Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio Engineering

‘’Production of Ethanol from Crude Glycerol Obtained from Transesterification Process Using Eschericha Coli’’

A final thesis submitted to the school of chemical and bio-engineering, Addis Ababa institute of technology, Addis Ababa University in partial fulfillment of the requirement for the degree of Master of Science in Process Engineering.

By:

Samson Adissu

Addis Ababa University
Addis Ababa, Ethiopia
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Table of Contents

List of tables

List of figures

List of Acronyms

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
$C_3H_8O_3$	Glycerol or glycerin
$C_6H_{12}O_6$	Glucose
CH_3CH_2OH	Ethanol
CO_2	Carbon dioxide
E.coli	Escherichia coil
FFA	Free Fatty Acid
FT-IR	Fourier transform Infrared spectra
g/L	Gram per liter
g/g	Gram per gram
glyDH	Enzyme
GTBE	Glycerol tertiary butyl ether
h^{-1}	1 divided by hour
H_2	Hydrogen
KOH	Potassium hydroxide
M	Mole per liter
mM	Milimole per liter

MONG	Matter organic non-glycerol
NaCl	Sodium Chloride
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
Nm	Nanometer
OD_{600}	Optical density at 600 nanometer wavelength

Abstract

As a by-product of biodiesel production, glycerol has now become an abundant and cheap source of carbon. Conversion of this glycerol to higher value products will increase the economic viability of the biodiesel production process. The aim of this project work was to produce Bio-ethanol using crud and purified glycerol obtained from by-product of biodiesel production. A full factorial experimental design was used to investigate the effects of glycerol concentration, inoculum concentration and agitation speed on the production of ethanol via pure and crude glycerol by means of fermentation using Escherichia coli 0157:H7. Escherichia coli 0157:H7 was obtained from Ethiopian Biodiversity institute, Addis Ababa, Ethiopia and the crude glycerol were obtained from the transesterification process of soybean oils. Chemicals and reagents used were bought from local market (Niway PLC, Addis Ababa). The production of ethanol from purified and crude glycerol was compared. Maximum ethanol yield was obtained with a crud glycerol concentration of 10 g/L was 0.38 g/g glycerol under 7.5 % (v/v) of inoculum and 75 rpm of agitation speed and 0.42 g/g of glycerol under 10 % (v/v) of inoculum and 150 rpm of agitation speed. The predicted optimum ethanol yield 0.361252 was observed at the process variables 10 g/L glycerol concentration, 85.57 rpm speed of agitation and 8.8 % (v/v) of inoculum concentration.

Keywords*: Bioethanol, Biofuel, Transesterification, Fermentation and Escherichia coli*

CHAPTER ONE

1. Introduction

1.1. Background

Ethanol fuel is currently produced on large scale using yeast fermentation of glucose obtained from corn crops and sucrose from sugar cane (Lin et al., 2006). Although much work has gone into improving ethanol yield from these processes, there remains a need for increase in throughput and decrease in costs (Wheals et al., 1999). The environmental advantages of ethanol over traditional fuels such as gasoline stem from its renewable nature since it is generated from plants that is recently fixing carbon dioxide, thus negating the greenhouse gas effect of combustion (Wheals et al., 1999). However, irrespective of process improvement, there remains a need to find non-edible feedstock for ethanol production.

Present day interest in biofuels has caused significant growth in the sector of biodiesel production. This has, in turn, significantly increased glycerol production, as it is the major by-product of biodiesel production (Pagliaro et al., 2007). For every 100 kg of biodiesel produced from the transesterification of oil, 10 kg of glycerol is produced (Yazdani et al., 2007). As a result of this surplus of glycerol, there has been a 10 fold decrease in glycerol price, creating the necessity for a method of treatment for this waste stream of biodiesel production (Yazdani et al., 2007). Glycerol has become an abundant source of carbon that is now equivalent in price to traditional fermentation sugar stocks. A cost analysis of ethanol production from glycerol demonstrates that ethanol from glycerol costs 0.17$/L versus 0.28$/L from corn (Yazdani et al., 2007). This is due mainly to complexity of the ethanol production process when corn is used as substrate versus the relative simplicity of the process when glycerol is used as substrate.

The capacity to ferment glycerol has been reported in species of multiple genera, including Klebsiella, Citrobacter, Enterobacter and Bacillus (Murarka et al., 2007). Microbial production of hydrogen and ethanol from crude glycerol has been successfully carried out using E. aerogenes (Ito et al., 2005). However, the usage of such micro-organisms on an industrial scale poses several issues, including the requirement of strict anaerobic conditions, requirement of supplementation with rich and cost ineffective nutrients and high sensitivity to contamination (Murarka et al., 2007). It has recently been reported that Escherichia coli (E. coli) produces ethanol and hydrogen from glycerol in a pH dependent manner, with an optimal pH for ethanol production of 7 (Dharmadi et al., 2006).

E. coli is highly amenable to use in industrial applications. For ethanol production, advantages include its capability to ferment a wide range of sugars and previous industrial use for the production of recombinant proteins. The fermentation of glucose by E. coli for the production of ethanol has been extensively studied. The fermentative pathway for glycerol is similar to that of glucose. In addition, the theoretical yield of ethanol from glycerol is higher than that from other traditional sugars, such as glucose (Yazdani et al., 2007). Chemical equations of ethanol production from glycerol are given in Equations.

The ability of E. coli to transform crude glycerol, waste of biodiesel production, into ethanol will allow for a zero waste process stream, leading to an increase in the economic viability of biofuels industry. The main aspect of this investigation is to study and compare the use of purified glycerol and crude glycerol as the carbon source for anaerobic growth of E. coli in order to produce ethanol. The comparison was carried out in two separate experiments. For purified glycerol and crude glycerol, the effect of carbon source is investigated by a comparative growth analysis of E. coli in the two substrates under anaerobic conditions. For purified glycerol and crude glycerol, the effects of initial glycerol concentration, supplement concentration and agitation speed on final ethanol concentration and dry mass are tested.

This research work is focused on the production of ethanol from pure and crude glycerol obtained from transesterification process.

$C_3H_8O_3 \longrightarrow CH_3CH_2OH + CH_2O_2 / (H_2 + CO_2)$……………………………...Equation 1.1

1.2. Statement of the problem

Environmental issues such as global warming, supply instability and fluctuating prices of fossil fuel have led to increasing interest in renewable resources. Converting biomass-derived oil to biofuels is being contemplated as a promising substitute for petroleum-based liquid fuel. Glycerol is one of the inevitable by-products of biodiesel fuel production. In the process of biodiesel production, 10 kg of crude glycerol is generated for every 100 kg of biodiesel produced by transesterification of vegetable oils or animal facts. Due to recent fast growth of the biodiesel industry and the resulting surplus of glycerol, the price of crude glycerol has dropped substantially The collapse in prices has led to the shutdown of many glycerol producing plants and affected the biodiesel industry itself. As a result, the produced glycerol is a waste stream instead of being a desirable co- product that could contribute in the economic viability of biodiesel production plants. Some biodiesel plants pay for the appropriate disposal of the glycerol since it cannot be disposed in the environment. Thus, improvement of bioconversion methods of low-price glycerol into higher value products is a good strategy to add economic viability to the biodiesel industry.

In Ethiopia the per capita consumption of petroleum is 0.52 bbl per day per 1000 people, this petroleum is imported at the expense of huge foreign exchanges. Ethiopia's over dependence on biomass fuel and the insignificant use of energy in the commercial, industrial and utility sector of the economy, clearly demonstrate the energy poverty of the country. Energy poverty evidently slows down the rural development to a great extent.

The government's interest in biofuels was later re-emphasized in the Growth and Transformation Plan (GTP). Over a five year period (2010–2015), Ethiopia's GTP foresees increasing the production of ethanol to 194.9 million liters, biodiesel to 1.6 million liters and an increase in blending facilities to 8 for ethanol and to 72 for biodiesel. The essential aspects of the focused projects include the establishment of more integrated ethanol distilleries, ethanol blending stations, engine modifications and replacement of wood fuels by the ethanol based stoves.

In view of these expectations, various biofuel developers have been promoted by the Ethiopian Government. Some of these sectors are received about 538,384 hr of land aiming at the cultivation of oil crops for biodiesel production but none of this start producing biodiesel. Instead of producing biodiesel they export the oilseed abroad. Bioethanol production from glycerol is not well known in our country Ethiopia.

1.3. Objectives

1.3.1. General objectives

The general objective of this study was product development of ethanol from crude glycerol obtained from byproduct of transesterification process by using E.coli.

1.3.2. Specific objectives

The specific objectives of this project work were to:

1. To produce biodiesel using soybean oil
2. To characterize the crude glycerol obtained from biodiesel production
3. To evaluate effect of glycerol concentration, inoculum concentration and agitation speed in lab scale bioreactor on the fermentation of pure and crud glycerol using *E. coli* to produce ethanol.

1.4. Significance of the study

In general the significance of this study can be seen from different perspectives. Such as;

- Utilization of glycerol as a by-product has more significant economic value instead of discarding it as a waste.
- Since the purification process of glycerol for pharmaceutical, food and beverage industries required intensive cost; almost all the glycerol produced from biodiesel synthesis has been discarded as waste to the environmental. This study has focused to solve this issue in reducing the environmental impact by converting to ethanol using E.coli.
- The annual energy supply to demand context of Ethiopia is increasing linearly and expected to continue simultaneously. This implies the need for renewable and sustainable energy option is necessarily important to inspire the energy demand by completely converting the glycerol to energy directly.
- It gives knowledge about the production of ethanol from glycerol by using Bio conversion method.
- Attract Investors to participate on the production of biodiesel in our country.

CHAPTER TWO

2. Literature Review

2.1. The Biofuels Industry

Present global energy requirements are fulfilled primarily via fossil fuel combustion, thus the world is dependent on a non-renewable resource for its energy needs. However, in response to growing environmental impacts and concern over dependence on oil from politically unstable regions, there has been increasing focus on the need for alternative sources of energy (Hansen et al., 2005). Research into sustainable and renewable fuel sources has led to advance development in the area of biofuels which are produced from renewable carbon sources. Today, there are three general platforms of feedstock for biofuel production: sugar (Service, 2007), oil (Marchetti et al., 2007) and syngas (Henstra et al., 2007). Examples of biofuel products from the first two platforms, which are presently more developed, include ethanol derived from fermentation of sugars from corn (Gray et al., 2006) and biodiesel produced by transesterification of used cooking oil (Marchetti et al., 2007).

2.1.1. Limiting Forces of Growth of the Biofuels Industry

Although the area of biofuels is promising, the most significant problem facing it today is a lack of economic feasibility (Yazdani et al., 2007). Ethanol is produced by fermentation of food stocks, such as corn, sugarcanes and sugar beets. In response to increasing global attention, many governments, including that of Canada and the United States of America, have highly subsidized ethanol production. The resulting boom in the industry in North America has caused substantial rises in the price of the feedstock, mainly corn. The side-effect on food prices demonstrates the existence of a need to find non-dietary carbon sources for the production of ethanol to prevent increasing scarcity of food for the human population.

