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PROCESS ENGINEERING STREAM

Optimization of Fermentation Condition and Product Characterization in Bioethanol Production from Waste Potato

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By: **Getachew Alemu**

A Thesis submitted to the Jimma University, Jimma Institute of Technology, School of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of Master of science in Chemical Engineering (Process Engineering)

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This is to certify that the thesis prepared by **Getachew Alemu**, entitled: "**Optimization of Fermentation Condition and Product Characterization in Bioethanol Production from Waste Potato**" and submitted in partial fulfillment of the requirement for the degree of Master of Science (Chemical Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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DECLARATION

I, Getachew Alemu, hereby declare that the work on which this thesis is based on and entitled: **Optimization of Fermentation Condition and Product Characterization in Bioethanol Production from Waste Potato** is my original work not submitted for another degree in this or any other university, and all resources of material used for this thesis had been duly acknowledged. The work was under the guidance of Dr. Kumsa Delessa, Assistant professor in Addis Ababa Institute of Technology (AAIT), Addis Ababa University and Mr. Mohammed Seid, Lecturer in School of Chemical Engineering in (JIT), Jimma University.

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Abstract

Fulfilling energy demand of generations in accordance with environmental policy and in a sustainable way is a very critical issue that requires a clear attention. For this reason, a promising solution is focusing on renewable energy sources. Bioethanol is one of these renewable energies that can be produced from fermentation of biomass such as waste foods. In the first part of the study, analytical methods were applied for characterization of waste potato which was used as a renewable carbon source for bioethanol production by fermentation. The value obtained from characterization of waste potato was relatively similar with standard and previous literature which implies the presence of component that helps to get possible ethanol production. The obtained result includes: 39.65% starch content, 34.71% of moisture content, 5.823% of ash content, 9.46% protein and 7.33% of fat content. The conversion of waste potato to ethanol was achieved mainly by four process steps: dry milling (physical pretreatment) of waste potato, acid hydrolysis of pretreated waste potato to convert starch into reducing sugar (glucose), fermentation of the sugars to ethanol using Saccharomyces cerevisiae and finally batch distillation of the fermented sugar into final product. In second part of the study, optimization of most significant parameters (temperature, pH and fermentation time) was carried out using Box-Behnken experimental design. The result of optimization indicated that optimal temperature of 32.199 °C, pH 4.066 and incubation time 72.082 hr. at this optimum condition, 34.5% ethanol yield was recorded. Finally, the properties of final product including functional groups FTIR analysis was done and compared with the standard physical and chemical characteristics of bioethanol

***Key words**: Bioethanol, waste potato, fermentation, optimization, characterization*

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of Variance
BBD	Box Bhenken Design
COD	Coefficients of Determination
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FTIR	Fourier Transfer Infrared Spectroscopy
GHG	Green House Gas
Kg/qt.	Kilogram Per Quintals
KJ/kg	Kilo Joule Per Kilogram
OD	Optical Density
pH	power of Hydrogen
PPW	Potato Peel Waste
PW	Potato Waste
rpm	revolution per minute
RSM	Response Surface Method
SG	Specific Gravity
SSF	Solid State Fermentation
TRS	Total Reducing Sugar

1. INTRODUCTION

1.1 Background

Most countries in the world are relying on fossil fuel to meet the increasing demand of energy for industrial process, heat and transportation. In many parts of the world, demand for ethanol as an alternative fuel source has regularly increased due to efforts in declining fossil fuel resources, increasing the overall amount of greenhouse gases emitted in atmosphere and increased gasoline price (Azad et al., 2014). Fossil fuels have powered an emergence of human kind to its current state of civilization. However, issues related to environmental pollution, security of supply and the rise in world population enforces the world to think for alternative renewable energy sources. Biofuel is one of this renewable energy sources which is obtained from biomass. Biofuel initiative has been backed by government policies in the quest for energy security through partially replacing the limited fossil fuels and reducing the threat to the environment from exhaust emissions and global warming (El-awad et al., 2011). Keeping in view all the advantage, biomass based fuel development technology should rapidly gain momentum for successfully attempting the production of bioethanol at commercial level (Science and Rai, 2010). This is considered as one of the most promising alternative fossil fuel that can ensure energy security and reduce environmental pollution problems.

Recently, the world science has focused on advanced change in biofuel production from biomass by addressing issues such as effective utilization of alternative biomass as feedstock, integrated co-generation facilities, commercialization of bioethanol production and integrated production systems capable of utilizing diverse feedstock under single platform Ethanol is a significant product of 21st century with its versatile usages and widely consumption across the globe (Nagabushana and Pak 8717, 2010). Initial stimulus for ethanol production in the mid-1970s was the drive to develop alternative and renewable supplies of energy in response to the oil embargoes of 1973 and 1979 (Uncu and In, 2009). The primary motive for producing fuel ethanol is to reduce a foreign exchange of oil import (Branco et al., 2019).

Renewable energy plays a great role in the protection of the environment and in fulfilling our energy needs (Hiben, 2013). Bioethanol is the most common biofuel which has been known as fuel vehicles since 1925 (Sheikh et al., 2016). Ethanol gasoline blends can be used as an alternative fuel for variable speed spark ignition up to 35% blends without engine modification. The gasoline fuel replacement is regulated by the amount of ethanol in the blend. However, problems arise, due to the presence of water in the blend because commercially available ethanol is seldom found in an anhydrous state. The commonly available ethanol grades contain between 10% and 20% water. Thus, there would be an economic incentive if the spark ignition engine could be run on industrial ethanol instead of anhydrous ethanol. It was found that the blend can be successfully used without phase separations within the tested temperature range.

Bioethanol can be produced through different bio refinery based processes. Nevertheless, as the first step, it is always necessary to find renewable raw materials with suitable compositions in terms of carbohydrates. According to the feedstock considered, there are three generations of bioethanol: first generation, where bioethanol is produced from human food/animal feed ingredients (e.g., potato, soybean, wheat, rice, corn, sugarcane, etc.); second generation bioethanol from lignocellulosic materials/agro-industrial residues (e.g., corn cob, wheat straw, sugar cane bagasse, agave bagasse, etc.) and third generation bioethanol produced from aquatic biomass (such as cyanobacteria, microalgae, and microalgae). Since starch is produced and retained intracellularly, it is necessary to increase its availability to microbial fermentation (Velazquez-lucio et al., 2018). Microorganisms meet their energy demand by converting carbon sources to byproducts such as: carbon dioxide, lactic acid, ethanol, etc. (Germec et al., 2019). However, as alcohol production from starchy materials remains not so feasible economically, the development of a more effective and high yield ethanol fermentation process is required to bring the necessary dramatic reduction of production costs. The sugary substrates available are comparatively expensive than molasses but can be easily used for ethanol production with some modification in the process. The starchy substrates are promising due to their economic viability and availability (Ghosal et al., 2013).

Bioethanol production includes: preparation of raw waste potato, starch hydrolysis and determination of optimal fermentation condition (pH, temperature, inoculum size, stirring rate, potato to powder rate of liquor and yeast concentration) (Mushimiyimana and Tallapragada, 2016). The yield of bioethanol production from waste potato can be improved by further study of optimal fermentation condition such as: different yeast concentration, pH, inoculum size, temperature and nitrogen sources (Izmirlioglu et al., 2012). Traditionally, various species of *Saccharomyces cerevisae* were used in fermentation processes, since they were known to be very effective for conversion of complex sugars to ethanol and other substances (Mahlia, 2017). Finally, distillation is performed to separate bioethanol from unwanted impurities based on temperature difference. The study and analysis of product is also required to be characterized in accordance with standard physical and chemical characteristics of bioethanol.

A vegetable biomass is used as feedstock for bioethanol production and it allows recycling of the CO_2 released during combustion (Oiwoh et al., 2018). Bioethanol production from starchy materials such as potato, sweet potato, corn flour is based on bioconversion of starch into sugar and then ethanol by fermentation process (Science and Subhash, 2015). Starch is composed of a complex carbohydrate which requires conversion into simple sugars before being converted to bioethanol product. A Starch containing crop such as: barely, corn, potatoes, wheat, sweet sorghum, rice and sweet potatoes are used for bioethanol production. They are considered as a cheap substrates for fuel ethanol production from biomass (Malik et al., 2018). Potato peel, carrot peel, onion peel and sugar beet peel are belongs to valuable biomass wastes which increased the yield of ethanol production by microbial fermentation of substrate (Mushimiyimana and Tallapragada, 2016).

Traditionally potato waste is used for producing low value animal feed, fertilizer or being raw material of biogas, which causes waste of abundant nutritive materials within it having the properties of antioxidant, antibacterial, apoptotic, chemo-preventive and anti-inflammatory. Current researches focus on several advanced developments of potato waste in food processing, phyto-pharmaceutical and biosynthesis industries, which increase the value of potato peel recycling (Wu, 2016)**.** Currently, potato (*solanum tubersum*) is an alternative starchy feedstock for bioethanol production which do not require complex pretreatment (Kilpimaa et al., 2014).

