hydrolysis process were acid concentration 1.5%, hydrolysis temperature 135^{o}C and hydrolysis time 60 minutes. At these operating conditions the maximum yield of glucose was found to be 49.6%. From this result obtained, it can be conclude that very high acid concentration, high hydrolysis temperature and very low reaction time have negative effect on the yield of glucose and very low acid concentration, medium hydrolysis temperature and very high hydrolysis time have positive effect on the yield of glucose.

Quadratic model was employed to correlate the operating variables with the response. From the analysis of variance acid concentration and time were found to have the most significant effect on productivity of glucose by using F-test ($p<0.05$). The maximum, observed, value of glucose productivity recorded was 49.6 % (w/w) and this is in a good agreement with the predicted value of 48.4 % (w/w). Based on this study, it is evident that the chosen method of optimization was

efficient, and reliable. From this, I can conclude that the selected model was adequate to fit the data of response variable.

Chemical characterization of the bio-ethanol produced was performed using FTIR. From the result obtained, it was observed that the ethanol produced from avocado seed contains OH, CO, CH_2, and CH_3 functional groups; which confirm the presence of ethanol in the product.

The world energy crisis is increasing day by day due to the increase in population and our more dependency on the resources which are non renewable and less on the renewable resources. Our main dependency for the energy needs is on the non renewable resources like as oil, coal and gas for over 80%. Due to which there is a need to find the alternative bio-ethanol such as biodiesel and bio-fuels in order to cope with the energy crisis. From my present study I can conclude that dependence on the petroleum reserves can be minimized by introducing alternative methods of ethanol production from avocado seed wastes. The ethanol production from the avocado seed waste can reduce the disposal load on the landfills and more over dependency on the non renewable resources. According to the results the ethanol can be available at the cheap rates which make it more valuable alternative bio-fuel than other petro diesel fuels.

5.2. Recommendation

Based on the current investigation the following recommendations are forwarded:

- In my study acid hydrolysis variables are optimized; it is recommended that, future studies should include optimization of pretreatment process, optimization of fermentation process and optimization of distillation process variables to obtain maximum yield of ethanol from avocado seed wastes.
- In my study I was used dilute acid hydrolysis as catalyst during hydrolysis; it is recommended that, future studies should another methods of glucose extraction such as enzymatic extraction method need to be done in order to investigate the variation that could be arise on the quality and quantity of the glucose yield as a result of using different extraction methods.
- An economic feasibility analysis of the overall conversion process is necessary for the purpose of commercialization.
- In my study I was characterized the final product bio-ethanol by using FTIR analysis; it is recommended that, future studies should characterize using GC-MS in order to investigate the variations that could be arise between these analysis methods.
- In this study to fermentation process I was use dextrose sugar, yeast extract, urea and hydrated magnesium sulfate for growth of yeast *saccharomyces cerevisiae;* it is recommended that, future studies should execute or develop the experiment of fermentation with another culture media which are less costs.

REFERRENCES

Amanda, A., Flórez[a], J. B., Mori[a], F. A., & Francisco, G. (2015). Acoustic Characterization of Sugarcane Bagasse Particleboard Panels (Saccharum officinarum L), *18*(4), 821–827.

Ayeni, A. O., Adeeyo, O. A., Oresegun, O. M., & Oladimeji, E. (2015). Compositional analysis of lignocellulosic materials : Evaluation of an economically viable method suitable for woody and non-woody biomass American Journal of Engineering Research (AJER), (4), 14–19.

Aysu, T., & Durak, H. (2015). In the Field (Persea americana) to produce bio-oil through supercritical liquefaction, 231–257. https://doi.org/10.1002/bbb

Braide, W., Kanu, I., Oranusi, U., & Adeleye, S. (2016). Production of bioethanol from agricultural waste. *Journal of Fundamental and Applied Sciences*, *8*(2), 372. https://doi.org/10.4314/jfas.v8i2.14

Chaves, M. A., & Borges, C. D. (2016). Avocado: characteristics, health benefits and uses, 747–754.