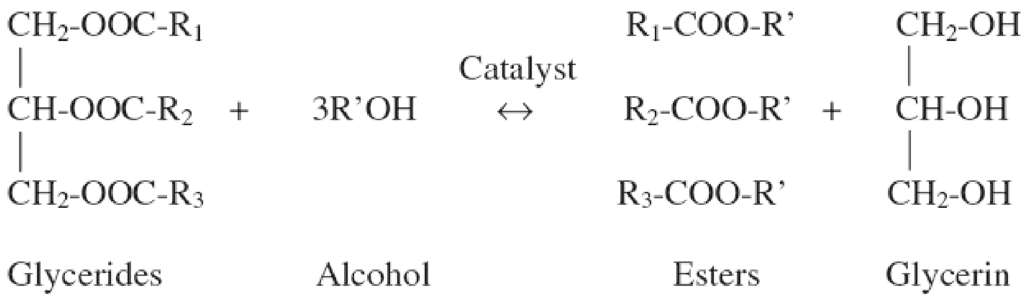
Biodiesel fuel consists of fatty acid methyl or ethyl esters which are produced from oil. This ‘diesel’ can be used directly in engines and is produced from vegetable oils and animal fats, making it an alternative to traditional petroleum based products. The production process requires the transesterification of these oils or fats with alcohol in the presence of a catalyst to produce esters (Miguel et al., 2001).

In a manner similar to the problems facing ethanol production, biodiesel production also lacks economic feasibility. As an example, the production cost for biodiesel that is sold at a market price of \$0.80/L from soybean is \$0.78/L (www.thejacobsen.com). The industry requires substantial government subsidies for success in North America.

The lack of economic viability is the major limiting force facing the growth of both the bioethanol and the biodiesel industries. One way to increase economic viability of both biofuels is to develop ‘bio-refineries’ (Kamm et al., 2007). Traditionally, this method involves the generation of small quantities of a high value product alongside the main product, off-setting production costs. However, the concept of bio-refineries could also be used to utilize the waste stream of biodiesel production as feedstock for ethanol production therefore working towards a zero waste process (Yazdani et al., 2007).

2.1.2. The biodiesel production process

Biodiesel is produced by transesterification of oils; thus the raw material for the production of biodiesel is renewable. The type of vegetable oil used in the process can vary and can be refined oil, crude oil or used frying oils (Marchetti et al., 2005). The process involves the reaction of the triglycerides with alcohol in the presence of a catalyst to produce esters, which constitutes biodiesel, and glycerol; this reaction is depicted in figure 3.1



$$\begin{array}{lcccccc} CH_2\text{-}OOC\text{-}R_1 & & & & R_1\text{-}COO\text{-}R' & & CH_2\text{-}OH \\ | & & & \text{Catalyst} & & & | \\ CH\text{-}OOC\text{-}R_2 & + & 3R'OH & \leftrightarrow & R_2\text{-}COO\text{-}R' & + & CH\text{-}OH \\ | & & & & & & | \\ CH_2\text{-}OOC\text{-}R_3 & & & & R_3\text{-}COO\text{-}R' & & CH_2\text{-}OH \\ \text{Glycerides} & & \text{Alcohol} & & \text{Esters} & & \text{Glycerin} \end{array}$$

Figure 2-1. Chemical reaction for the production of biodiesel (Miguel et al., 2001)

The overall reaction depicted above actually consists of a sequence of three reactions, all of which are reversible. The reactions involve the step wise reduction of triglycerides to diglycerides then monoglycerides and finally to glycerol; with each step producing esters as well. The stoichiometric balance for the reaction requires a ratio of alcohol to oil of 3 to 1; however excess alcohol favours the direction of the reaction towards the desired product (Marchetti et al., 2005).

The variables that affect the yield of biodiesel depends on six important reaction conditions: reaction temperature, type of oil, ratio of alcohol to oil, amount and type of catalyst and mixing speed (Marchetti et al., 2005). This catalyst can be a base, acid or lipase.

Base catalysts are typically sodium hydroxide or potassium hydroxide; base catalysts can be used with alcohols methanol or ethanol and any kind of oil, be it refined, crude or frying. Typically, for base catalysts, the ratio of alcohol to oil is typically 6:1, amount of catalyst can vary from 0.5% to 1% (Ma et al., 1999; Barnwal et al., 2005) and reaction temperature is 60 °C (Barnwal et al., 2005; Zhang et al., 2003). The alkali process is less corrosive and more efficient than using an acid but is sensitive to the purity of the oil and water content; if too much water is involved then the reaction can produce soap instead of biodiesel.

For acid catalysts, the most typically used one is sulphuric acid (Freedman et al., 1984; Harrington et al., 1985). The yield of the reaction is high but the reaction itself is very slow, taking more than a day for completion. For example; conversion of more than 99% has been reported using a ratio of 1 mol % sulphuric acid to oil of 30 to 1 for 50 hours at 65 °C (Freedman et al., 1986). Using acid catalysts is a better choice if the oil has high free fatty acid content.

Lipase enzymes have been recently found to have the ability to catalyze transesterification and esterification of reactions. Lipases have been used to catalyze the production of biodiesel from waste cooking oil and use of lipases presents an excellent alternative to use of acids or bases due to ease of glycerol recovery and high purity of the biodiesel produced; research is currently underway to produce immobilized enzymes for this reaction (Perez, 2003).

The process of biodiesel production requires alcohol that must be recovered to optimize the process; this is especially true in the case of methanol, which is toxic. For both acid and base catalysts, an excess of alcohol is used to push the reaction towards favoring ester production.

2.2. Glycerol Conversion for increasing economic viability

2.2.1. Glycerol as waste of biodiesel production

Not long ago, glycerol was considered a valuable product, with processes designed for its production. If the price of glycerol had remained at the level it was in 2004, the glycerol produced during the biodiesel process would have served as an important source of revenue

(Yazdani et al., 2007). However, the recent growth in the biodiesel industry has been so severe that a surplus of glycerol is now present in the market, causing prices to drop substantially, as much as 10 fold (Yazdani et al., 2007). The industry that produces glycerol as waste is primarily the biodiesel industry (Thompson et al., 2006).

Due to a surplus of glycerol in the market, glycerol producing plants owned by companies such as Dow Chemical have shut down, and crude glycerol is now considered a waste, with an associated disposal cost (McCoy, 2006; Hazimah et al., 2003).

Glycerol occurs in oil, particularly vegetable oil, at a level of 10% by mass (Pagliaro et al., 2007). Thus for every 100 kg of biodiesel produced, there are approximately 10 kg of glycerol generated (Yazdani et al., 2007). The glycerol generated during the process is crude and requires further processing, which is cost intensive and difficult, for the production of pure commercially viable glycerol. The characterization of the crude glycerol produced during the biodiesel manufacturing process depends on the oil feedstock that was used, as well as the biodiesel production process method utilized, focusing primarily on the type of catalyst (Marchetti et al., 2005).

The nutritional value of crude glycerol has also been evaluated to investigate potential for use as supplement in animal feed (Pagliaro et al., 2007). This analysis focused on the nutritional content of the parent oil and the crude glycerol produced from the biodiesel production process. To date, the nutritional value of the glycerol remains unclear however the glycerol analysis indicates that it consists mainly of carbohydrates and could thus be combined with a high protein meal for use as feed supplement (Thompson et al., 2006). Crude glycerol contains unreacted glycerides and may contain soap, mainly when WVO is used as source oil. It also contains ash which is mainly the sodium from the catalyst. In addition, the excess alcohol from the biodiesel production process will end up in the glycerol layer. The process for the refinement of crude glycerol involves filtration, chemical additives and fractional vacuum distillation.

Glycerol has applicability in a variety of markets: It includes the food industry, personal care and drugs and pharmaceuticals to name a few (Pagliaro et al., 2008). These industries do however necessitate pure glycerol and are currently saturated with supply. Biodiesel producers that operate on large scale refine the crude glycerol that is produced and sell it to the applicable industries, listed above. However, the process of purifying the crude glycerol produced during biodiesel production is expensive and cost-prohibitive for biodiesel plants that are on the smaller scale.

Development of processes that utilize glycerol, and especially crude glycerol, would be beneficial not only to the biodiesel industry but also to other industries that produce glycerol as a major by-product or waste, as many industries do, including the oleochemical industry which generate waste streams consisting of 55-90 % glycerol (Hazimah et al., 2003).

2.2.2. Transformation of Glycerol to high value products

There are several methods of converting glycerol into higher value products, based on both chemical and biological transformations.

2.2.2.1. Chemical methods for glycerol conversion

Chemical methods of glycerol transformation have the drawbacks of problems related to low product specificity and the requirement of extreme pressure and extreme temperatures. In addition, chemical catalysis requires high purity glycerol, eliminating the potential of using crude glycerol waste as reactant (Yazdani et al., 2007). Chemical methods do, however, have the potential for synthesis of a large variety of products.

Selective oxidation is difficult with glycerol due to the reactive characteristics of the hydroxyl groups; from selective oxidation of the primary hydroxyl group in glycerol, there is potential for the production of glyceric acid and further on, tartronic acid (McMorn et al., 1999). Both of these compounds are commercially useful. Selective oxidation of the secondary hydroxyl group in glycerol yields dihydroxyacetone (DHA), the main active ingredient in the preparation of all sunless tanning beauty products. The production of DHA from glycerol is very simple; there is direct conversion of glycerol to DHA via anodic oxidation when an electrical potential is applied in the presence of 2,6,6- Tetramethylpiperidinyloxy (TEMPO), a catalyst (Hunt, 1999; McCoy, 2006). Oxidation of all three hydroxyl groups, done in the presence of CeBiPt/C catalyst under either basic or acidic conditions, produces polyketomalonate (PKM), a molecule used in household detergents (Pagliaro et al., 2007).

There are many molecules that are favorably added to fuel to improve performance in combustion engines. Pure glycerol cannot be used as an additive due to properties of polymerization at high temperature, which would lead to engine clogging; however, derivatives of glycerol have potential for applicability. An example of such a molecule is glycerol tertiary butyl ether (GTBE); when added to diesel fuel, which contains 30 to 40% aromatic compounds as standard, GTBE can lead to significant reduction in the emission of particulate matter, hydrocarbons, unregulated aldehydes and carbon monoxide (Liotta et al., 1994). The synthesis of alkyl esters from glycerol is easy and involves the reaction of glycerol with isobutylene in the presence of an acid catalyst. At present, methyl tertiary butyl ether (MTBE), a toxic compound, is used as the main additive; GTBE derived from glycerol has the potential to replace it (Pagliaro et al., 2007).

Conversion of glycerol to propylene glycol (1,2-propanediol) via a two-step process has resulted in the manufacturing of an anti-freeze product which contains 70% propylene glycol and 30% glycerol and can thus be produced and sold by existing biodiesel facilities (Boswell, 2005). The reaction involves the formation of acetol at atmospheric pressure followed by hydrogenation at high temperature (200 °C) and pressure (1000 kPa) (Dasari et al., 2005). The yield and cost of producing propylene glycol from glycerol in this fashion is significantly less than that of production utilizing petroleum products as starting material.

Other chemicals that can be produced from glycerol include Acrolein, a molecule that is an intermediate in the reaction pathways for many industrially produced chemicals, specifically in the production of detergents, absorbent polymers and acrylic acid esters (Girke et al., 2005). Acrolein is produced from glycerol is high yields via glycerol dehydration by passing a glycerol-water gas mixture over an acidic solid catalyst at specific temperature and catalyst conditions. This process has not gained popularity as more economically favourable methods for the production of acrolein exist (Ott et al., 2006).

Glycerol can also be used for the production of syngas (synthesis gas), containing hydrogen and carbon monoxide. This is done under relatively mild conditions at temperatures between 225 and 300 °C in a single reactor using platinum based catalyst (Soares et al., 2006). Although the reaction to produce syngas from glycerol is highly endothermic, the energy balance is favourable when this reaction is coupled with the conversion of syngas to alkanes, a reaction that is highly exothermic. Using these coupled reactions, there is an overall gain of - 30 kcal per mole of glycerol (Pagliaro et al., 2007).

2.2.2.2. Biological conversion of glycerol to ethanol

Biological methods of glycerol transformation present an alternative that may be considered favorable, when the nature of the substrate is considered. Due to decrease in market value, glycerol is at a current price of 5.6 cents/kg, making it competitive with common sugars used in biological processing for the production of chemicals and fuels (Yazdani et al., 2007). The usage of biological methods would help avoid some of the problems associated with chemical methods while still offering the possibility of generating a wide variety of products.