This cheaply available waste potato contain a considerable amount of starchy material required for optimum growth of yeast which is used for bioethanol production (Mushimiyimana and Tallapragada, 2016).

One of the major problems in potato production is a postharvest loss which covers (20-25%). The quantity of postharvest losses at producer, local trader, whole seller and retailer level are 21.724, 1.838, 3.406 and 4.07 kg/qt, respectively (Tadesse and Bakala, 2018). The conventional technique of removing potato peel in processing plant and potato consumption in public kitchen, student cafeteria and hotels are the main causes of losses of starchy mash. Potato peel waste has been utilized in a variety of traditional applications for local animal feed (Liang and Mcdonald, 2014).

1.2 Statement of the problem

Nowadays the energy needs of the world is rapidly growing and leading to the high consumption of non-renewable energy resources found in nature. The excessive consumption of non-renewable energy has brought a negative impact on environment whose consequence is reported as various disasters day to day via mass media throughout the world. Countries at different economic and development levels are trying to overcome this problem by developing different technologies that convert renewable energy sources into usable form.

Hence improper management of waste agricultural biomass is contributing towards climate change, water and soil contamination, and local air pollution. Since organic waste is an unavoidable product and economies of developing countries need that all materials and resources be used to their full potential, management of organic waste is a particularly serious issue. Therefore, there exists a great need to find alternative solutions to treatment of organic wastes.

Ethiopia is facing new macroeconomic difficulties i.e. drought, oil price surge and hyperinflation. Particularly oil price surge is a serious issue for Ethiopia as oil importing country by exchange of hard currency. This draws the attention of nations to look for alternative fuel that is environmentally friendly. For this reason, production of bioethanol from food waste is believed to be high potential biofuel that can reduce problem related to oil shortage.

So far, an effort has been made to produce bioethanol from waste potato by identifying optimum condition in acid hydrolysis process. However, more attention was given to hydrolysis rather than condition in fermentation process (Alemayehu, 2015). Ethanol production by fermentation is significantly constrained by the pretreatment cost of the raw material (for example straw), which accounts for more than half of the production cost. Accordingly, selection of cheap, abundantly available and starchy raw material (waste potato) is important for economic feasibility of the producing plant. Fermentation process is highly affected by processing parameters (like temperature, pH and fermentation time), thus in addition to looking for cheap raw material, process optimization is also required for attaining maximum yield of the product.

1.3 Objectives

1.3.1 General objective

The main objective of this research was to optimize fermentation condition for bioethanol production from waste potato and to characterize raw materials and bioethanol after fermentation process.

1.3.2 Specific objectives

The specific objectives of this research were:

- ✓ To characterize the physical properties of waste potato
- ✓ To identify the optimum fermentation operating conditions (Temperature, pH and fermentation time).
- ✓ To investigate a combined effect of fermentation and distillation process on bioethanol yield
- ✓ To characterize bioethanol properties (density, specific gravity, viscosity, pH, boiling point, flash point, fire point and the functional group)

1.4 Significance of the Study

Globally, the production of ecologically sustainable biofuels is a growing interest. The worldwide economy is mainly relying on rising oil price which has become burning issue. The rising oil price results in increasing demands for alternative energy sources. Plants grown to make ethanol for bio-fuel draw CO_2 out of the atmosphere for photosynthesis, causing a recycling process that result in less accumulation of CO_2 in the atmosphere. Thus, bio-fuel does not contribute to global warming in the same way that petroleum does. This leads to an alternative energy sources that fulfill the vision of a full transition to renewable energy. More healthy people working in better conditions, enjoying cleaner planet and a death reduction of millions people due to sensitiveness to pollutants can be achieved by using renewable energy sources. Bioethanol can be used as an alternative fuel to gasoline. Ethiopia has planned to reduce oil imports from foreign due to its huge potential of polluting the environment. Therefore, optimal production of bioethanol from starchy raw material is very important to substitute petroleum derived fuel and reduce greenhouse gas emissions. Fermentation of starchy materials leading to the production of biofuel is economical and should be practiced in developing countries. Therefore, to study and set optimum value of all significant conditions in the process is found to be essential to get a maximum yield of product in bioethanol production.

1.5 Scope of the study

This study was covered production of bioethanol from a preparation and analysis of raw waste potatoes to final product. The focus area of the research involves the study of fermentation parameters, determination yield after distillation and evaluation of product properties. Finally, the obtained result was discussed and the conclusion was draw based on the result. The left necessary things which need additional study and investigation related to this topic, were recommending those to future researchers.

2. REVIEW OF LITERATURE

2.1 Introduction

During last decade, demand for energy has been increasing while the issues of environmental protection become an important area of study. Society has realized that the use of renewable energy sources should be practiced by reducing dependence on foreign oil and non-renewable energy source (Izmirlioglu et al., 2012).

Bioenergy is a useable energy that is converted from biomass source that creates a healthy environment through reduction of pressure on finite natural resources, reduction of greenhouse gas emission via fossil fuel substitution, reduction of landfill waste and provision of thermal, electrical and mechanical energy services (Hiben, 2013). Bioenergy benefits farmer as an improvement of marginal crops and additional source of income from energy byproducts. Types of bioenergy are bioethanol, biogas, biodiesel etc. But the focusing area of these review will focus on bioethanol (Izmirlioglu et al., 2012).

Bioenergy plays a great role in keeping global energy demand by ensuring dire need of renewable and sustainable liquid fuel that helps make future commitment to the energy solution (Mushimiyimana and Tallapragada, 2016). The alternative energy sources such as ethanol, methane and hydrogen are being considered to meet the energy requirements of the country (Ahamad and Murthy, 2016)

Application of agro-industrial waste and co-products in bioprocess provides an alternative way to replace the refined and expensive raw materials (Fochesato et al., 2018). Feed-stocks are typically grouped under "biomass" which includes municipal solid waste, wood, agricultural residues, food processing waste, woody biomass from small diameter trees and energy crops (Ojewumi et al., 2018). It is essential that feedstock accounts for about (20-50)% of total production costs (Liang and Mcdonald, 2014). The conversion of biomass to ethanol can be made economically attractive and have a numerous potential feedstock. Agronomic research is underway on improving dedicated energy crops such as hybrid willow, hybrid poplar, and switch grass. One project looks at genetic improvement of switch grass to optimize its conversion. Grasses grow quickly while tree crops such as willow require a 22-year rotation.

The first harvest is in year 4 and subsequent harvests required every 3 years. Hybrid poplar trees require 6-10 years to reach their first harvest (Rendleman, 2014).

2.2 Bioethanol

Ethanol fermented from biomass based materials for fuel is considered as bioethanol (Azad et al., 2014). Bioethanol is one of the most promising alternatives to fossil fuels that can ensure energy security and address environmental pollution problems, which is produced from agricultural residues, lignocellulosic and starchy biomass (Thenmozhi and Victoria, 2013). The lignocellulosic biomass is cheaper, available in plenty and its conversion to bioethanol involves many steps and expensive. In this context, the starchy substrates are used for bioethanol production due to their economic viability as well as availability in large quantities in the world (Malik et al., 2018).

Bioethanol production is usually accomplished by microbial conversion of carbohydrates present in agricultural residues and chemical synthesis of petrochemical substrates. Owing to depleting reserves and competing industrial needs of petrochemical feedstock, there is a global emphasis in bioethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable starch conversion technology. The most common way of producing bioethanol is fermentation of feedstock's which are rich in sugar or starch such as sugar cane, sugar beet, sweet sorghum, potato, cassava etc. (Bekele et al., 2015).

2.3. Uses and properties of ethanol

2.3.1 Uses of ethanol

Ethanol has an advantage of lowering various noxious emissions (carbon monoxide, hydrocarbons, sulfur oxides, nitrogen oxides and particulates) when compared to straight gasoline. Nevertheless, The extent of emission reduction depends on a number of variables mainly engine characteristics, the way ethanol is used and emission control system features (Zuurbier, 2008).

Ethanol is mixed with petrol and is used as motor fuel. This mixture is called power alcohol. As the ethanol molecule contains oxygen, it allows the engine to more completely combust the fuel,

resulting in fewer emissions and thereby reducing the occurrence of environmental pollution. Ethanol is used for preparation of chloroform, iodoform, ethanoic acid, ethanol, ethyl ethanoate etc., Ethyl alcohol is also used as hypnotic (induces sleep), a mixture of ethanol and water has lower freezing point than water. This mixture is known as antifreeze and is used as radiators of vehicles on cold countries and at hill situations. Bioethanol is renewable and oxygenated ethanol that reduces emission of carbon dioxide and aromatic compounds. Bioethanol also has other advantages such as: lower carbon (IV) oxide emission, lower dust emission, biodegradable. It is also the only liquid made from biomass that can be used as transportation fuels and does not cause greenhouse gas effect (Mushimiyimana and Tallapragada, 2016). Use of bioethanol as a renewable transportation fuel helps to minimize the amounts of fossil derived carbon dioxide (CO_2) to the Earth's atmosphere (Subhash et al., 2016). It has a low melting point, so it can be added to liquids as antifreeze and can be added to gasoline as an anti-knocking agent (Vazirzadeh and Robati, 2013).