Cristina, A., Istiana, N., & Dwijananti, P. (2017). Ethanol Production from Fermentation of Arum Manis Mango Seeds (Mangifera Indica L .) using Saccharomyces cerevisiae. https://doi.org/10.15294/jbat.v6i1.8139

C. D. Blasi, G. Signorelli, C. Di Russo, G. Rea, Product distribution from pyrolysis of wood and agricultural residues, *Industrial Engineering Chemistry Research*, *38(6)*, 1999, 2216-2224.

Danish, M., Naqvi, M., Farooq, U., & Naqvi, S. (2015). Characterization of South Asian agricultural residues for potential utilization in future “ energy mix .” *Energy Procedia*, *75*, 2974–2980. https://doi.org/10.1016/j.egypro.2015.07.604

Danmaliki, G. I., Muhammad, A. M., Shamsuddeen, A. A., & Usman, B. J. (2016). Bioethanol Production from Banana Peels Bioethanol Production from Banana Peels, (June). https://doi.org/10.9790/2402-1006025662

Eriksson, N. (2013). Production of four selected renewable aromatic chemicals.

Fermentation, Y. (2011). Ethanol from Fermentation, *6*, 1–12.

Harmsen, P., & Huijgen, W. (2010). Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass, (September), 1–49.

Hirschnitz-Gabers, M., & Gosens, J. (2015). Producing bio-ethanol from residues and wastes. *Recreate POLYCE BRIEF*, (2).

Hong, K. O. H. M. A. Y. (2013). PREPARATION AND CHARACTERIZATION OF, (May).

Hossain, A. B. M. S., & Fazliny, A. R. (2010). Creation of alternative energy by bio-ethanol production from pineapple waste and the usage of its properties for engine, *4*(X), 813–819.

Journal, B. (2011). Production of cellulase by, *91*, 1119–1127.

Kam, Y. C., Hii, S. L., Sim, C. Y., Gaik, L., & Ong, A. (2016). Schizophyllum commune Lipase Production on Pretreated Sugarcane Bagasse and Its Effectiveness, *2016*.

Karp, S. G., Woiciechowski, A. L., Soccol, V. T., & Soccol, R. (2013). Pretreatment Strategies for Delignification of Sugarcane Bagasse : A Review, *56*(August), 679–689.

Limayem, A., & Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production : Current perspectives , potential issues and future prospects. *Progress in Energy and Combustion Science*, *38*(4), 449–467. https://doi.org/10.1016/j.pecs.2012.03.002

Mahawan, M. A., Tenorio, M. F. N., & Gomez, J. A. (2015). Characterization of Flour from Avocado Seed Kernel, *3*(4), 34–40.

Nairobi, O. F., Food, O. F., Nutrition, S., Extraction, S., From, P., Seed, A., & By, W. (2006). 2..0\0, 1–20.

Nichols, N. N., Sharma, L. N., Mowery, R. A., Chambliss, C. K., Walsum, G. P. Van, Dien, B. S., & Iten, L. B. (2008). Enzyme and Microbial Technology Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate ☪, *42*, 624–630. https://doi.org/10.1016/j.enzmictec.2008.02.008

Onuki, S., Leeuwen, J. Van, Jenks, W. S., & Grewell, D. A. (2008). Ethanol production ,

purification , and analysis techniques : a review.

Otulugbu, K. (2012). Production of Ethanol From Cellulose. *Plastic Technology*, 1–46.

Parameters, P., Kernel, M. S., Application, C., Advisor, M. K., & Yimam, A. (2015). ADDIS ABABA UNIVERSITY ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT) SCHOOL OF CHEMICAL AND BIO- ENGINEERING, (June).

Potential for Bioethanol Production from Wet Coffee Pulp Waste in. (2014).