In comparison to aerobic metabolism, relatively few microbes are capable of metabolizing glycerol anaerobically (Neidhardt et al., 2005; Schuller, 2003). However, anaerobic fermentation is however more economically favourable due to l ower operational and capital costs. For the microorganisms that have been identified with the ability to ferment glycerol, the fermentation of glycerol is linked to the production of highly reduced compounds (Bouvet, 1995). The compound produced during fermentation is specific to the microbe utilized. The compounds currently identified to be capable of being produced from glycerol fermentation by various microbes include 1,3-propanediol, butanol, formate, propionic acid, succinic acid and ethanol (Yazdani et al., 2007).

When compared to common sugars such as glucose, glycerol contains highly reduced carbon atoms (Nielsen et al., 2003). As a result, cellular conversion of glycerol to compounds such as pyruvate generates twice as many reducing equivalents as those produced by the conversion of glucose. This means that glycerol fermentation enables higher yields in fuels and reduced chemicals than common sugars do. Production of compounds such as ethanol is limited in glucose based fermentation due to a shortage of reducing equivalents, presenting a clear advantage for the use of glycerol as substrate instead (Yazdani et al., 2007). Thus glycerol has the possibility for use as carbon source for the production of both ethanol and other high value products.

Fermentation requires the absence of external electron acceptors such as oxygen. The utilization of glycerol as carbon source until now has been limited due to a lack of microorganisms capable of fermenting it anearobically. However, the ability to metabolize glycerol by fermentation has been reported in several species of the genera of E.coli, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium*, and *Anaerobiospirillum* (Yazdani et al., 2007).

2.2.3. Biochemistry of glycerol fermentation by E. coli

It has recently been found that *E. coli* has the capability to metabolize glycerol anaerobically (Dharmadi et al., 2006). A potential reason for no previous observation of this phenomenon is the prevention of the fermentation by fast accumulation of hydrogen, due to the resulting redox imbalance (Murarka et al. 2008). Hydrogen is an inhibitor of the process and the recycling of hydrogen produced during glycerol fermentation acts as negative feedback into the process. The role of hydrogen as an inhibitor was identified when fermentation of glycerol was conducted with *E. coli*; the mutant was capable of maintaining the redox balance even in the presence of hydrogen, something the wild type strain MG1655 was unable to do (Murarka et al., 2008).

2.2.3.1. Metabolic pathways of glycerol fermentation

During fermentation of traditional sugars such as glucose, the synthesis of acetate is required in the metabolic pathway (Hasona et al., 2004; Sawers et al., 2004). However, for the fermentation of glycerol, the synthesis of ethanol becomes the necessary requirement, with the acetate pathway becoming optional (Murarka et al., 2008). There are two major functions that a metabolic pathway must fulfill: maintaining redox balance in the cell and generation of ATP to provide energy for the cell using substrate-level phosphorylation (Murarka et al., 2008). In glycerol fermentation, it is the pathway of synthesis of ethanol which fulfills these requirements. This pathway, which generates ethanol, results in the net production of 1 ATP per molecule of glycerol metabolized (Unden et al., 2004). ATP yield during fermentation of traditional sugars is limited by the redox balance requirements, producing for example only 0.9 ATP per molecule of xylose metabolized (Unden et al., 2004). Thus, ATP yield from glycerol metabolism is superior.

The conversion of glycerol to other metabolites such as succinate would also result in a balance of redox potential in the cell. However, the amount of ATP generated per molecule of glycerol metabolized would be decreased in comparison to the production of ethanol (Unden et al., 2004). This may present the potential reason for the low levels of succinate production that are seen in glycerol fermentation processes. There is also no significant effect observed when the conversion of fumarate to succinate is blocked, demonstrating that the ethanol production pathway is the metabolic determinant of glycerol fermentation, ensuring generation of ATP and maintaining redox balance in the absence of external electron acceptors (Murarka et al., 2008).

2.3. Ethanol as fuel

Current production for ethanol used as fuel is by yeast fermentation of sugars and then separation by distillation (Lyon et al., 1995; Laluce, 1991). The major producer of ethanol fuel in the world is Brazil at 10.5 billion litres of hydrous and 6.5 billion litres of anhydrous produced from sugar c ane in 1998 (Wheals et al., 1999). In that year, 0.24 billion litres of anhydrous ethanol was produced in Canada, primarily from corn.

The production of ethanol from maize can be by wet or dry milling (Pimentel, 1991). For wet milling, the grain is first steeped in water with Sulphur dioxide and then ground. The starch is separated from the co-products. The starch is then broken down into smaller sugars by cooking and addition of α-amylase enzyme (Wheals et al., 1999). The sugars broken down to glucose are then fermented to ethanol using yeast. Dry milling entails initial grinding of entire corn kernels into flour (referred to as meal). In this case, the starch and other components are not separated and the milled kernels are mixed with water, treated with enzymes and placed into fermenters. The waste is centrifuged, dried and potentially used for animal feed. Dry milling plants are generally smaller than wet milling plants and require less capital investment. As a result, all ethanol plants that have come online in the recent past have been dry milling plants (Wang et al., 2007). In the steps following, the process is the same as that of wet milling. When using yeast, it is important that the sugar mixture leaving the milling process be sterile.

2.3.1. Comparing Economics of Pathways of Ethanol Production

Ethanol produced by fermentation has a co-product due to the pathways. The advantage of glycerol fermentation is clear when ethanol is produced with either hydrogen or formate as the co-product. A comparison of the manufacturing facilities and costs associated with the feedstock demonstrates that ethanol production from corn requires a more complex facility and thus more capital investment (Yazdani et al., 2007). The operational costs of ethanol production from glycerol are also 40% lower than from corn (Shapouri et al., 2006).

When the co-produced product such as hydrogen or formate is assigned value (as it will, since hydrogen is a useful fuel), the use of glycerol becomes even more economically favorable (Yazdani et al., 2007). The same arguments can be made when considering the production of the other higher value compounds possible from glycerol fermentation using different microbes.

2.4. Literature Summary

As glycerol prices drop, the use of waste glycerol streams to produce higher value products could serve as a potential path to economic viability for the biofuels industry. Biodiesel production produces large quantities of crude glycerol; due to availability and low cost, this glycerol in turn serves as a very attractive carbon source for use in fermentation processes. Compared to traditional carbon sources of fermentation such as glucose, the reduced nature of carbon atoms in glycerol enables the production of highly reduced products. When a microorganism such as *E. coli* is capable of utilizing glycerol, the process becomes increasingly attractive, given the feasibility of engineering *E. coli* to suit industrial needs.

The ability of *E. coli* to ferment glycerol opens the doors to developing a platform for production of high value reduced products produced at higher yields from glycerol than by traditional sugar stocks. The production of ethanol with a co- product such as hydrogen presents obvious advantages when facing the increasing interest in renewable energy sources. The development of *E. coli* based fermentation of glycerol for the production of ethanol facilitates the development of the biofuels industry by increasing economic viability of biodiesel production and providing a non-food carbon source for the production of bio-ethanol. Fermentative utilization of glycerol by *E. coli* is a new path to biofuels and biochemical.

CHAPTER THREE

3. Methodology

3.1. Raw material source and equipment's

In order to produce bioethanol from crude glycerol obtained from biodiesel production. *Escherichia coli* 0157:H7 was obtained from Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia holding it by ice bag. The crude glycerol were obtained from the transesterification process of soybean oils. Chemicals and reagents used were: Ethanol 96%, Tryptone, yeast extract, Nutrient broth, and agar powder NaCl 99%, CaO 96%, NaOH 99%, H_3PO_4 99%, $KHSO_4$ 99%, HCl 35% bought from local market (Niway PLC, Addis Ababa). All chemicals were used in microbial medium for analytical methods were laboratory and analytical grades.

The equipment's used for this thesis work were analytical balance, flask, test-tube, incubator shaker, autoclave, UV Spectrophotometer, Density meter, pH meter, micropipette, Fourier transfer infrared spectrophotometer, Rotary evaporator, moisture content analyzer, Density meter, centrifuge, digital magnetic stirrer, water bath fitted with thermostat and separation funnel.

3.2. Experimental procedure for biodiesel production

Glycerol samples were obtained from batch transesterification of soybean oils and ethanol, CaO was used as a catalyst, the reaction was carried out in 500-mL flasks placed in an Excella E24 incubator Shaker set at 60°C and at 240 rpm for 60 min. After 1 hr on the shaker bath content of the flask was transferred into a separator funnel and allowed to settle overnight. The two layers were separated, the top layer was biodiesel and the bottom darker layer was the by-product glycerol. Finally, removal of the crud glycerol from the biodiesel was done.

3.3. Purification of Crude Glycerol

Crude glycerol samples obtained from the Production of biodiesel were transferred to purification process as it contains impurities like ash, ethanol, water and residuals. The purification was carried out by simple distillation at 78^oC for 20 min to remove ethanol which was not recovered during biodiesel manufacturing process, then after further purification was continued with phosphoric acid. These acid was added to the glycerol and placed in the shaker bath at 37 ^oC for one hour, then the content of the flask was transferred to the separator funnel and allowed for settling to separate the middle glycerol layer to which 20 mL of 50% NaOH were added for decolourization and centrifuged to remove the impurities from the sample. The purified glycerol was used for culture media.

3.4. Characterization of crude glycerol

Characterization of glycerol was conducted at the school of chemical and Bio-Engineering Laboratory at the Addis Ababa University, Addis Ababa institute of technology.

After the purification process, crude glycerol turns as usable for the experimental work and the glycerol can be tasted its presence by adding a pinch of $KHSO_4$ to 5 mL of purified glycerol and heated for a short time of period.

3.4.1. Determination of viscosity

Viscosity taste was conducted and reported in centistokes (cs) using SV-10 vibro viscometers according to ASTM standard method (ASTM D445) at 40°C with Grant Model TXF200 water bath (Grant Instrument Company, Cambridge Ltd) at AAiT school of chemical and Bio-Engineering, Addis Ababa.

3.4.2. Determination of pH value

The pH was measured using JENWAY 3505 pH meter, pH electrode was standardized with buffer solution, the electrode immersed into the samples and the pH value was recorded.

3.4.3. Determination of conductivity

The conductivity was measured using conductivity meter, the conductivity electrode immersed into the glycerol layer and reported as $\mu S\ cm^{-1}$.

3.4.4. Determination of total Chemical oxygen demand (Dichromate Method)

Total chemical oxygen demand was conducted using HI 839800 COD reactor and NANOCOLOR 400 D COD photometer. The reactor was first heated for 30 min, 2 ml of glycerol sample was added to COD reagent and inserted into the reactor for 2 hours. The sample was cooled by atmospheric air for 15 min, then the COD value was recorded from COD photometer.

3.4.5. Determination of moisture content

A moisture content was measured using MB45A moisture content analyzer.