2.3.2 Properties of ethanol

(i) Physical properties

Ethanol is a volatile, clear colorless liquid with a strong characteristic odor. It has specific smell, burning taste, a smokeless blue flame that is not always visible in normal light and used in finger nail polish remover. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding; rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight. Ethanol is a versatile solvent which is miscible with water and with many organic solvents.

(i) Chemical properties

Ethanol is classified as primary alcohol which means hydroxyl group of the carbon is attached to at least two hydrogen atoms and it has the following chemical properties.

Ester formation: under acid catalyzed conditions, ethanol reacts with carboxylic acids to produce ethyl esters and water:

$$RCOOH + HOCH_2CH_3 \rightarrow RCOOCH_2CH_3 + H_2O \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.1)$$

Oxidation: Ethanol can be oxidized as acetaldehyde and acetic acid. In the human body these oxidation reaction is catalyzed by enzymes. Direct oxidation with chromic acid is given as follows:

$$C_2H_5OH + 2[O] \rightarrow CH_3COOH + H_2O \quad (2.2)$$

Chlorination: when exposed to chlorine ethanol is oxidized and its alpha carbon chlorinated to form the chloral compound.

$$4CL + C_2H_5OH \rightarrow CCL_3CHO + HCl \quad (2.3)$$

Halogenation: ethanol reacts with hydrogen halides to produce ethyl halides such as ethyl chloride and ethyl bromide:

$$CH_3CH_2OH + HCl \rightarrow CH_3CH_2Cl + H_20 \quad (2.4)$$

$$CH_3CH_2OH + HBr \rightarrow CH_3CH_2Br + H_20 \quad (2.5)$$

Acid base chemistry: Ethanol can be quantitatively converted to its conjugate base, the ethoxide ion ($CH_3CH_2O^-$) by reacting with alkali metal such as sodium:

$$2CH_3CH_2OH + 2Na \rightarrow 2CH_3CH_2ONa + H_2 \quad (2.6)$$

2.4. Bioethanol Application as a Fuel

Bioethanol is a product of fermentation that has been utilized as biofuel, a beverage and industrial alcohol. Most of produced ethanol (73%) is utilized as fuel, while percentage of beverage and industrial alcohol are 17% and 10% respectively. The alcohol in fuel ethanol is chemically identical to ethanol used for other purposes such as distilled spirit beverages and industrial products (Uncu and In, 2009). Moreover bioethanol is already being used or blended with gasoline for transportation purpose. When blending ethanol with gasoline, the fuel mixture is oxygenated so that it burns more completely thereby reducing emission of unburned gas that causes environmental pollution. Many countries have implemented, or are in the process of implementing, programs providing for the addition of ethanol to gasoline. Fuel ethanol production has increased remarkably due to the global demand to reduce oil importation, thereby contributing towards boosting rural economies and improving air quality.

Ethanol in general and bioethanol in particular are considered as chemical compounds with high potentials to replace fossil fuels used in vehicles. Bioethanol has a high octane number and for the same reason it is used to increase octane number in spark ignition engines.

Though the earlier combustion powered transportation vehicles were fuelled with ethanol and crude oil derivatives have provided the vast majority of transportation fuels throughout the 20th and 21st centuries (Byadgi and Kalburgi, 2016).

2.5 World Ethanol Production

World demand for fuel ethanol production has significantly growing in recent years for use of gasoline blends. The world ethanol production in 2010 was 13, 298 in million gallons, but according to statistical data for renewable energy source by the end of 2016 increased to 15,330 gallons per year (Berhane, 2016).

According to a statistic depict of fuel ethanol production of different countries USA is a world leading fuel ethanol producer by generating 15.8 billion gallons in total. With more than seven billion gallons, Brazil was ranked second. The increment for the consumption of cleaner and reformulated gasoline has been largely responsible for growing of ethanol production plant in the world (Izmirlioglu et al., 2012).

Table 2.1: Annual fuel ethanol production of country (2012-2016) (in millions of liquid gallons per year) (López-vásquez et al., 2019).

Rank (2017)	Country/Region	2012	2013	2014	2015	2016
1	United states	13,300	13,300	14,300	14,806	15,330
2	Brazil	5,577	6,267	6,190	7,093	7,295
3	Europe	1,179	1,371	1,445	1,387	1,377
4	China	555	696	635	813	845
5	Canada	449	523	510	436	436
	Rest of the world	752	1.272	1,490	1,147	1,301
	World total	21,812	23,429	24,570	25,682	26,094

Source: renewable fuel association

2.6 Ethanol Production in Ethiopia

Ethiopia is a developing country with high population density and large rural population rely on land for their livelihood. Ethiopia has joined hands with Eugen Schmitt Company, a German company, to construct ethanol plant in Wonji Shoa Factory. When the plant is constructed it was said to have the capacity to produce 60,000 liters of ethanol per day using molasses, by-product of sugar, from Wonji Shoa sugar factory, Gashaw Aychiluhim, corporate communication executive office of the Ethiopian Sugar Corporation, explained.

Table 2.2: Annual Ethanol productions in Ethiopia in liters

Year	Fincha Sugar Factory	Methara Sugar Factory	Total
2004/05	1,636,047	-	1,636,047
2005/06	6,847,816	-	6,847,816
2006/07	6,066,860	-	6,066,860
2007/08	5,330,337	-	5,330,337
2008/09	5,878,516	-	5,878,516
2009/10	7,116,585	-	7,116,585
2010/11	7,127,895	6,373,775	13,501,670
2011/12	6,794,000	7,658,000	14,452,000
2012/13	7,620,500	7,063,000	14,683,000
2013/14	11,678,000	7,767,000	19,445,000
2014/15	10,999,000	8,806,000	19,805,000

Source: Fana Broadcasting Corporation http://www.2merkato.com/news/alert

2.7 Waste Potato as a Feedstock for Bioethanol Production

The potato (Solanum tuberosum) is a potential feedstock for ethanol production due to its high starch content (approximately 80%) and a yield that is two to three times higher than that of fermentable sugar (López-vásquez et al., 2019). It grows up to 100 cm tall and produces a tuber which is rich in starch and ranked as the world's fourth most important food crop, after maize, wheat and rice. The potato belongs to the Solanaceae or nightshade family of flowering plants, shares the genus solanum with at least 1000 other species including tomato and eggplant (Tadesse and Tsegaye, 2018).

Potatoes are a high value crop and a (5 – 20) % byproduct ends as a waste during cultivation. Moreover in potato processing industry approximately 18% of potatoes are generated as waste. Potato peel waste is a zero value by product, which occurs in big amounts after industrial potato processing and can range from (15-40)% of initial product mass, depending on the peeling method (Dash et al., 2017). Potato peels have been exploited as natural antioxidants in food system due to its high content of polyphenols, antioxidants in biological systems, screened as low cost solid substrates for microbial production of enzymes to be used either in food applications or in other industrial sectors. The problem of management of the potato peel waste requires an integrated and ecofriendly solution (Adegunloye and Oparinde, 2017). Potato peel utilization for bioethanol production is a considerable option as 58% of the dry weight is starch (Biotechnol and Tripathi, 2018).

Starch is polysaccharide composed of amylose and amylopectin both of which are glucose units (Mushimiyimana and Tallapragada, 2016). Amylopectin is highly branched by short chains which (70-80) % is a starch by composition.

Amylose is a linear polysaccharide formed by alpha 1, 4-linkage glucose residues is the major component of starch (20-30)% (Izmirlioglu et al., 2012). It is widely employed as versatile excipients in various firm dosages such as diluents, disintegrates, and binding agents in tablet formulations (Bekele, 2016).

A further work is required on optimization of operational condition for the production of bioethanol from waste potato (Malik et al., 2018).

Table 2.3: The chemical composition of waste potato for suitability in the production of bioethanol (Ghosal et al., 2013).

Constituents	Amount (%)
Moisture	37.20
Ash	2.00
Starch	34.26
Protein	19.87
Fat	6.3

2.7.1. Potato Production in the World

Potatoes were first cultivated around 200 B.C. by the Inca Indians in Peru. At that time, potatoes served a wide variety of uses, such as healing broken bones and measuring time. Nearly 4,000 varieties can be found in the Andes. The Spanish brought potatoes to Europe in the 16th century. European consumers were reluctant to adopt the potato. However, due to the sheer practicality of the potato adaptability, generally plentiful crops and relatively long shelf life, combined with the nutritional value it was soon widely accepted and consumed. Since the early 1960s, the growth in potato production area has rapidly overtaken all other food crops in developing countries. It is a fundamental element in the food security for millions of people across South America, Africa, and Asia, including Central Asia (Tadesse and Tsegaye, 2018). Potato production is important in fulfilling energy needs beside consumption as a food crop since sugary substrates and cellulosic materials are either expensive or involves many steps for bioethanol production. Therefore, crops which contained greater amount of starch are usually consumed for the bioethanol production (Khan et al., 2012).