Pretreatment, S., Lignocellulose, O. F., Of, H., In, S., & To, M. (2010). Simultaneous pretreatment of lignocellulose and hydrolysis of starch in mixtures to sugars, *5*(Demirbas 2005), 2457–2469.

Produkcji, W., & Na, B. (2010). ENVIRONMENTAL IMPACT OF BIOETHANOL PRODUCTION, *2*(1).

Reddy, L., Obulam, V., Sarathi, R., & Wee, Y. (2011). Production of ethanol from mango (Mangifera indica L .) peel by Saccharomyces cerevisiae CFTRI101. *African Journal of Biotechnology*, *10*(20), 4183–4189. https://doi.org/10.5897/AJB10.2286.

Teixeira, E. D. M., Luzia, A., Ro, D., Carvalho, F. De, & Aprigio, A. (2005). Preparation and Characterisation of Thermoplastic Starches from Cassava Starch , Cassava Root and Cassava Bagasse, 266–275. https://doi.org/10.1002/masy.200551133

Tekaligne, T. M., Woldu, A. R., Tsigie, Y. A., Science, C., Box, P. O., & Dar, B. (2015). Pretreatment Hydrolysis of the Pretreated Samples, *8*(1), 1–13.

Ververis, C. (2007). Cellulose , hemicelluloses , lignin and ash content of some organic materials and their suitability for use as paper pulp supplements, *98*, 296–301. https://doi.org/10.1016/j.biortech.2006.01.007

Weatherby, L. S. (1934). Composition of Avocado Seed, 132–134.

Woldu, A. R., & Tsigie, Y. A. (2015). Optimization of Hydrolysis for Reduced Sugar Determination from Avocado Seed Wastes, *3*(1), 1–10.

Amanda, A., Flórez[a], J. B., Mori[a], F. A., & Francisco, G. (2015). Acoustic Characterization of Sugarcane Bagasse Particleboard Panels (Saccharum officinarum L), *18*(4), 821–827.

Ayeni, A. O., Adeeyo, O. A., Oresegun, O. M., & Oladimeji, E. (2015). Compositional analysis of lignocellulosic materials : Evaluation of an economically viable method suitable for woody and non-woody biomass American Journal of Engineering Research (AJER), (4), 14–19.

Aysu, T., & Durak, H. (2015). In the Field (Persea americana) to produce bio-oil through supercritical liquefaction, 231–257. https://doi.org/10.1002/bbb

Badger, P. C. (2002). Ethanol From Cellulose : A General Review, 17–21.

Braide, W., Kanu, I., Oranusi, U., & Adeleye, S. (2016). Production of bioethanol from agricultural waste. *Journal of Fundamental and Applied Sciences*, *8*(2), 372. https://doi.org/10.4314/jfas.v8i2.14

Chaves, M. A., & Borges, C. D. (2016). Avocado: characteristics, health benefits and uses, 747–754.

Cristina, A., Istiana, N., & Dwijananti, P. (2017). Ethanol Production from Fermentation of Arum Manis Mango Seeds (Mangifera Indica L .) using Saccharomyces cerevisiae. https://doi.org/10.15294/jbat.v6i1.8139

Danmaliki, G. I., Muhammad, A. M., Shamsuddeen, A. A., & Usman, B. J. (2016). Bioethanol Production from Banana Peels Bioethanol Production from Banana Peels, (June). https://doi.org/10.9790/2402-1006025662

Eriksson, N. (2013). Production of four selected renewable aromatic chemicals.

Fermentation, Y. (2011). Ethanol from Fermentation, *6*, 1–12.

Harmsen, P., & Huijgen, W. (2010). Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass, (September), 1–49.

Hirschnitz-Gabers, M., & Gosens, J. (2015). Producing bio-ethanol from residues and wastes. *Recreate POLYCE BRIEF*, (2).

Hong, K. O. H. M. A. Y. (2013). PREPARATION AND CHARACTERIZATION OF, (May).