3.4.6. Determination of free fatty acid

Free fatty Acid or Acid value of the crude glycerol was determined according to AOAC Official method 972.28 (AOAC, 2000). Five gram of crude glycerol sample was placed in 250ml Erlenmeyer flasks and 100ml of neutralized ethanol and 2ml of phenolphthalein indicator were added. The mixture was vigorously steered and titrated with standard 0.1N NaOH solution until slight pink color persists for 30 seconds. This was done in duplicate and the volume the titrant (NaOH) was recorded for acid value/free fatty acid calculation. The free fatty acid was then calculated with the formula:

$$\%\ \mathrm{FFA(as\ oleic acid)} = \frac{\mathrm{V \times N \times 28.2}}{\mathrm{W}} \text{ ……………………………………………..Equation 3.1}$$

Where, %FFA = percent free fatty acid expressed as oleic acid

3.4.7. Determination of glycerol concentration (% v/v)

The glycerol layer contains glycerol, ethanol and the catalyst CaO, this mixture was heated at 80°C for 1 hour to remove the ethanol, then centrifuged for 15 minute using universal 320 R centrifuge at School of Chemical and Bio-Engineering laboratory, AAiT, and the supernatant was measure.

$$\%\ \text{glycerolconcentration} = \text{crude glycerol} - \text{ethanol} - \text{catalyst} \text{ ………………..Equation 3.2}$$

3.5. Microorganism and maintenance

Escherichia coli 0157:H7 obtained from Ethiopian Biodiversity institute was used all the way throughout this study. The strains were kept in 5ml tube filled with 2ml of Brain Heart Infusion 5/6, Horse blood serum 1/6 and Glycerol 1/10g/L broth solution stock solution at -4^{0}C. Plates were prepared using LB medium (Tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L) containing 1.5% agar. Plates were incubated at 37$^{\mathrm{O}}$C for 24 hours and then a single colony was selected from these agar plates were used to generate 80% glycerol stock solutions which were then stored at - 80 °C. The medium was autoclaved at 121 ^{0}C, 15psi for 15 min before use.

At the beginning of each reactor run, a sterile pipet tip was used to transfer cells from glycerol stock into 5 ml of Luria Bertani media broth and incubated overnight at 37 °C. During the exponential growth phase, a sample was plated onto Luria Burtani agar plates and incubated at 37 °C in anaerobic state inside an anaerobic jar for 24 hours.

3.6. Inoculums and Culture medium

10 ml of 5 g/L yeast extract and 10 g/L tryptone filled with 10 g/L purified glycerol as carbon source and inoculated with a single colony taken from the anaerobically grown agar plate with in hungate tubes adjusted to pH 7 to serve as experiment inoculum. The hungate tubes were incubated at 37 °C for 24 hours.

3.7. Kinetics of batch fermentation

500 ml flask filled with 200 ml Sterilized initial glycerol concentration of 10g/L glycerol plus LB medium in the absent of Trypton was prepared in pH 7, 10% of concentrated cell was inoculated into the reactor flask, the reactor content was purged with nitrogen gas for 2 min to achieve anaerobic condition, then sealed with rubber stoppers and aluminum cap. Fermentation condition was set up at 37 ^{0}C with incubator shaker (Excella E24 incubator Shaker Series) at 150 rpm for 40 hours, for every 2 hours, through 5 ml syringe liquid sample of 3 ml was collected from the reactor, biomass was measured by correlating optical density with dry mass was taken in UV-VIS Double Beam PC 8 Scanning Auto Spectrophotometer at 600nm at AAiT, School of chemical and Bio-Engineering, Addis Ababa. The sample was then centrifuged at 6,000 RPM for 10 mins, the pellet was collected and washed twice with 35 ml distilled water. To verify the correlation between absorbance and dry mass, the pellet was then dried at 105 °C for 24 hours and then weighed.

3.8. Experimental medium

Batch fermentation was performed anaerobically in sealed flasks next to a modified hungate method (Miller et al., 1974). The flasks were initially flushed with nitrogen and then placed in a shaker set at agitations of 0, 75 and 150 rpm, as required for the experiment.

The experiment was conducted in 2 blocks with pure and crude glycerol as the carbon source. For each block, a full factorial design for 3 factors at 3 levels with 2 replicates was employed. The factors considered were initial glycerol concentration (10, 20 and 30 g/L), inoculum concentration (5, 7.5 and 10 g/L) and mixing speed (0, 75 and 150 RPM).

The temperature was controlled at 37°C and the crude glycerol pH was adjusted to pH 7 by addition of 1 M HCl. and initial pH of 7.

3.9. Experimental Design

In this experiment, three quantitative variables were expected to influence ethanol production yield, in order to evaluate the effect of glycerol concentration, inoculum concentration and agitation speed. This study were conducted using a full factorial design with 2 replicates to reduce variability in the data collection. Table 3.1 shows the experimental set-up, this design considered the interaction effects among the variables that affected the response based on the contribution percentage of the tested variables. The experimental data analyses were performed using Design Expert software version 7.0.

Table 3-1. Experimental factors and levels

Factors	Level		
	Low	medium	High
glycerol concentration	10 g/L	20 g/L	30 g/L
inoculum concentration	5 %	7.5 %	10%
agitation speed	0 rpm	75 rpm	150 rpm

3.10. Ethanol separation

Distillation was the final step in the production of ethanol. It is the purifications steps. Distillation is the method used to separate mixture of liquids based on their boiling points different. However, to achieve high purification, several distillations are required. In this experiment separation was achieved by RVO 400 rotary evaporator distillation at a temperature of 85 ^{0}C and 40 rpm for 2 hours at AAiT, School of chemical and Bio-Engineering, Addis Ababa.

3.11. Product conformation

The end product as the result of fermentation was tested its concentration and functional group.

3.11.1. Measuring the Concentration of Alcohol

After the ethanol was separated, the concentration of ethanol samples was measured and reported in (% v/v) DMA 4100 M Density meter at AAiT, School of chemical and Bio-Engineering, Addis Ababa.

3.11.2. FT-IR Spectrum for Ethanol

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

CHAPTER FOUR

4. Result and Discussion

4.1. Crude Glycerol

The crude glycerol was characterized as follows: 80% glycerol, 1.7% free fatty acid, 12.3% unreacted ethanol, 3.4% MONG, 8.65 cs viscosity at 40°C and 10.5 pH.

4.2. Kinetics of batch fermentation

The growth curve for *E. coli* growth with glycerol as carbon source is shown in Figure 4.1.

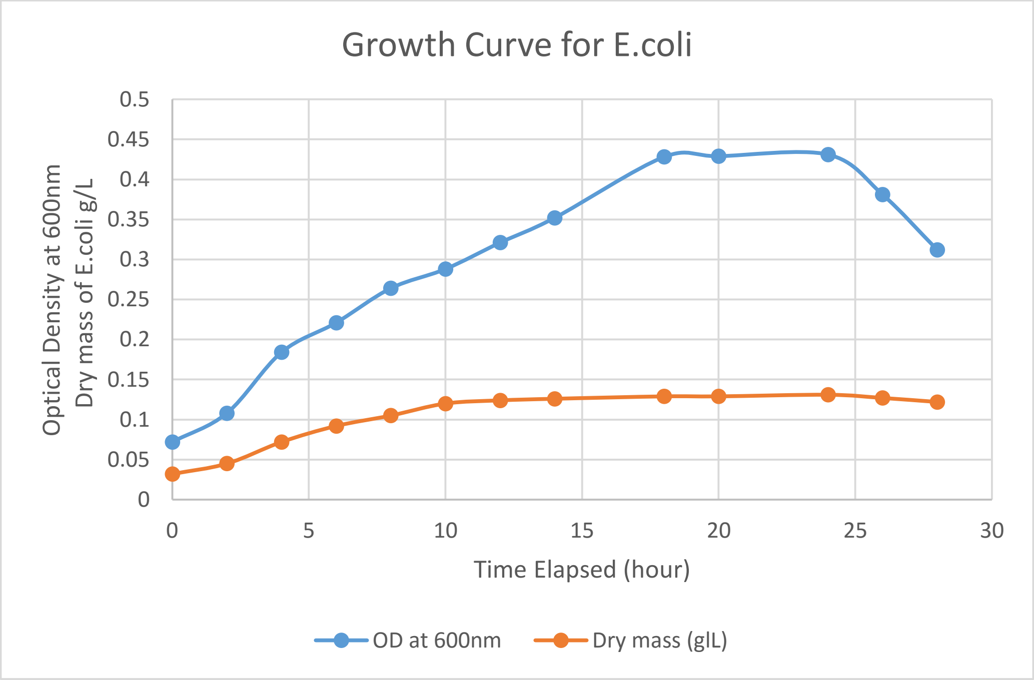


Figure 4-1. Growth curve for dry mass of E. coli with respect to for 10 g/L glycerol within 24 hour fermentation

This experiment is carried out to study the kinetic growth of microorganism. E.coli is selected as the cell and being cultivated inside a shake flask. The growth of microorganism in shake flask is a simple method of fermentation. The nutrients for the microorganism are being supplied by the media which contain the carbon sources. The flask is shaken during the cultivation to mix the cell and the media; increase the homogeneity between these two and also to provide aeration for the cells. The culture is gone through the fermentation process for 24 hours. Within that period, the biomass/cell sample is taken out for every 2 hours to analyze the concentration of the cell (g/L), the cell dry weight and the glucose concentration.

In order to analyze the concentration of the cell inside the flask, absorbance reading for the optical density is taken from the spectrophotometer. The higher the absorbance reading means higher number of cell presence inside the flask at a particular time. As for this experiment, the absorbance reading is increase from the beginning of the experiment until the 24st hour. It can be explained that the number of cell increase throughout the cultivation indicating that the cell is growing. In the other hand, the decrease in cell number after the 24th hour indicating that the cell growth has reach its deceleration phase where the growth of the cell is started to slow down. The decelerating growth phase is where the culture is in a transient state. During this stage there are feed/back mechanisms that regulate the bacterial enzymes involved in key metabolic steps to enable the bacteria to withstand starvation. There is much turnover of protein for the culture to cope with this period of low substrate availability. In cell growth, the cell will go through several phases like lag, exponential, deceleration, stationary and death phase.

In cell cultivation, the cells themselves need food or carbon sources like glycerol for growth. In batch fermentation for example in this experiment, the glycerol can be the limiting factor for the cell growth or we called it as substrate limiting growth. For this condition, the exponential growth rate is first order kinetics and cited in Equation (4.1):

$$\frac{dX}{dT} = \mu_{net} X \quad \text{……………………………………………………………………….Equation 4.1}$$

Integration of Equation (4.1) yields Equation (4.2):

$$\ln \frac{X}{X_0} = \mu_{net} t \quad \text{……………………………………………………………………Equation 4.2}$$

Thus, the growth rate is evaluated by linearizing the exponential portion of the growth curve shown in Figure 4.1. The net growth rate for glycerol was 0.45 h^{-1} and a maximum dry mass of 0.131g/L was reached at 24 hours.

4.3. Glycerol vs. Crude glycerol

A comparison was made to evaluate the effect of utilizing crude glycerol in place of pure glycerol on fermentation yields of ethanol.

The effect of glycerol concentration, mixing speed and inoculum concentration was also evaluated. A sample comparison between crude and pure glycerol is presented in figures 4.2 and 4.3. These figures show the final ethanol concentrations for pure and crude glycerol substrate at 10, 20 and 30 g/L glycerol with 5, 7.5 and 10 % inoculum concentration at a mixing speed of 150 rpm.

From figures 4.3 and 4.4, it is seen that the final ethanol concentration is lower for an initial glycerol concentration of 20 g/L as compared to that with 10 or 30 g/L at the same conditions with respect to inoculum concentration and mixing speed. However, it is to be noted that although the absolute ethanol concentration is lower, the yield for 20 g/L initial glycerol concentration is higher than that of 30 g/L as ethanol yield is calculated as final ethanol concentration divided by initial glycerol concentration.