Table 2.4: Top five Potato Producing Countries in the world

Rank	Country	Potato productions in metric tones	% of world total
1	China	85,860,000	23.3
2	India	45,000,000	12.2
3	Russian federation	29,532,530	8.0

4	Ukraine	23,250,200	6.3
5	United states	19,165,865	5.2

Sources: FAOSTAT data, 2014 (last accessed by Top 5 of anything: January 2014).

2.7.2 Potato Production in the Africa

Potato production arrived late in Africa around 20th century. In recent decades, production has been in continual expansion, rising from 2 million tons in 1960 to a record 16.7 million tons in 2007. Potatoes are grown under a wide range of conditions from irrigated commercial farms in Egypt and South Africa to intensively cultivated tropical highland zones of Eastern and Central Africa, where it is mainly a small farmer's crop.

Table 2.5: Top Potato Producing Countries in Africa

Rank	Country	Potato Production (tones)
1	Egypt	4,500,00
2	Algeria	4,219,476
3	Malawi	3,255,780
4	Kenya	2,915,067
5	Morocco	1,560,000
6	Rwanda	1,300,000
7	Nigeria	843, 000
8	Mali	800, 000
9	Uganda	650, 000
10	Angola	615, 000
11	Ethiopia	525, 000

Source: (http://www.fao.org/potato2008/en/world/africa.html,June, 2015)

2.7.3 Potato Production in the Ethiopia

A German immigrant is credited with introducing potato to Ethiopia in 1858. Over Open Access Journal of Chemistry in 2018/19 the following decades, farmers in Ethiopia's highlands began cultivating the new tuber known as denech as an "insurance policy" against cereal crop failures.

Among African countries, Ethiopia has possibly the greatest potential for potato production: 70 percent of its arable land mainly in highland areas above 1500 m is believed suitable for potato (Tadesse and Tsegaye, 2018).

A good harvesting technology is recommended for farmers to increase benefit generated from potato production and reduce loss. Farmers use traditional tools (sharp spades, hoe, sometimes pull the potato with hand) to harvest potato. The loss at harvest is estimated at 20% in Alata Wondo Area. To protect quick wilting, farmers cover the harvested potato with leaves which is a good practice (Emana and Nigussie, 2011).

2.7.4 Potato Varieties

There are literally thousands of different varieties of potatoes grown around the world depending on different criteria. In Ethiopia there is no clear data about the varieties of potatoes (Tadesse and Tsegaye, 2018).

Red Potatoes: small to medium; round to long shape; white or tan skin; white flesh. Its Texture is medium starch; slightly creamy, slightly dense; thin, delicate skin Its Flavor is subtly sweet; mild; low sugar content. It is preferred uses are mashing, salads, steaming/boiling and frying.

Figure 2.1: Appearance of red potato

White potato; Its Appearance ranges from small to medium; round or slightly oblong; smooth, thin red skin; white flesh. Its Texture is waxy, moist and smooth; creamy. Its Flavor is subtly sweet; mild medium sugar content. Its preferred uses are Roasting, mashing, salads, soups/stews. White potatoes hold their shape well after cooking.

Their delicate, thin skins add just the right amount of texture to a velvety mashed potato dish without the need for peeling. Grilling whites brings out a more full-bodied flavor.

Figure 2.2: Appearance of white potato

2.8 Bioethanol Production Process

Ethanol can be produced either by fermentation of sugar containing feeds, starchy fed materials or lignocellulosic materials or chemically by hydration of ethylene which is derived from crude oil or natural gas. This includes preparation of raw materials, pretreatment, hydrolysis, fermentation, distillation and dehydration.

2.8.1 Raw material preparation

Bioethanol can be produced from various feedstocks including sugar containing, lignocellulosic and starchy feedstock which can be categorized as follows: Starch containing feedstock (e.g. potato, wheat, corn, rice, barley, etc.), Sucrose containing feedstock (e.g. molasses, sugar cane, sugar beet and sweet sorghum) and Lignocellulose containing feedstock (e.g. wood, straw and grass).

2.8.2 Pretreatment

Pretreatment: is a process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps (Faria, 2013).

Pretreatment techniques are mainly classified as physical (e.g., grinding and milling), chemical (e.g., alkali, acid, ammonia percolation), physiochemical (e.g., steam explosion, ammonia fiber expansion) and biological (e.g., fungi and *Actinomycetes*) (Safarian and Unnthorsson, 2018). It is

carried out to increase the surface area and accessibility of the plant fiber to enzymes and thus, achieve high sugar yield for ethanol fermentation (Otulugbu, 2012). To be efficient any selected pretreatment method should improve the availability of monomeric sugars, prevent their degradation, avoid inhibitor formation and be low cost. Pretreatment methods can be classified as physical, chemical, physiochemical, biological methods and their combinations (Dimos et al., 2019). Waste potatoes are required to disperse in acid, alkali and hydrothermal pretreatment for the control of untreated sample. The residue is dispersed separately in distilled water, 1% sulfuric acid solution (v/v), 1% sodium hydroxide solution (w/v) at solid to liquid ratio of 1:10 before being heated at 121 ^{0}C for 30 minutes. A pH of acid and alkali sample is adjusted to 7. Finally, all samples are required to dry in an oven at 40 ^{0}C to get a balanced constant weight (Chniti, 2018).

2.8.3 Hydrolysis

Hydrolysis is a process of breaking down starch into fermentable sugars and required before fermentation of starchy materials. Hydrolysis is carried out at high temperatures (90 – 110) ^{0}C and it is possible to use low temperature which helps to save energy.

To convert starch into fermentable sugar, either acid hydrolysis or enzymatic hydrolysis should be done (Islamic, 2017). Both acid and enzymatic hydrolysis method have benefits and limitations. The limitation of acid hydrolysis includes: need of expensive constructional materials, neutralization before fermentation and inhibition of byproducts on yeast (such as 5-hydroxymethylfufural). Enzymatic hydrolysis requires high enzyme cost despite high conversion yield of glucose (Izmirlioglu et al., 2012).

Traditional methods developed in the 19th century and at the beginning of the 20th century, produced glucose from cellulose by usage of acid. Dilute acid thermal hydrolysis is the most extensively used treatment in biomass ethanol research and it is considered more cost effective with a shorter reaction time than current hydrolysis methods used. Hence, it can be concluded that acid hydrolysis is a simple method for starch hydrolysis since the resources are easily available and cheap. Acid hydrolysis is perhaps currently seen as the most technologically matured method of sugar release from biomass.

Depending on the concentration of the acid and the other parameters dilute acid maybe used at high temperature and pressure while concentrated acids may be used at very low temperature and pressure. In the case when sulfuric acid can be concentrated (25-80)% or dilute (3-8)%, it is used under hydrothermal conditions resulting in shorter reaction times, lower costs, and higher capacity to hydrolyze polymers and oligosaccharides to monosaccharaides (Otulugbu, 2012).

Higher concentrations of these chemicals decrease reaction times, avoid the use of enzymes, and while the use of low concentrations makes necessary higher temperature and pressure values to achieve favorable hydrolysis efficiencies (Velazquez-lucio et al., 2018).

2.8.4 Filtration

Filtration is a process used in mixture separation of different phase (solid, liquid, and gases) and components. Rotary vacuum filter is essential type of filtration which consists drum rotating in a tube of liquid to be filtered. The technique is well suited to slurries and liquids with a high solid content, which could clog other forms of filter. The drum is pre-coated with a filter aid, typically of diatomaceous earth (DE) or Perlite.

Generally, the main process in a rotary vacuum drum filter is continuous filtration whereby solids are separated from liquids through a filter medium by a vacuum.

The filter cloth is one of the most important components on a filter and is typically made of weaving polymer yarns. The best selections of cloth can increase the performance of filtration. Initially, slurry is pumped into the trough and as the drum rotates, it is partially submerged in the slurry. The vacuum draws liquid and air through the filter media and out the shaft hence forming a layer of cake. An agitator is used to regulate the slurry if the texture is coarse and it is settling rapidly. Solids that are trapped on the surface of the drum are washed and dried after 2/3 of revolution, removing all the free moisture. The rotary vacuum drum filter is a continuous and automatic operation, so the operating cost is low and the process can be easily modified.

2.8.5 Fermentation

Fermentation is metabolic process of microorganisms in which energy is obtained by breaking down organic compounds and it is an essential aspect of bioethanol production. In ethanol

fermentation, energy is derived from sugar and either yeast or bacteria is used to produce ethanol and carbon dioxide. Batch, semi-batch and continuous fermentation processes from any material that contains sugar is used in ethanol industry. Sugars (from molasses, sugar canes, fruits and sugar beets) can change into ethanol directly. Starches (from waste potatoes, cassava, root crops and corn) must first hydrolyze into fermentable sugars before fermentation. Cellulose (from wood, agricultural residues and paper) must be converted into sugars, by the action of acid minerals (Flayeh, 2017). However, the main disadvantages of this process is most of starchy crops are food crops and tend to increase cost of production. In order to make fermentation method cost effective and meet the great demand for ethanol using inexpensive raw materials such as agricultural wastes, municipal and industrial wastes as a feedstock is highly recommended for bioethanol production (Bekele et al., 2015).