Hossain, A. B. M. S., & Fazliny, A. R. (2010). Creation of alternative energy by bio-ethanol production from pineapple waste and the usage of its properties for engine, *4*(X), 813–819.

Journal, B. (2011). Production of cellulase by, *91*, 1119–1127.

Kam, Y. C., Hii, S. L., Sim, C. Y., Gaik, L., & Ong, A. (2016). Schizophyllum commune Lipase Production on Pretreated Sugarcane Bagasse and Its Effectiveness, *2016*.

Karp, S. G., Woiciechowski, A. L., Soccol, V. T., & Soccol, R. (2013). Pretreatment Strategies for Delignification of Sugarcane Bagasse : A Review, *56*(August), 679–689.

Limayem, A., & Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production : Current perspectives , potential issues and future prospects. *Progress in Energy and Combustion Science*, *38*(4), 449–467. https://doi.org/10.1016/j.pecs.2012.03.002

Mahawan, M. A., Tenorio, M. F. N., & Gomez, J. A. (2015). Characterization of Flour from Avocado Seed Kernel, *3*(4), 34–40.

Nairobi, O. F., Food, O. F., Nutrition, S., Extraction, S., From, P., Seed, A., & By, W. (2006). 2..0\0, 1–20.

Nichols, N. N., Sharma, L. N., Mowery, R. A., Chambliss, C. K., Walsum, G. P. Van, Dien, B. S., & Iten, L. B. (2008). Enzyme and Microbial Technology Fungal metabolism of fermentation inhibitors present in corn stover dilute acid hydrolysate ☆, *42*, 624–630. https://doi.org/10.1016/j.enzmictec.2008.02.008

Onuki, S., Leeuwen, J. Van, Jenks, W. S., & Grewell, D. A. (2008). Ethanol production , purification , and analysis techniques : a review.

Otulugbu, K. (2012). Production of Ethanol From Cellulose. *Plastic Technology*, 1–46.

Parameters, P., Kernel, M. S., Application, C., Advisor, M. K., & Yimam, A. (2015). ADDIS ABABA UNIVERSITY ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT) SCHOOL OF CHEMICAL AND BIO- ENGINEERING, (June).

Potential for Bioethanol Production from Wet Coffee Pulp Waste in. (2014).

Pretreatment, S., Lignocellulose, O. F., Of, H., In, S., & To, M. (2010). Simultaneous pretreatment of lignocellulose and hydrolysis of starch in mixtures to sugars, *5*(Demirbas 2005), 2457–2469.

Produkcji, W., & Na, B. (2010). ENVIRONMENTAL IMPACT OF BIOETHANOL PRODUCTION, *2*(1).

Reddy, L., Obulam, V., Sarathi, R., & Wee, Y. (2011). Production of ethanol from mango (Mangifera indica L .) peel by Saccharomyces cerevisiae CFTRI101. *African Journal of Biotechnology*, *10*(20), 4183–4189. https://doi.org/10.5897/AJB10.2286

Sn, W. (2008). SUGARCANE BAGASSE : HOW EASY IS IT TO MEASURE ITS CONSTITUENTS ?, 266–273.

Teixeira, E. D. M., Luzia, A., Ro, D., Carvalho, F. De, & Aprigio, A. (2005). Preparation and Characterisation of Thermoplastic Starches from Cassava Starch , Cassava Root and Cassava Bagasse, 266–275. https://doi.org/10.1002/masy.200551133

Tekaligne, T. M., Woldu, A. R., Tsigie, Y. A., Science, C., Box, P. O., & Dar, B. (2015). Pretreatment Hydrolysis of the Pretreated Samples, *8*(1), 1–13.

Ververis, C. (2007). Cellulose , hemicelluloses , lignin and ash content of some organic materials and their suitability for use as paper pulp supplements, *98*, 296–301. https://doi.org/10.1016/j.biortech.2006.01.007

Weatherby, L. S. (1934). Composition of Avocado Seed, 132–134.