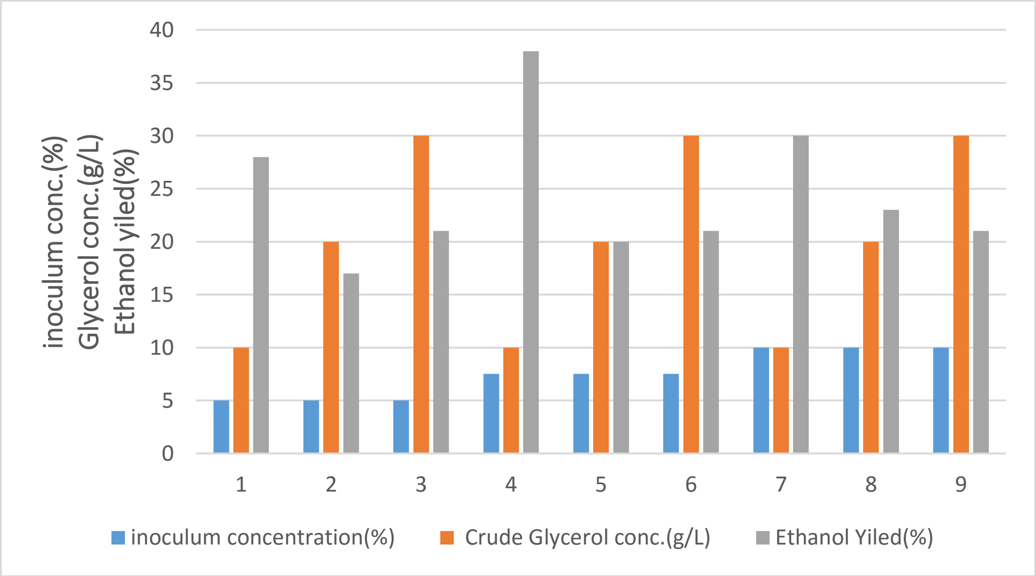


Figure 4-2. Final ethanol concentration crude glycerol at 75 RPM

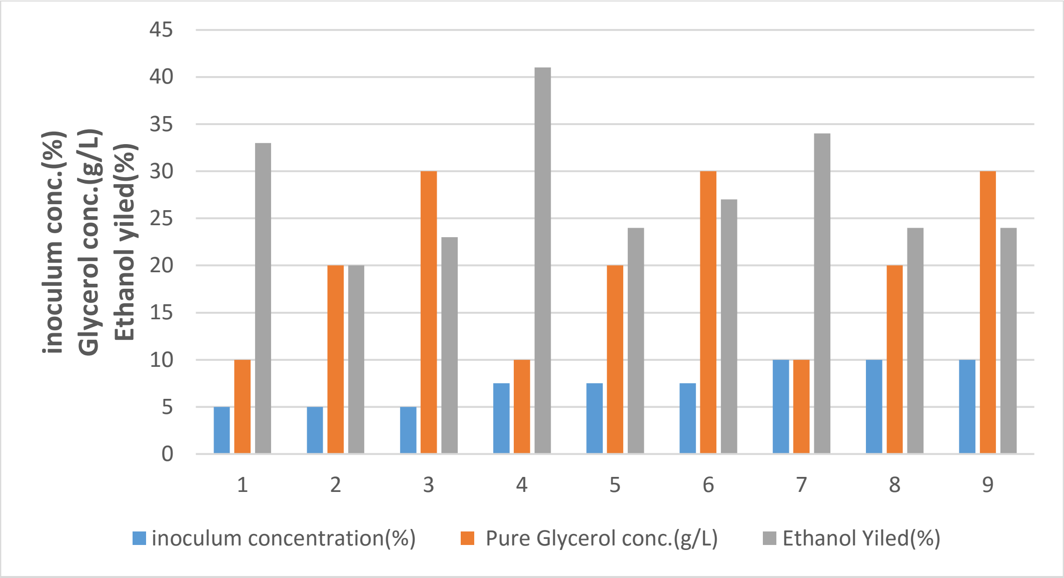


Figure 4-3. Final ethanol concentration crude glycerol at 75 RPMEthanol Yield

The yield of ethanol was determined using DMA 4100 M Density meter at AAiT, School of chemical and Bio-Engineering, Addis Ababa.

Table 4-1. Ethanol Yield for glycerol type, Glycerol concentration, Inoculum Concentration and speed of agitation

Agitation speed (RPM)		**Glycerol type concentration (g/L)**					
		Pure			**Crude**		
		Inoculum concentration (%)			10		
0	5	0.24	20	30	10	20	30
0	7.5	0.34	0.17	0.17	0.23	0.16	0.15
75	10	0.32	0.17	0.21	0.36	0.16	0.27
	5	0.33	0.10	0.18	0.25	0.11	0.14
75	7.5	0.41	0.20	0.23	0.28	0.17	0.21
150	10	0.34	0.24	0.27	0.38	0.20	0.21

	5	0.11	0.23	0.24	0.30	0.24	0.21
150	7.5	0.31	0.14	0.21	0.20	0.12	0.24
	10	0.42	0.22	0.24	0.35	0.14	0.24

Table 4.1 shows the effects of glycerol type and initial concentration, inoculum concentration and agitation speed on ethanol yield. Feedstock usually account for a third of the production costs, making the ethanol yield a focus for optimization (Dien et al., 2003). A maximum ethanol yield overall of 0.42 was observed with 10 g/L pure glycerol, 10 % inoculum and 150 rpm. For crude glycerol, the maximum yield observed was 0.38 for 10 g/L crude glycerol, 7.5 % inoculum and 75 rpm. It is noted that the yields with 10 g/L crude glycerol and 7.5 % inoculum were comparable for all mixing speeds.

Overall, the ethanol yield is greatest for initial glycerol concentrations of 10 g/L. In addition, all cases but two, 0 rpm at 7.5% inoculum and 150 rpm at 5 and 7.5% inoculum, the yield at 10 g/L glycerol is higher for pure glycerol than for crude glycerol.

For all pure glycerol and crude glycerol, the yield is higher with 7.5 % inoculum but higher with 10 % inoculum at 150 rpm for pure glycerol.

4.4. FT-IR Spectrum for Ethanol

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

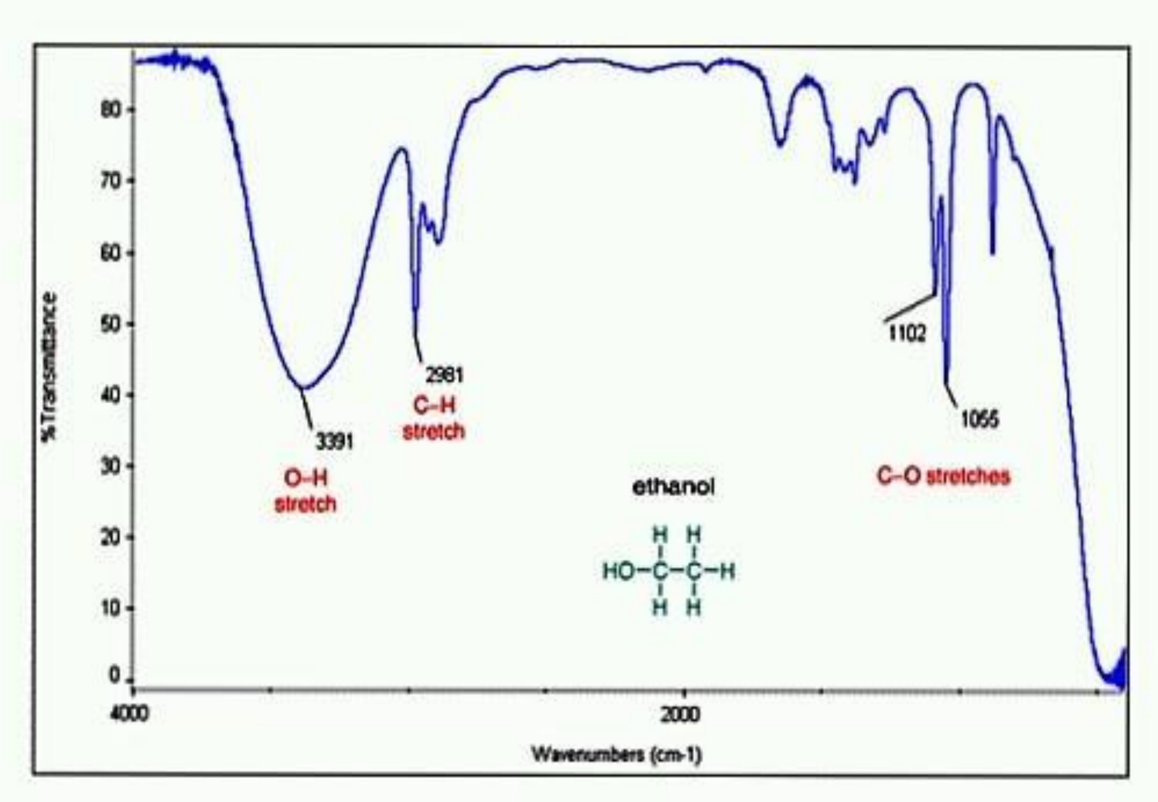


Figure 4-4. Ethanol Standard spectrum graph

Figure 4.4. , shows O-H, C-H, C-C and C-O stretch of ethanol. The broadest peak in the IR spectrum of ethanol comes from OH stretches, similar to O-H bonds in water. This absorption at 3400cm^{-1} corresponds to a wavelength of about 2.9µm. Other strong stretching modes are C-H at 3000cm^{-1}, C-C at 1102cm^{-1} and C-O at 1050cm^{-1}, corresponding to 3.3µm and 9.5µm, respectively.

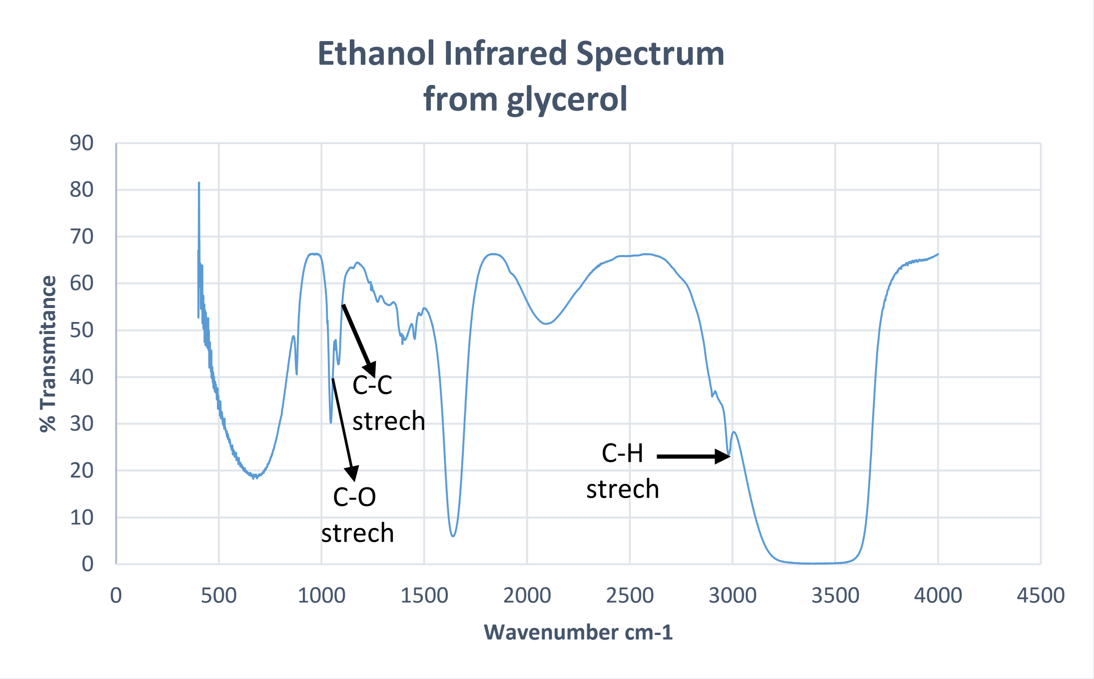


Figure 4-5. FT-IR Spectrum analysis of ethanol from Glycerol

IR spectrum of ethanol produced from Glycerol is demonstrated in Figure 4.5. And it showed the same trend like the standard IR graph of ethanol. The molecular formula of ethanol is CH_3CH_2OH. The existing bonds are O-H, C-H, C-C and C-O in molecular formula of ethanol.