The efficiency of the process and thus bioethanol yield also depend upon the efficient utilization of the released sugars and thereby their conversion to ethanol by different microbes (Rastogi and Shrivastava, 2018).

Cost effective ethanol production from renewable resources is essential since it primarily depends on the cost of the carbon sources and nutrients used in the fermentation media. Therefore, techno-economic analysis of a fermentation process plays a critical role to see whether or not the process is cost effective. Accordingly, investigations of economically suitable alternatives for raw materials and bioprocess technology platforms have gained momentum in the industrial and academic sectors (Germec et al., 2019).

Until now, as reported by their fermentable sugar composition, required optimal fermentation processes, and methods/strategies for their resulting hydrolysates have been examined. Before the bioethanol fermentation process, acid hydrolysis as a pre-treatment step of these byproducts has been carried out by using dilute range solutions of sulfuric acid (in particular), and phosphoric acid, or hydrochloric acid (Biotechnol and Tripathi, 2018).

Saccharomyces cerevisiae, the yeast act as the major vehicle to run the whole fermentation process, can increase amounts of sugars in the medium, when all required nutrients are provided inadequate amounts (Salehi et al., 2018). There are manifold fermentation approaches that are being practiced by laboratories and industries nowadays to manufacture bioethanol and

maximize the yield, such as separate hydrolysis and fermentation (SSF), simultaneous saccharification and fermentation (SSCF) and solid state fermentation (Dijken et al., 1993).

The rates of hexose consumption, bioethanol production and yeast population growth are correlated by a kinetic model. New yeast cells occur catalytically from the substrate with a specific growth rate during yeast growth in fermentation medium. This process can be expressed by the kinetic formula shown below in Equations (2.7) and (2.8).

$$rx = \mu X \dots\dots\dots\dots\dots\dots\dots\dots\dots (2.7)$$

$$\frac{dX}{dt} = \mu X \dots\dots\dots\dots\dots\dots\dots\dots\dots (2.8)$$

Where r_x or dX/dt is the rate of cell growth, μ is the specific growth rate (h^{-1}), X is cell concentration (gL^{-1}) and t is time (h). According to the Monod Equation, the relationship between the limiting substrate concentration, S and μ are as shown in Equation (2.9).

$$\mu = \frac{\mu_{max} S}{K_s + S} \dots\dots\dots\dots\dots\dots\dots\dots\dots (2.9)$$

Where S is limiting substrate concentration (gL^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), Ks is the saturation constant (gL^{-1}).According to Line weaver-Burk method, K_s and μ_{max} can be predicted by taking reciprocals of both sides of equality sign as shown in Equation (2.10).

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} S + \frac{1}{\mu_{max}} \dots\dots\dots\dots\dots\dots\dots\dots\dots (2.10)$$

Here 1/μ versus 1/S will allow K_s to be evaluated while the intercept is $1/\mu_{max}$ (Mahlia and Indra, 2017).

2.8.6 Distillation

Distillation is the most dominant and recognized industrial purification technique of ethanol. In the early days of ethanol distillation, the basic ethanol production design came from beverage industry where there is no need of all water removal. It utilizes the differences of volatilities of components in a mixture and necessary to produce fuel grade ethanol, as anhydrous ethanol is necessary for blending with gasoline (Fedenko, 2011). Distillation is used to remove water but alcohol content of distillation product is limited to 95 (%v/v) due to formation of water ethanol azeotrope (Kilpimaa et al., 2014). Fuel ethanol, however must be pure through dehydration using a technique called azeotropic distillation (Rendleman, 2014).

Several column configurations can be used for distillation process both in batch and continuous. In batch mode, batch extractive distillation is a process where the mixture to be separated is charged into the still whereas entrainer is fed continuously. A typical extractive distillation process, which includes an extractive distillation column where the solute, A, is obtained as the distillate and the mixture of raffinate and solvent is exists from the bottom is called continuous extractive distillation. A solvent recovery column comes next where the purified raffinate is obtained as distillate and the solvent is recovered from the bottom and recycled to the extractive distillation column. The advantages of batch distillation over continuous distillation are its simplicity due to just one column to control and have great flexibility, where many different materials can be distilled in the same equipment (Fidkowski and Industries, 2015).

The basic principle is that by heating a mixture, low boiling point components are concentrated in the vapor phase. By condensing this vapor, more concentrated less volatile compounds is obtained in liquid phase. However, it contains several problems.

One is separation of volatile compounds and the second is its cost. In ethanol production, a distillation tower is designed to separate water and ethanol effectively. Water is obtained from the bottom of the tower and ethanol is obtained from the top of the tower.

It is expected that impurities with similar boiling points to ethanol lodges in ethanol even after distillation. Distillation consumes energy cost due to its repetition of vaporization and condensation (Onuki et al., 2008).

2.8.7 Dehydration

Bioethanol, if it is used as a fuel, must be anhydrous. Most of the water can be removed by distillation, but the alcohol content of the distillation product is limited to 95 volume % due to the formation of a water ethanol azeotrope. There are many dehydration processes to remove the water from an azeotropic mixture. The first process is called azeotropic distillation, in which auxiliary substance, benzene or cyclohexane, is added to the mixture. The formed azeotropic mixture is stronger than the original one, and anhydrous ethanol can be formed from the mixture on the top of the column. The second method is called extractive distillation, in which a ternary component is added to the mixture. Ternary component will increase the relative volatility of ethanol (Kilpimaa et al., 2014).

Distillation based methods are very energy intensive. There exist also some modified, less energy-intensive methods, e.g. membranes. Through membranes water can be separated from water ethanol mixture without distillation. Water molecules penetrate the membrane and ethanol molecules concentrate to the other side of the membrane. Pervaporation is combination of membrane permeation and evaporation, and it can be used for dehydration. Adsorption is also a usable method especially in case of the azeotropic mixtures (Kilpimaa et al., 2014).

2.9 Factors Affecting Fermentation Process

Until now, as reported by their fermentable sugar composition, required optimal fermentation processes, and methods/strategies for their resulting hydrolysates have been examined. Before the bioethanol fermentation process, acid hydrolysis as a pre-treatment step of these byproducts is carried out by using dilute range solutions of sulfuric acid (in particular), and phosphoric acid, or hydrochloric acid (Biotechnol and Tripathi, 2018).

Optimization of significant operational conditions is an important stage to develop efficient and cost effective production. To optimize fermentation condition it is crucial that considering a condition that have a significant effects (such as: pH, temperature, yeast concentration, fermentation time and rotation rate). Experimental design and response surface method are helpful in the analysis of results by visualizing the relationships of the experimental variables, response and selection of optimal value (Nguyen, 2018).

Different fermentation conditions are required to be compared in order to get maximum yield of ethanol and concentration. Amongst them the optimized ethanol production parameters includes: (pH 5.0, temperature 30 ^{0}C, initial moisture content 87%, fermentation time period of six days, $(NH_4)_2SO_4$ 0.2%, inoculum size 20% for yeast, 2% for bacteria and 4% for *Aspergillus niger* (Bekele et al., 2015).

2.9.1 Temperature

Temperature is an important fermentation parameter that influences the growth rate of microorganisms and rate of ethanol production. Too high temperature kills yeast and slows yeast activity if it is lower than enough. Therefore, to set a correct value of temperature at which a system works accurately is required to get a higher yield. As heat energy is liberated during fermentation of sugar by yeasts there is always an increase in temperature and cooling of fermenter is required to use temperature at tolerant strains of (30 – 35) ºC. However, the optimum fermentation temperature at a low alcohol concentration is often slightly higher (38 ºC), but alcohol tolerance is improved at reduced temperature. Fermentations of beer and wine are conducted below 20 ^{0}C (Mohammed, 2017).

2.9.2 pH

Most alcoholic yeast fermentations are conducted below pH 4.5. Yeast cultures can grow over a wide range from 3 to 8 with an optimum for growth generally in the slight acidic range. Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures. Thus, the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth and physio-chemical effect on waste product pathways (Berhane, 2016).

Control of pH during the fermentation process is important for the Growth of harmful bacteria that is retarded by acid solutions and the growth of yeast only in an (slightly) acid solution.

The development of bacteria is severely repressed at pH value under 5. The acid most commonly used is sulfuric acid, although any mineral acid is perfectly suitable. Further, yeast can tolerate as low pH as 2 without permanent damage (Bekele et al., 2015).

2.9.3 Fermentation time

Optimum fermentation time is a time at which a fermentation process becomes economical and produces a required conversion of fermentable sugar into alcohol. The effect of fermentation time is identified using a flask containing 1.0% Waste potato and PPW hydrolysate inoculated with 1.0% (v/v) yeast isolates then incubated for different period.

2.9.4 Inoculum size

The size of inoculum in ethanol fermentation has a great importance for completion of the fermentation process. A 3-10% (v/v) inoculum of *saccharomyces cerevisiae* has been reported for maximum bioethanol concentration from various substrates (Dash et al., 2017).