Woldu, A. R., & Tsigie, Y. A. (2015). Optimization of Hydrolysis for Reduced Sugar Determination from Avocado Seed Wastes, *3*(1), 1–10.

APPENDICES

Appendix A: Laboratory photos

Fig.1. Sample ready for pretreatment

Fig 2. Sample ready for hydrolysis

Fig 3. Vertical autoclave

Fig 4. Pretreated avocado seed

Fig 5. Filtered avocado seed

Fig 6. hydrolyzates Samples

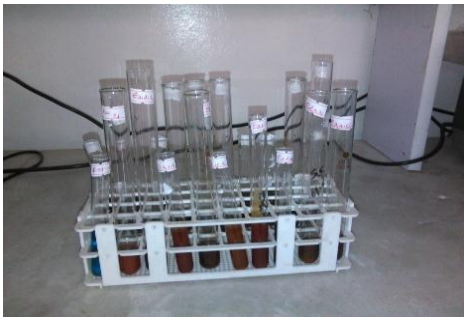


Fig 7. Sample ready to measure absorbance

Fig 8. Benedict's solution

Fig 9. Standard glucose solution

Fig 10. Prepared media

Fig 11. Sample ready for fermentation

Fig 10. Fermentation setup

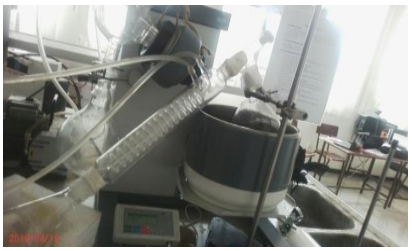


Fig 11. *Distillations*

Fig.14. produced ethanol sample

Appendix B: Properties of Ethanol

Molecular formula	C_2H_5OH
Molecular mass	46.07 g/mol
Appearance	Colorless liquid
Water solubility	(Between –117°C and 78°C)
Density	0.789 kg/l
Boiling temperature	78.5°C (173°F)
Freezing point	–117°C
Flash point	12.8°C (Lowest temperature of ignition)
Ignition temperature	425°C
Explosion limits	The lowest 3.5% (v/v) Upper 19% (v/v)
Vapor pressure at 38°C	50 mm Hg
Higher heating value (at 20°C)	29,800 KJ/kg

Lower heating value (at 20°C)	21,090 KJ/kg
Specific heat	Kcal/Kg 60°C
Acidity (p*K*a)	15.9
Viscosity	1.200 maps. s (20°C)
Octane number	99

Appendix C: concentrations and yield of glucose during hydrolysis

We have slop (m= 0.02751) and y intercept (b= -0.04394) then we can calculate the concentrations of unknown samples as follow :

$$C1 = \frac{Absorbance1 + 0.04394}{0.02751} = \frac{0.561 + 0.04394}{0.02751} = 21.95 mg/ml$$

$$C2 = \frac{Absorbance2 + 0.04394}{0.02751} = \frac{0.205 + 0.04394}{0.02751} = 9.049 mg/ml$$

$$C3 = \frac{Absorbance\ 3 + 0.04394}{0.02751} = 0.45 + 0.04399/0.02751 = 21.94 mg/ml$$