4.5. Statistical Analysis of the Experimental Results

This study were conducted using a full factorial design with 2 replicates to reduce variability in the data collection. The experimental data analyses were performed using Design Expert software version 7.0.

4.5.1. Analysis of variance (ANOVA)

Table 4-2. Analysis of variance table for ethanol production from crude glycerol

Source	***Sum of squares***	***Degree of Freedom***	***Mean square***	***F- value***	***P-value***	
Block	48.17	1	48.17			
Model	2249.01	9	249.89	12.97	< 0.0001	Significant
A-substrate concentration	560.11	1	560.11	29.08	< 0.0001	
B-inoculum concentration	200.69	1	200.69	10.42	0.0024	
C-speed agitation	81.00	1	81.00	4.20	0.0464	
AB	51.04	1	51.04	2.65	0.1109	
AC	100.04	1	100.04	5.19	0.0277	
BC	234.38	1	234.38	12.17	0.0011	
A^2	645.33	1	645.33	33.50	< 0.0001	
B^2	200.08	1	200.08	10.39	0.0024	

C^2	176.33	1	176.33	9.15	0.0042	
Residual	828.32	43	19.26			
Cor Total	3125.50	53				

The analysis of variance of the quadratic regression model was a significant model, from evident of Fisher's F test with a very low probability value [(P-model > F) =0.0001]. From Table 4.2 it was observed that the Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AC, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient for the linear effect of substrate concentration, inoculum concentration and speed of agitation was highly significant. It was also observed that there is an interaction effect between substrate and speed of agitation, inoculum concentration and speed of agitation.

Table 4-3. Model Adequacy Measures

Std. Dev.	4.39	R-Squared	0.7308
Mean	23.50	Adj R-Squared	0.6745
C.V. %	18.68	Pred R-Squared	0.5492
PRESS	1387.25	Adeq Precision	12.656

The "Pred R-Squared" of 0.7308 is in reasonable agreement with the "Adj R-Squared" of 0.6745 it is less than 0.2. "Adeq Precision" measures the signal to disturbance ratio due to random error. A ratio greater than 4 is desirable. Here ratio of 12.656 indicates an adequate signal. Therefore, this model can be used to navigate the design space.

The regression coefficients and the corresponding 95% CI (Confidence Interval) High and Low were presented in table 4.3 above. If zero was in the range High and Low 95% Confidence interval, the factors has no effect. From the 95% CI High and Low values of each model term, it could be concluded that the regression coefficients of substrate concentration, inoculum concentration, speed of agitation have highly significant effect in ethanol production.

Table 4-4. Regression coefficient and the corresponding 95 % CI High and Low

Factor Intercept	Coefficient Estimate	Df	Standard Error	95 % CI Low	95 % CI High	VIF
pure glycerol	23.89	1	1.58	20.70	27.08	
crude glycerol	0.94	1				
A-substrate concentration	-0.94					
B-inoculum concentration	-3.94	1	0.73	-5.42	-2.47	1.00
C-speed of agitation	2.36	1	0.73	0.89	3.84	1.00
AB	1.50	1	0.73	0.025	2.98	1.00
AC	-1.46	1	0.90	-3.27	0.35	1.00
BC	2.04	1	0.90	0.23	3.85	1.00
A^2	3.13	1	0.90	1.32	4.93	1.00
B^2	7.33	1	1.27	4.78	9.89	1.00
C^2	-4.08	1	1.27	-6.64	-1.53	1.00
Intercept	-3.83	1	1.27	-6.39	-1.28	1.00

4.5.2. Development of Model Equation

A model equation is a representative equation in which it represents the whole model with a single mathimatical relation that helps to maximize response yield and the operating conditions.

Final Equation in Terms of Coded Factors:

$$\text{ethanol yiled} = +23.89 - 3.94 * \mathrm{A} + 2.36 * \mathrm{B} + 1.50 * \mathrm{C} - 1.46 * \mathrm{A} * \mathrm{B} + 2.04 * \mathrm{A} * \mathrm{B} + 7.33 * \mathrm{A}^2 - 4.08 * \mathrm{B}^2 - 3.83 * \mathrm{C}{}^\wedge 2$$

Final Equation in Terms of Actual Factors:

$$\begin{aligned}\text{ethanol yiled} = {} & +16.65278 - 3.09444 * \text{substrate concentration} + 10.66111 \\ & * \text{inoculum concentration} - 0.057222 * \text{speed of agitation} - 0.058333 \\ & * \text{substrate concentration} * \text{inoculum concentration} + 2.72222\text{E} - 0.03 \\ & * \text{substrate concentration} * \text{speed of agitation} + 0.016667 \\ & * \text{inoculum concentration} * \text{speed of agitation} + 0.073333 \\ & * \text{substrate concentration}^2 - 0.65333 * \text{inoculum concentration}^2 - 6.8141\text{E} \\ & - 004 * \text{speed of agitation}\hat{}2\end{aligned}$$

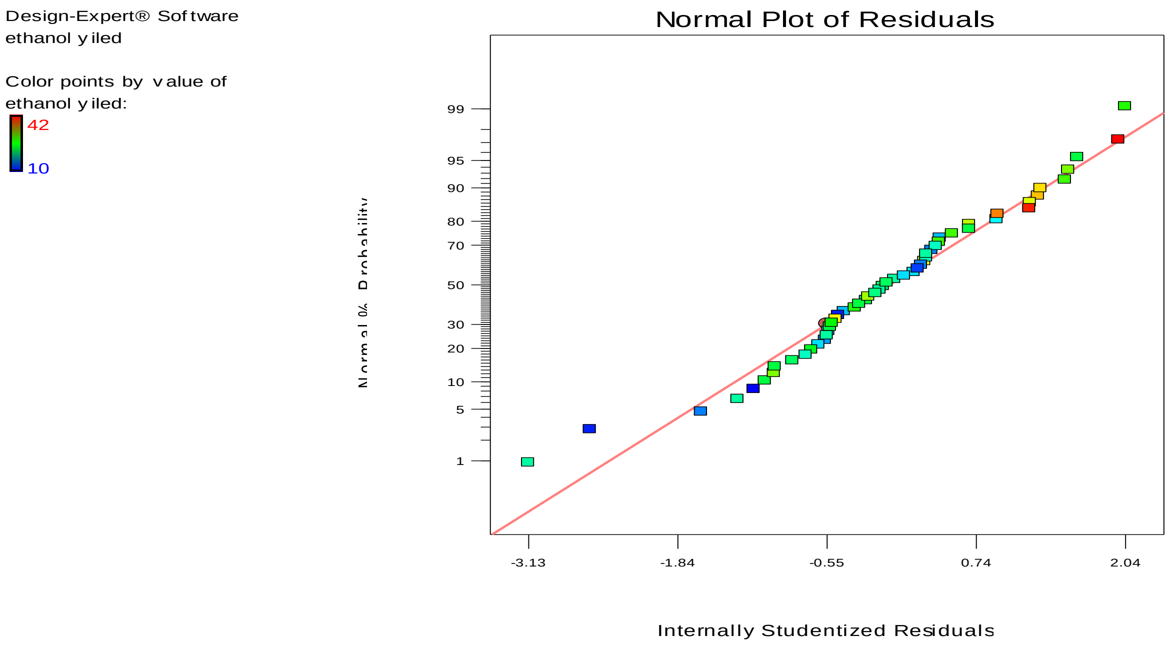


Figure 4-6. Normal Plots of Residual

From figure 4.6. shows, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the 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.

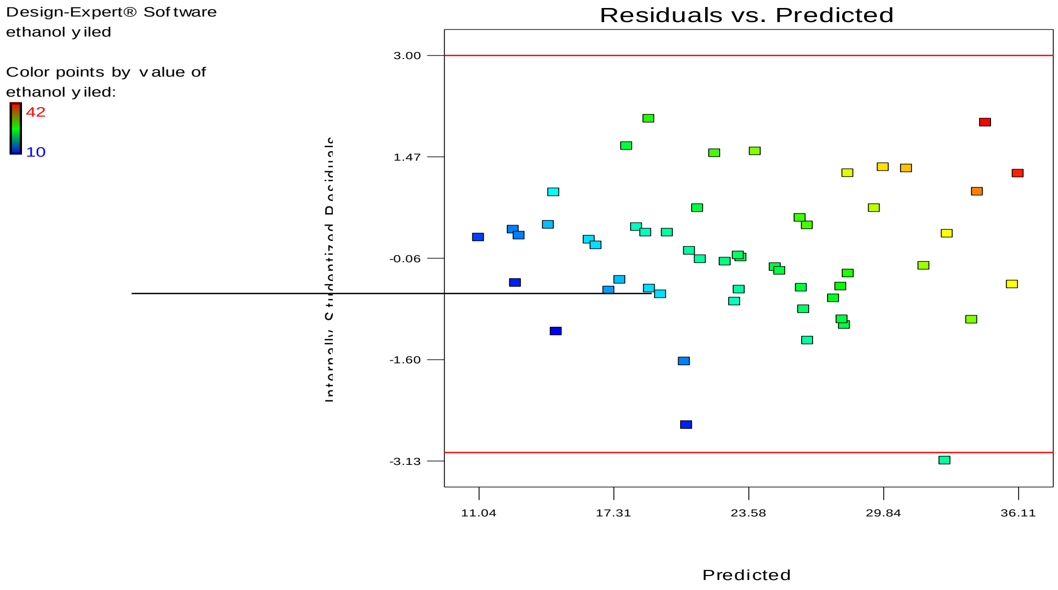


Figure 4-7. Residual versus Predicted value

The plot shows random scatter which justifying no need for an alteration to minimize personal error.

4.5.3. Effect of individual factors on yield

The factors used in this analysis were substrate concentration, inoculum concentration and speed of agitation. These factors had a great impact on the production of ethanol and analyzed individually to determine their effects.

4.5.3.1. Effect of substrate concentration on yield

Figure 4.7 shows that the substrate concentration on the ethanol yield. Maximum ethanol concentration was observed at the minimum glycerol concentration 10 g/L. The ethanol concentration decrease as initial substrate concentration increased. High concentrations of glycerol, inhibits the growth of bacterial cells.