2.9.5 Substrate concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. The amount of ethanol produced is proportional to the amount of sugar added; thus, high sugar concentrations are desired. However, too high sugar concentrations can inhibit metabolism due to increased osmotic pressure. Very low levels of sugar, on the other hand, may limit the rate of ethanol production (Berhane, 2016).

2.10 Definition of Important Biofuel Properties

2.10.1 Density and Specific Gravity

Specific gravity is a measure of the density of liquid fuels. It is the ratio of the density of the fuel at 15.6 °C to the density of water at the same temperature. The density of water at 15.6 °C is 1 kg/L, so the specific gravity of a fuel is equal to its density in kg/L. Density of liquids decreases slightly with increasing temperatures. Therefore, densities must be measured at the standard temperature of 15.6 °C or must be corrected to that temperature (Mathani et al., 2010).

2.10.2 Viscosity

Viscosity is measure of the resistance to flow of a fluid under gravity, it is important to note that viscosity critically depends on temperature and numerically value of viscosity has no significance or meaning unless the temperature of the test is specified.

So in determining any viscosity of fuel the temperature during the test must always be state. If viscosity is too low, the fuel will flow too easily and will not maintain a lubricating film between moving and stationary parts in the carburetor or pump. If viscosity is too high, may not be able to atomize the fuel into small enough droplets to achieve good vaporization and combustion (Kheiralla et al., 2011).

2.10.3 Flash and Fire Point

The flash point varies with fuel volatility but is not related to engine performance. Rather, the flash point relates to safety precautions that must be taken when handling a fuel. The flash point is the lowest temperature to which a fuel must be heated to produce an ignitable vapor air mixture above the liquid fuel when exposed to an open flame. At temperatures below the flash point, not enough fuel evaporates to form a combustible mixture. Insurance companies and governmental agencies classify fuels according to their flash points and use these points in setting minimum standards for the handling and storage of fuels. Gasoline's have flash points well below the freezing point of water and can readily ignite in the presence of a spark or flame. It is important to realize that the value of the flash point is not a physical constant but is the result of a flash point test and is dependent on the apparatus and procedure used. The fire point is the lowest temperature at which application of an ignition source causes the vapors of a test specimen of the sample to ignite and sustain burning for a minimum 5 sec under specific conditions of test (Mathani et al., 2010).

2.11 Bioethanol Analysis using Fourier Transfer Infrared Spectroscopy

In the electromagnetic spectrum, infrared region lies between visible and microwave region. The energy associated with molecular vibration fall in the infrared region as they are lower than electronic transition. A molecular vibration absorbs infrared radiation if the vibration causes change in dipole moment. FTIR is used to identify organic molecules. Absorption by the molecules is measured against wavelength. An FTIR uses an interferometer which generates radiation; absorption of wavelength brings change in interferogram which gets detected.

An interferogram is a time domain signal and is converted to frequency domain signal though Fourier Transformation. FTIR plots are usually % transmittance or absorbance versus wave number. Absorption bands in 4000 - 1500 cm^{-1} are generally due to functional groups, peaks below this region are due to complex vibrations of several atoms. The sample can be used in different forms (solid, liquid or gaseous) depending upon the instrumentation used (Biotechnol and Tripathi, 2018).

Table 2.6: Typical vibrational frequencies of functional groups

Bond	Molecules	Wavelength (cm^{-1})
C-O	Alcohols, Ethers, Esters, Carboxylic Acids, etc	1300-1000
C=O	Aldehydes, Ketones, Esters, Carboxylic Acids	1750-1680
C=O	Amides	1680-1630
N=H (stretching)	Amines and Amides	3500-3100
-N-H (bending)	Amines and Amides	1640-1550
O-H	Alcohols	3650-3200
C-N	Amines	1350-1000
S-H	Mercaptans	2550

2.12 Critique of Existing Literature Relevant to Study

An extensive study on the production of bioethanol was presented in above reviews, which also proposes different improvement regarding methods of production, use of various yeasts and optimization on different process parameters. This helps what is known in a title, how well this

knowledge is established and where future research might be directed. This finally results in improved yield of bioethanol produced from waste potato (Niculescu et al., 2019).

Table 2.7: Comparison summary of previous literature with respects to this work

Title	Previous work	Reference of previous work	This work
Bioethanol production from waste potatoes using co-culture of *Saccharomyces cerevisiae* and *Aspergillus niger*	Production of bioethanol from waste potatoes was done by employing (SSF) and simultaneous saccharification and fermentation (SiSF) using co-culture of *Saccharomyces cerevisiae* and *Aspergillus niger*.	(Science and Subhash, 2015)	Production of bioethanol from waste potato was carried out through separate hydrolysis and fermentation using yeast *saccharomayces cerevisae*
Production of bioethanol from waste potatoes	Only optimization of temperatures and incubation periods were discussed to get maximum yield of bioethanol in solid state fermentation.	(Science and Rai, 2010)	Optimization of Temperature, pH and incubation time were studied by considering both individual and interaction effect among parameters
Optimization of Hydrolysis for Production of Bioethanol From Waste Potatoes and Potato Peels	Waste potato was analyzed using ANOVA in the form of hydrolysate solution of and the optimization was done on hydrolysis parameters	(Alemayehu, 2015)	Detail proximate analysis of waste potatoes mixture was done before hydrolysate formation and optimization was done on fermentation operating condition

3. MATERIALS AND METHODS

3.1 Experimental Framework

This research is conducted to obtain a maximum yield of bioethanol by optimizing fermentation condition. All experiments in this study were planned according to this framework.

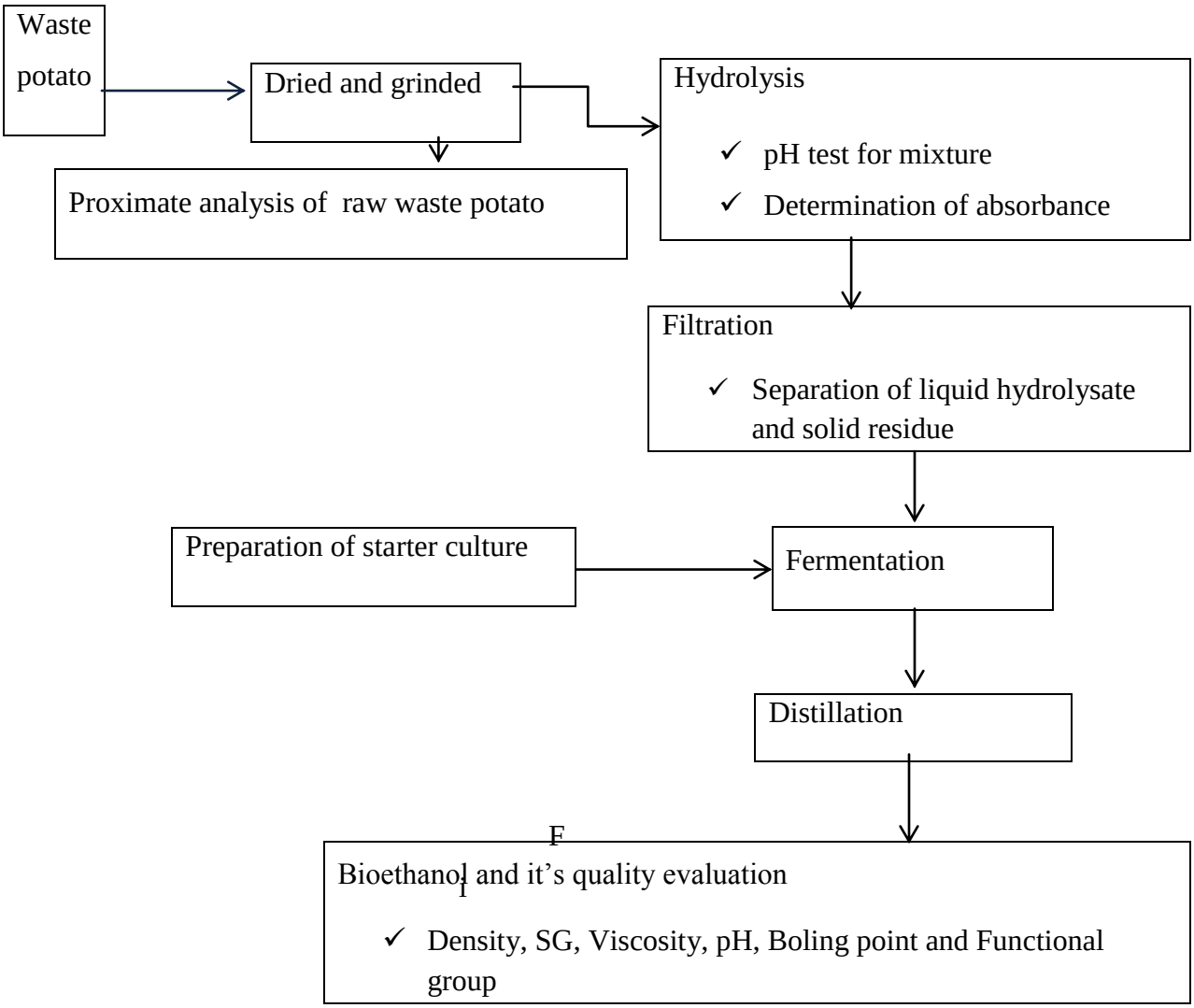

Figure 3.1 Experimental Framework of the study

3.2 Materials

Raw materials: Raw waste potato was collected from market of Jimma, Merkato (Atkilt tera).