$$C4 = \frac{0.230 + 0.04394}{0.02751} = 20.65 mg/ml$$

$$C5 = \frac{0.620 + 0.04394}{0.02751} = 23.17\ mg/ml$$

$$C6 = \frac{0.210 + 0.04394}{0.02751} = 21.08\ mg/ml$$

$$C7 = \frac{0.571 + 0.04394}{0.02751} = 22.79\ mg/ml$$

$$C8 = \frac{0.189\ 3 + 0.04394}{0.02751} = 21.67\ mg/ml$$

$$C9 = \frac{0.691 + 0.04394}{0.02751} = 26.54\ mg/ml$$

$$C10 = \frac{0.370 + 0.04394}{0.02751} = 23.86\ mg/ml$$

$$C11 = \frac{0.39 + 0.04394}{0.02751} = 22.90\ mg/ml$$

$$C12 = \frac{0.311 + 0.04394}{0.02751} = 23.75\ mg/ml$$

$$C13 = \frac{0.47 + 0.04394}{0.02751} = 23.11 mg/ml$$

$$C14 = \frac{0.421 + 0.04394}{0.02751} = 24.24\ mg/ml$$

$$C15 = \frac{0.58 + 0.04394}{0.02751} = 25.89\ mg/ml$$

$$C16 = \frac{0.551 + 0.04394}{0.02751} = 25.20\ mg/ml$$

$$C17 = \frac{0.59 + 0.04394}{0.02751} = 24.98 mg/ml$$

$$C18 = \frac{0.601 + 0.04394}{0.02751} = 25.91 mg/ml$$

$$C19 = \frac{0.578 + 0.04394}{0.02751} = 25.63\ mg/ml$$

$$C20 = \frac{0.569 + 0.04394}{0.02751} = 225.31\ mg/ml$$

$$Yield = \frac{gram\ of\ glucose\ produced}{raw\ material\ used} * 100$$

Raw material used = $\frac{gram\ of\ sample\ used}{ml\ solution} = \frac{5.58g}{104.23ml}$ = 53.5mg/ml

Y1 = $\frac{21.95mg/ml}{53.5mg/ml} * 100$ = 41%

Y2 = $\frac{21.67mg/ml}{53.5mg/ml} * 100$ = 40.5%

Y3 = $\frac{21.94mg/ml}{53.51mg/ml} * 100$ = 41%

Y4 = $\frac{20.65mg/ml}{53.51mg/ml} * 100$ = 38.6%

Y5 = $\frac{23.17mg/ml}{53.51mg/ml} * 100$ = 43.3%

Y6 = $\frac{21.08mg/ml}{53.51mg/ml} * 100$ = 39.4 %

Y7 = $\frac{22.79mg/ml}{53.51mg/ml} * 100$ = 42.6%

Y8 = $\frac{21.67mg/ml}{53.51mg/ml} * 100$ = 40.5%

Y9 = $\frac{26.54mg/ml}{53.51mg/ml} * 100$ = 49.6%

Y10 = $\frac{23.86\ mg/ml}{53.51mg/ml} * 100$ = 44.6%

Y11 = $\frac{22.90mg/ml}{53.51mg/ml} * 100$ = 42.8%

Y12 = $\frac{23.75mg/ml}{53.51mg/ml} * 100$ = 44.1%

Y13 = $\frac{23.11\ mg/ml}{53.51mg/ml} * 100$ = 43.2%

Y14 = $\frac{24.24mg/ml}{53.51mg/ml} * 100$ = 45.3%

Y15 = $\frac{25.89mg/ml}{53.51mg/ml} * 100$ = 48.4%

Y16 = $\frac{25.20\ mg/ml}{53.51mg/ml} * 100$ = 47.1%

Y20 = $\frac{25.31mg/ml}{53.51mg/ml} * 100$ = 47.3%

Appendix D: ***Regression coefficients and the corresponding 95% CI High and Low***

Factors	Coefficient of estimate	Standard error	95% CI low	95% CI high
Intercept	47.32	0.33	46.59	48.06
A-acid con.	-1.31	0.31	-1.99	-0.63
B-temper.	0.060	0.31	-0.62	0.74
C-time	0.76	0.31	0.080	1.44
AB	-0.11	0.34	-0.87	0.65
AC	-0.49	0.34	-1.25	0.27

BC	0.19	0.34	-0.57	0.95
A^2	-0.036	0.58	-1.33	1.26
B^2	-3.69	0.58	-4.98	-2.39
C^2	-2.89	0.58	-4.18	-1.59