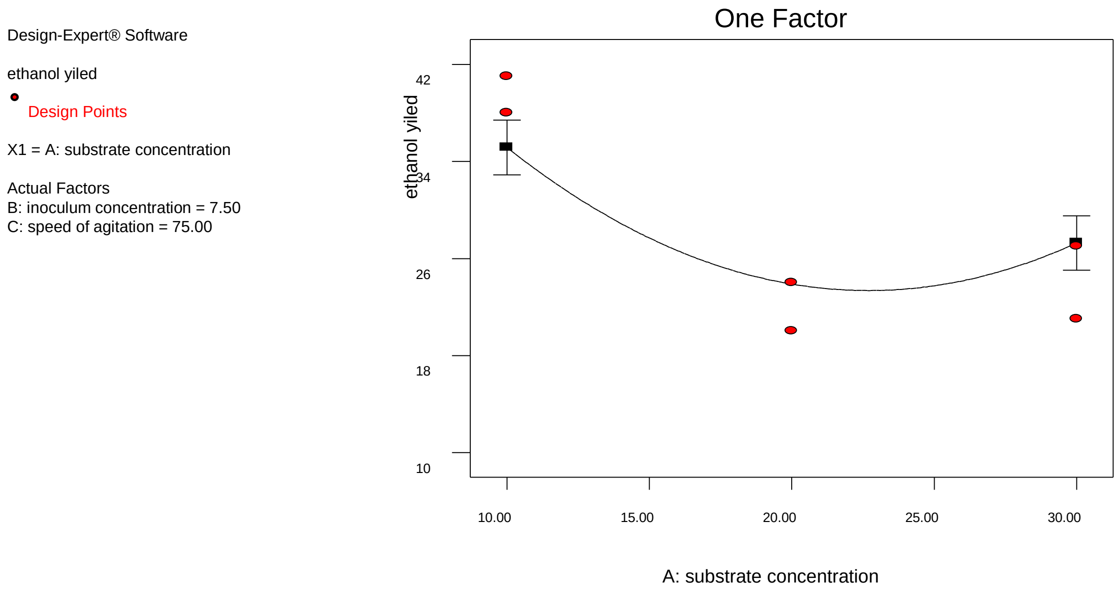


Figure 4-8. Effect of substrate concentration on ethanol yield

4.5.3.2. Effect of inoculum concentration on ethanol yield

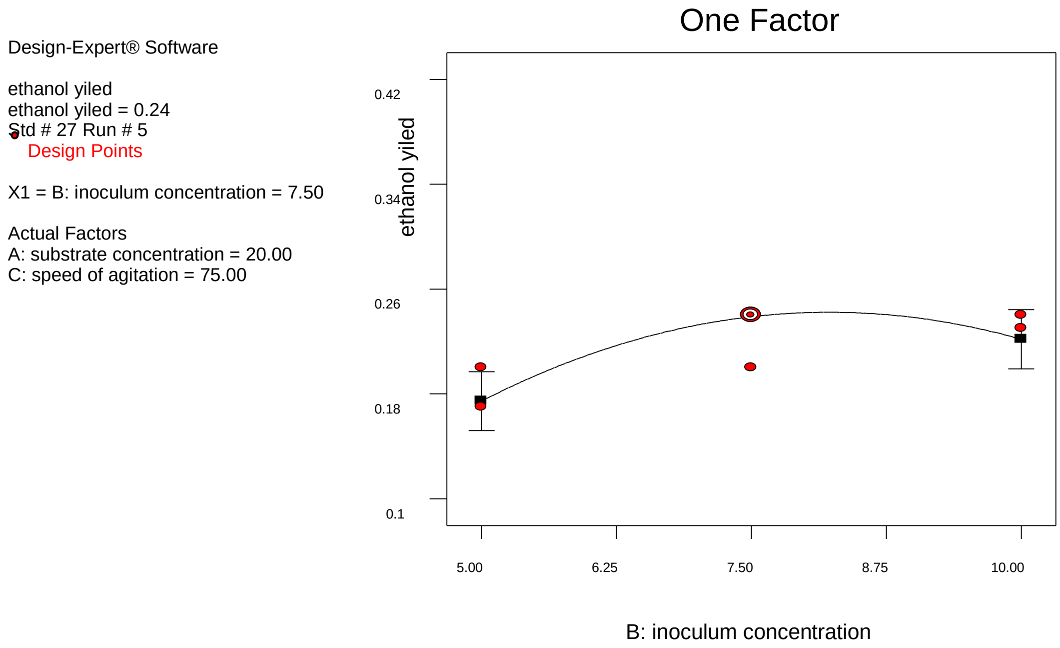


Figure 4-9. Effect of inoculum concentration on ethanol yield

The size of inoculum in ethanol fermentation is of great importance in completing the fermentation process. The amount of ethanol produced gradually increased with the increase in the inoculum size at 7.5% (v/v) inoculum it was found that maximum ethanol production was achieved. Further increase in inoculum size did not result in the considerable enhancement of ethanol production (Figure 4.8).

4.5.3.3. Effect of speed of agitation on ethanol yield

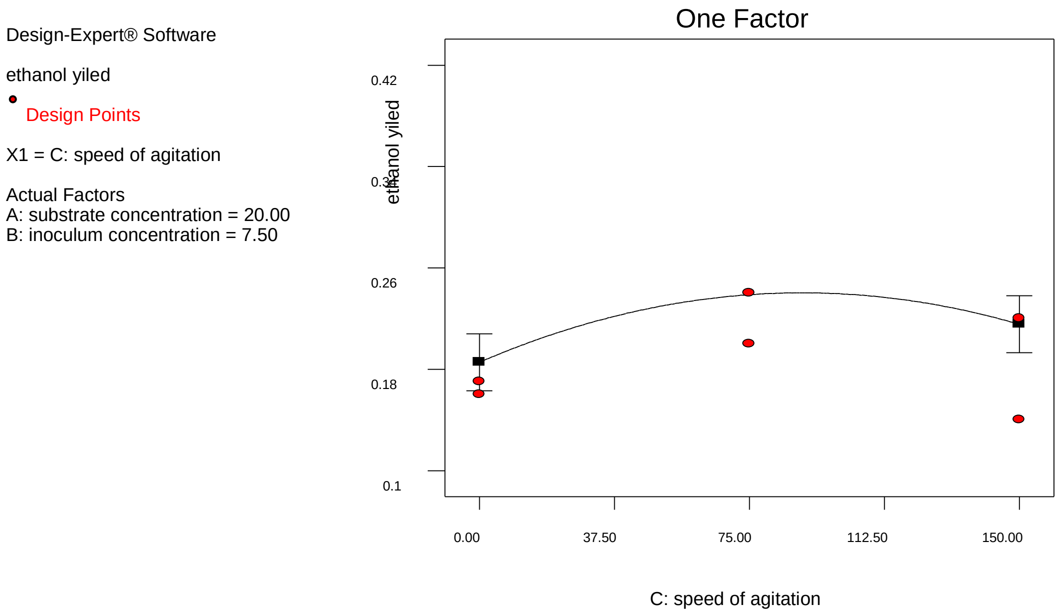


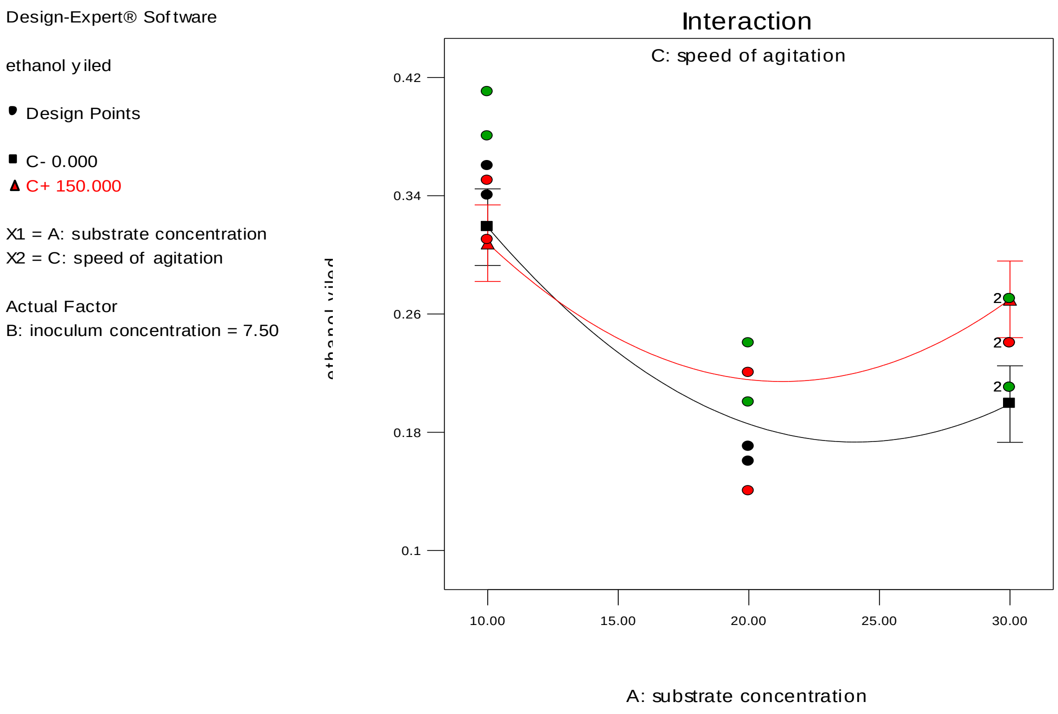
Figure 4-10. Effect of speed of agitation on ethanol yield

At lower level of mixing ethanol yield was small, increasing speed of agitation also increase ethanol yield . Agitation is important for adequate mixing, mass transfer and heat transfer. It assists mass transfer between the different phases present in the culture, also maintains homogeneous chemical and physical conditions in the culture by continuous mixing. gitation creates shear forces, which affect microorganisms, causing morphological changes, variation in their growth and product formation and also damaging the cell structure(Kongkiattikajorn et al.,2007)

4.5.4. Interaction effect of Factors on ethanol yield

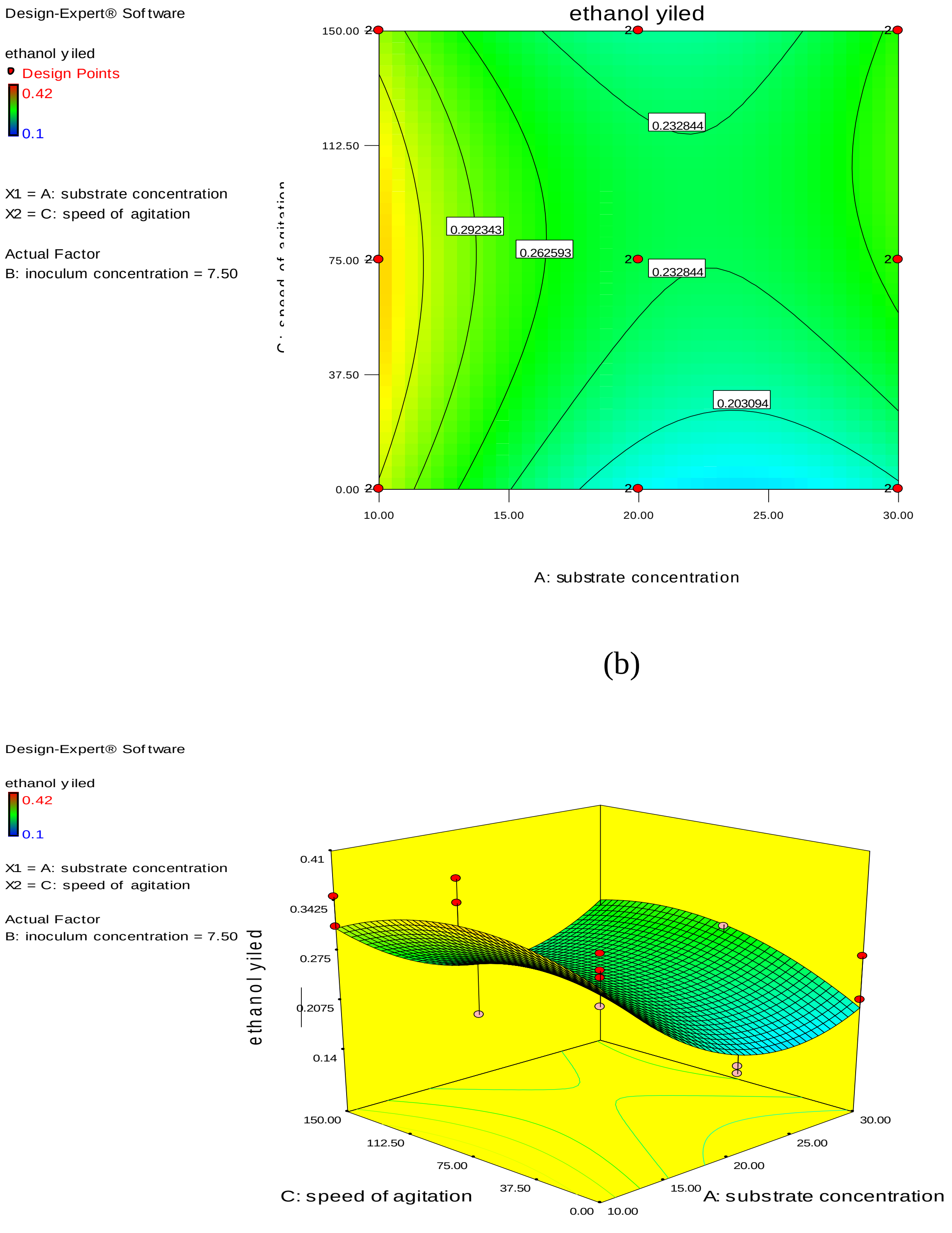
An interaction effect is a combination effect of two or more individual factors that can be possibly affects the response in the same or different way. Interaction effects should always under consideration if and only if the P-value of the combined factors found to be less than 0.05 or 5% (i.e. the probability of the model).