List and Uses of Equipment: the apparatus used to perform the experimental work with their corresponding functions include: stainless steel knife for size reduction; oven to dry and rotary mill was used to ground a dried waste potato; sieves to sieve the crushed sample to the particle size of 2 mm; desiccator to bring down temperature at room condition; measuring cylinder to measure the solutions and distilled water; beaker to contain solution; test tubes to hold samples; magnetic stirrer to stir iodine solution; balance to measure the sample ingredient; spectroscopy to measure the absorbance based on given concentration; dropper Cuvette to transfer solution to iodine solution; safety material to protect skin and prevent eye damage during solution preparation; parafilm to wipe a cuvette containing solution; erlenmeyer flask to put a prepared waste potato sample ratio to sulfuric acid; autoclave to keep a solution prepared for hydrolysis at a given temperature and time in accordance with experimental design; volumetric flask in sugar content determination; spectrophotometer to measure optical density of glucose concentration; shaker incubator for media preparation during fermentation; aluminum flask to cover a media of culture media and hydrolyzate solution; vessels to hold samples for distillation and Thermometer to determine liquid temperature.

Chemicals and Ingredients: The chemical used in this experimental work include: benedicts solution, starch solution, distilled water, sulfuric acid, D-glucose and anhydrous glucose. The ingredients used in fermentation during media preparation were includes: yeast extracts, urea, dextrose, peptone, $MgSO_4.7H_2O$ 1.0g and distilled water. In fermentation cultured media and hydrolyzate media were added into a solution with a ratio of 1:10 (% w/w).

3.3 Methods

3.3.1 Characterization of waste potato

The experiments on proximity analysis were takes place at Addis Ababa Institute of Technology and additionally the starch content of powder waste potato was determined at Burrayu Kebron Food Complex Factory.

i) Moisture content determination

Moisture content of waste potato was determined using oven dry method. A known chopped potato samples were put on a pre-weighed aluminum foil (W_1) and record the weight of sample and aluminum foil (W_2).

Then, keep in an oven at a temperature of 100 ^{0}C for 8 hours until constant weight recorded. After this dry sample with aluminum foil is (W_3) and the difference in sample became a moisture loss. Finally, percentage moisture was determined using the following formula (Science and Subhash, 2015).

$$\% \text{ Moisture } = \frac{(W2-W1)}{(W2-W3)} \text{x } 100 \ldots\ldots (3.1)$$

ii) Total ash content determination

Total ash was determined to obtain the amount of organic and minerals present in the waste potato. An empty crucible was weighed and 10 g of dried milled sample of waste potato was added to the crucible and weighed. The crucible with sample was taken to already heated furnace. The temperature of the furnace was gradually increased from 250 ^{0}C to 450 ^{0}C after every 20 minutes to avoid incomplete ashing. After one hour of ashing, the crucible was removed from the furnace with a tong and placed in desiccators for cooling. The crucible with the sample (now ash) was cooled for one hour and then weighed.

W_1= mass of crucible

W_2 = mass of crucible and sample

W_3 = mass of crucible and cooled dried sample

$$\% \, Totalash \; = \; (\frac{(W_3 - W_1)}{(W_2 - W_1)}) \, X100 \ldots\ldots (3.2)$$

iii) Total Reducing Sugar determination

Standard glucose dilution series solution was prepared at different concentration of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 (% w/v). 1 ml of each of the standard glucose solution is added into labeled test tubes, each containing 6 ml of benedict's solution and mixed by shaking.

The labeled test tubes were heated in 90 ^{o}C water bath for 3 minutes the red color was obtained. The test tubes removed from the water bath and filtered the samples using filter paper to remove any red precipitate formed when reducing sugar in the samples reacted with benedict reagent.

The absorbance of the samples was read using spectrophotometer. Finally, calibration curve to show the % of absorbance and for the calculation of concentration of unknown sample.

Finally, the absorbance was measured using a spectrophotometer at a wavelength of 540 lamda (Umo et al., 2013).

Table 3.1: Calibrated results of absorbance for glucose concentration

Concentration (g/ml)	Absorbance (%)
0.000	0.000
0.2	0.169
0.4	0.416
0.6	0.483
0.8	0.785
1.0	0.852
1.2	0.975
1.4	1.100

iv) Protein content determination

Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure.

Before starting the determination of protein content in a prepared waste potato sample, preparation of the following reagents were found to be essential:

Kjedahl catalyst: a 1: 9 (% v/v) proportion mixture of copper sulfate and potassium sulfate, 96 % sulfuric acid solution, 40 % NaOH solution, 0.2 N HCL solution and 4 % H_3BO_3. Firstly a 1.0 ml waste potato sample was placed in digestion flask (Wilson, 1990). Then 5 g of Kjedahl catalyst and 200 ml of 96 % concentrated H_2SO_4 were added. Prepare a tube containing the above chemical except sample as blank, heat gently and briskly until solution clears in an inclined position. Next a sample was cooled for 30 minutes and 60 ml of distilled water was added cautiously. Immediately flask was connected to digestion bulb on condenser with tip immersed in standard acid and mixed with 5 drops of indicator in receiver. Again flask was rotated to mix content thoroughly and heated until all NH_3 was distilled. Finally before the tip of condenser washed; excess standard acid was distilled with standard NaOH solution and the receiver was removed (Aoac, 2000).

$$\text{Protein } (\%) = \frac{\left((A - B)x\ N\ x\ 14\ x\ 6.25\right)}{(W)} \ldots\ldots\ldots (3.3)$$

Where A = volume (ml) of 0.2 N HCL used in sample titration, B = volume (ml) of 0.2 N HCL used in blank titration, N = Normality of HCL, W = Weight (g) of sample, 14 = Atomic weight of nitrogen and 6.25 = the protein-nitrogen conversion factor for fish and its by-products.

v) Fat content determination

Before starting the determination for content of fat in a prepared waste potato sample petroleum ether reagent was used (Wilson, 1990). Firstly the weight of bottle was made stable by putting and lidding in the incubator at 105 °C overnight. Then 4 g of waste potato sample was weighed to paper filter and wrapped. A waste sample potato was taken into extraction thimble and transferred into soxhlet. The bottle was filled about 250 ml and taken into a heating mantle. The soxhlet apparatus was connected and turn on the water to cool before switching on the heating mantle for about 14 hours. Vacuum condenser was used to evaporate the solvent.

The bottle was incubated at (80 – 90) ℃ until solvent was completely evaporated and dried. Finally the bottle was transferred to the desiccator with partially covered lid for cool (Aoac, 2000).

$$\text{Fat (\%)} = \frac{(\text{weight of fat})}{(\text{weight of potato sample})} \text{x } 100 \ldots\ldots\ldots (3.4)$$

vi) Crude fiber content determination

Two grams of defatted sample were treated successively with boiling solution of H_2SO_4 of 0.26 N and KOH of 0.23 N. The residue was then separated by filtration, washed and transferred into a crucible then placed into an oven adjusted to 105 ℃ for 18 hours. The crucible with the sample was weighed and ashed in a muffle furnace at 500 ℃ and weighed. The crude fiber was calculated using the following equation:

$$\text{Fiber Content} = \frac{(W_2 - W_1)}{W_3} \text{x } 100 \ldots\ldots\ldots (3.5)$$

Where:

W_1 = Weight of crucible with sample before ashing

W_2 = Weight of crucible with sample after ashing

W_3 = Weight of sample

vii) Carbohydrate content determination

The sum of moisture, fat, protein, fiber and ash contents was subtracted from 100 to obtain the total carbohydrates (Saed and El-waseif, 2018).

$$\text{Carbohydrates} = 100 - (\text{ash \%} + \text{moisture \%} + \text{protein \%} + \text{fat \%} + \text{fiber \%}). (3.6)$$

3.3.2 Experimental procedures

i) Preparation of waste potato sample

In waste potato sample preparation first all the samples were washed with tap water, then the sample size were reduced manually using a stainless steel knife and dried in an oven at a temperature of 105 ^{0}C which was taken place for about 8 hours in order to prevent decomposition of waste potato during storage. Finally, the finished samples were grinded for further size reduction and sieving.

ii) Pretreatment

A 0.5 mm reduced size of waste potato was dispersed in distilled water. A ratio of solid powder of waste potato to liquid mixture of water was 1:15 (% w/w) and being heated at 121 ^{0}C for about 30 minutes (Chniti, 2018).

iii) Acid hydrolysis

A 1 to 15 (% w/w) concentrated solution of waste potato mash was prepared in distilled water. The pH of mixture was 5.85 and it was reduced to 4.5 by using H_2SO_4 to bring about required pH. Then prepared mixture was autoclaved at 121 ^{0}C for 60 minutes. Finally, the samples were cooled down for about 40 minutes and prepared for next analysis.

$$(C_6H_{10}O_5)_n + nH_2O + \text{Heat} \longrightarrow nC_6H_{12}O_6 \ldots\ldots\ldots\ldots (3.7)$$

Starch Water Glucose

iv) Preparation of starter culture

A 3 g yeast extract of saccharomyces cerevisiae, 10 g of peptone and 20 g of dextrose were dissolved in 200 ml of distilled water. The media was sterilized at 121 ^{o}C for 15 minutes until favorable condition were achieved.

v) Fermentation

The reactor and all the equipment's that were used for fermentation purposes were sterilized. The sterilization was carried out at a temperature of 121 ℃, for 15 minutes.