4.5.4.1. Effect of substrate concentration and agitation speed on ethanol yield



(a)

substrate concentration and agitation speed affects the yield interactively. This means, a proportionate increment of these factors causeed an increment in the yield simultaneously. The figure below entailed the increment in agitation speed and decrease substrate concentration from 30 to 10 the yield was increased simultaneously to 0.318 a single interactive value. This figured out, both the factors affect the yield simultaneously and finally interacted to a single value of yield.



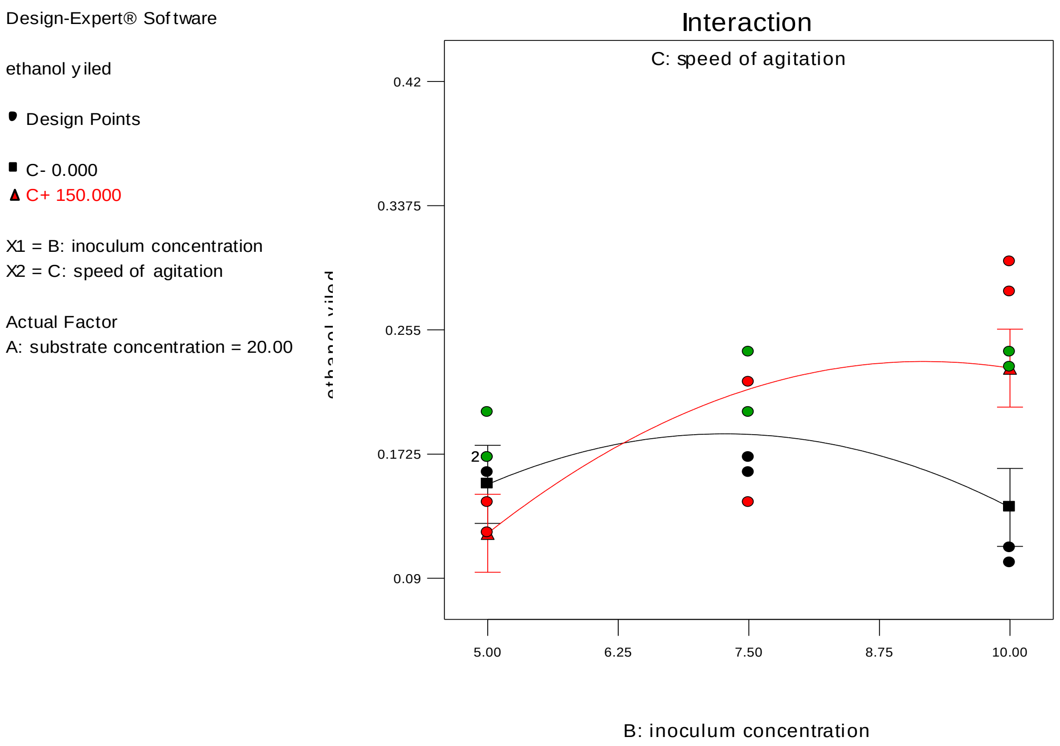
(b)

(c)

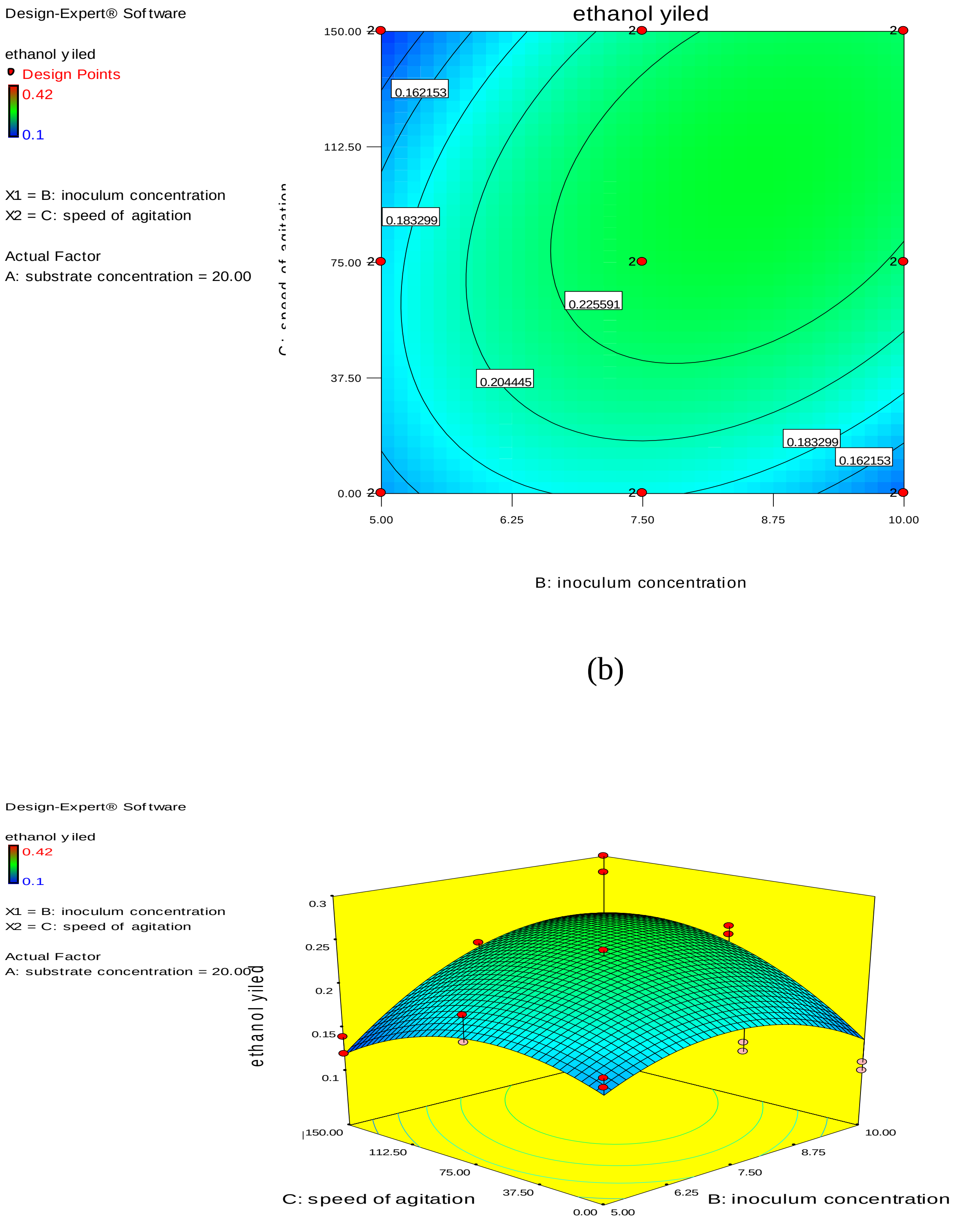
Figure 4-11. Interaction (a) Contour (b) and 3D (c) plots substrate concentration and agitation speed

4.5.4.2. Effect of inoculum concentration and agitation speed on ethanol yield

Inoculum concentration and agitation speed affects the yield interactively. This means, a equivalent increment of these factors causeed an increment in the yield simultaneously. The figure below caused both in agitation speed andinoculum concentration decrease from 10 to 7.5 the yield was increased concurrently to 0.24 with two interactive value. This figured out, both the factors affect the yield simultaneously.



(a)



(b)

(c)

Figure 4-12. Interaction (a) Contour (b) and 3D (c) plots inoculum concentration and agitation speed

4.5.5. Optimization of Process Variables

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. It also has important applications in the design, development, and formulation of new products, as well as in the improvement of existing product designs. The optimization of those factors for the production of ethanol production from crud glycerol are summarized as follows:

Table 4-5. Optimization Criteria for optimum ethanol yield

Constraint Name	Goal	Lower Limit	Upper Limit
Substrate Concentration	In Range	10	30
Inoculum concentration	In Range	5	10
Speed of agitation	In Range	0	150
Ethanol Yield	Maximize	0.1	0.42

The optimum possible solutions for the production of bioethanol are presented in table 4.5. The predicted optimum ethanol yield 0.361252 was observed at the process variables 10 g/L glycerol concentration, 85.57 rpm speed of agitation and 8.8 % (v/v) of inoculum concentration.

Table 4-6. Optimum possible solutions

Number	**substrate concentration**	**inoculum concentration**	**speed of agitation**	**ethanol yiled**	**Desirability**	
1	**10.00**	**8.80**	**85.57**	**0.361252**	**0.816**	**selected**
2	10.00	8.71	84.29	0.361198	0.816	
3	10.00	8.88	84.28	0.36118	0.816	
4	10.00	8.92	86.51	0.361171	0.816	
5	10.00	8.90	92.82	0.360957	0.815	
6	10.00	8.51	83.93	0.360731	0.815	
7	10.00	8.40	79.76	0.360325	0.814	
8	10.00	9.23	100.95	0.359567	0.811	
9	10.00	8.27	54.88	0.355673	0.799	
10	10.00	7.35	41.25	0.344762	0.765	
11	30.00	8.35	120.14	0.284952	0.578	
12	30.00	8.03	110.07	0.284128	0.575	
13	30.00	8.71	130.41	0.283999	0.575	
14	30.00	8.04	100.32	0.282687	0.571	
15	30.00	7.70	116.17	0.282465	0.570	
16	30.00	7.63	86.20	0.277808	0.556	

CHAPTER FIVE

5. Conclusion and Recommendation

5.1. Conclusion

This work investigated the potential of crude glycerol use for production of ethanol via anaerobic fermentation. The variables of interest were agitation speed, initial glycerol concentration and inoculum concentration. Maximum yield of 0.38 gm ethanol per gm of glycerol was obtained at 10 g/L crude glycerol, 7.5 % (v/v) inoculum concentration and 75 rpm; this corresponded to an ethanol concentration of 3.8 g/L. The maximum yield of ethanol for pure glycerol was 0.42, observed with 10 g/L pure glycerol, 10 % (v/v) inoculum concentration and 150 rpm; thus maximum yield for crude glycerol was 90% that of pure glycerol overall. Analysis of variance demonstrated that all three variables, as well as the interactions had an effect on the final ethanol concentrations; however, the variables that were significant were inoculum concentration, agitation speed and initial glycerol concentration. The use of crude glycerol in the place of pure did not have a significant effect on the yields of ethanol. This study demonstrates the feasibility of utilizing crude glycerol as substrate for anaerobic fermentation for the production of ethanol by *E. coli*.

5.2. Recommendation

- It is recommended that regeneration of the byproduct which can be used for biogas should be studied.
- Effects of other parameters on the growth of E.coli also suggested for further study.
- Government should have to create connection with the researchers and investors in order to be understand the study have a significant value add for the biodiesel production plant.

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