Then prepared sample and media were mixed in the 500 ml flasks with the ratio of 10% (1% cultured media with 10% hydrolysate sample). Finally, it was placed on shaking incubator at different temperature, pH, fermentation time and stirring rate of 200 rpm. Fermentation condition was analyzed by taking a three central points on experiment. Usually, the method for determining optimal condition in fermentation processes is varying one parameter while keeping others at a constant level.

This results in a high number of experiments rather than focusing on essential runs of experiment. Optimization using factorial design and response surface method can overcome such drawback by investigating different optimal point for determination of maximum yield (Menoncin et al., 2007).

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 + \text{Energy} \ldots\ldots (3.8)$$

Glucose Ethanol Carbon dioxide

vi) Distillation

After fermentation, to get a distillate yield batch distillation was carried out using batch distillation apparatus. About 200 ml of top fermented broth was transferred to a bottom flask. Then, it was placed on a heating mantle for about 2 hours for each run, which was fixed to a distillation and enclosed in a tap water. This helps to know the combined effect of overall fermentation process and distillation on yield of bioethanol. The condenser was connected with an apparatus to cool down the vapor ethanol and increased temperature of another flask was fixed to a distillation column set temperature of 78 ^{0}C. Finally, a distillate were collected since 78 ^{0}C is a standard temperature for bioethanol production (Richardson's and Coulson, 2002).

3.3.3 Experimental Design

The experiments were designed to determine the effect of fermentation condition on the yield of bioethanol produced from waste potato. A fully randomized experimental design was conducted to determine the optimal point.

Randomization ensures that the conditions in one run neither based on the condition of the previous runs nor predict the conditions in the subsequent runs. Randomization is essential for drawing conclusions from the experiment, in correct, unambiguous and defensible manner.

Temperature, pH and Fermentation time were taken as experimental factor and ANOVA was performed using Design expert® (V.11.0.0) trial version.

The response surface methodology (RSM) was extensively used for experimental data analysis as this model predicts experimental modifications like changes in operational conditions, various processing steps, which ultimately help in designing an experimental setup with minimum requirements and maximum yields (Dash et al., 2017).

The experiment was done through a combination of values for actual design factors on each levels from Design Expert (Douglas, 2001). A Response Surface Method was employed to provide a scope for improvement and optimization of the designed response which is influenced by various variables (Tripathi, 2018).

For statistical calculations the variable x_i was coded X_i according the following equation:

$$X_i, = \frac{xi - xo}{\Delta x}, I = 1;\ 2;\ 3 \ldots k \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.9)$$

WhereX_i is coded (dimensionless) value of the variable x_i, x_0 is the value of x_i at the center point and Δx is the step change.

The response variable was fitted to the following second-order polynomial model which is generally able to describe relationship between the responses and independent variables. A second order polynomial model was selected by taking into account the benefit of order and hierarchy. The order of the polynomial model is kept as low as possible. Some transformations can be used to keep the model to be of first order. If this is not satisfactory, then second order polynomial is tried. A model is said to be hierarchical if it contains the terms x, x^2, x^3, etc. in a hierarchy. It is expected that all polynomial models should have this property because only hierarchical models are invariant under linear transformation. This requirement is more attractive from mathematics point of view (Helwig and E, 2017).

$$Y = b_o + \sum_{i-1}^{n} b_i X_i + \sum_{i-1}^{n} b_{ii} X_i^2 + \sum_{i-1}^{n} \sum_{j-i+1}^{n-1} b_{ij} X_i X_j \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.10)$$

Where Y is the predicted response, b_0 the offset term, b_i the linear effect, b_{ii} the square effect, and b_{ij} is the interaction effect. It is a polynomial regression model in one variable and is called as second order model or quadratic model.

This design consisted of fifteen randomized runs with three replicates at the central point to minimize the error. Each variable was studied over three levels: low, middle and high. The levels of each variable were selected based on the expected experiment trials and available literatures. The factors and levels are given in table 3.2.

Table 3.2: The levels of variables chosen for the trials

Variables	Levels		
	Lower	Medium	Upper
Temperature, ⁰C	25	31	37
pH	4	4.5	5
Incubation time, hr.	72	84	96

Box-Behnken designs (BBD) are a class of rotatable or nearly rotatable second-order designs based on three level incomplete factorial designs. BBD is slightly more efficient than the central composite design but much more efficient than the three level full factorial designs where the efficiency of one experimental design is defined as the number of coefficients in the estimated model divided by the number of experiments. Another advantage of the BBD is that it does not contain combinations for which all factors are simultaneously at their highest or lowest levels. So these designs are useful in avoiding experiments performed under extreme conditions, for which unsatisfactory results might occur (Ferreira et al., 2007).

According to BBD the total number of experiment can be calculated as:

$$N = K^2 + K + Cp \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . (3.11)$$

Where k is a number of factors, and Cp is a central replication point. The above table shows the 15 experimental runs that are arranged according to BBD.

Therefore, the response in all above runs was registered and the significance was analyzed using Design Expert software. In RSM the process response which is required to be discussed from ANOVA table in detail is a bioethanol yield.

3.3.4 Data Analysis

The significance of important condition includes: Temperature, pH and fermentation time on the process was discussed in further at 150 rpm stirring rate and 20% yeast concentration. In addition to the results and conclusion of ANOVA table a further discussion relating to regression model was explained using a correct relation between yield and significant fermentation condition.

i) Temperature

To determine the optimum temperature for maximum yield of bioethanol after hydrolysis process, each flask containing 100 ml sample of hydrolysate were inoculated with 10% (v/v) yeast isolates and incubated at a different temperature in between 25 and 37 under stationary conditions. A 10% yeast concentration was selected with a stirring rate of 150 rpm.

ii) pH

To determine the optimum pH for maximum yield of bioethanol after hydrolysis process, each flask containing 100 ml sample of hydrolysate were inoculated with 10% (v/v) yeast isolates and incubated over a selected point of temperature with a pH in between 4 and 5 under stationary condition. A required fermentation process for 10% yeast concentration was achieved at a stirring rate of 150 rpm.

iii) Fermentation time

To determine the optimum fermentation time for maximum yield of bioethanol after hydrolysis process, each flask containing 100 ml sample of hydrolysate were inoculated with 10% (v/v) yeast isolates, incubated over a selected point of temperature and pH with different incubation period in between 72 hours and 96 hours at a stationary conditions. A required fermentation process for 10% yeast concentration was achieved at a stirring rate of 150 rpm.

Table 3.3 Input values of fermentation parameters

Std	Run	Factor 1: Temperature (^{o}C)	Factor 2: pH	Factor 3: Fermentation time (hr.)
5	1	25	4.5	96
6	2	31	4.5	84
8	3	37	5	84
3	4	25	4.5	72
12	5	31	4	96
11	6	37	4.5	72
14	7	31	5	72
1	8	37	4.5	96
15	9	25	4	84
7	10	37	4	84
9	11	31	4	72
2	12	25	5	84
10	13	31	4.5	84
5	14	31	4.5	84
6	15	31	5	96

3.3.5 Bioethanol Characterization

Ethanol analysis techniques have been developed to improve the value of ethanol. The IR spectra of the solutions were collected over the range from 500 to 4000 cm^{-1} using a Bruker Vertex v70 model spectrophotometer. The nominal resolution was 1 cm^{-1}. In order to increase the signal to noise ratio, for every sample 32 scans were averaged (Corsetti et al., 2015). Infrared spectroscopy (IR) was used for quality assurance of ethanol (Onuki et al., 2008).

The following qualities of produced bioethanol were assessed by determining the quality attributes such as: density, pH test, viscosity, functional group, boiling point, flash point, fire

point and specific gravity. The alcohol volume by volume percentage was measured using the following equation:

$$\%\ \text{Yield of Bioethanol}\ \left(\frac{v}{v}\right) = \frac{(\text{volume of distillate})}{(\text{volume of used glucose mixture})} \ldots\ldots (3.12)$$

i) Density and specific gravity test

Empty pycnometer was weighed. The pycnometer was filled with sample (ethanol) the excess was wiped off, the density was recorded. Secondly distilled water was filled into the pycnometer, weighed and recorded. Specific gravity was calculated using its formula:

$$\text{Specific gravity} = \frac{\text{Density of bioethanol}}{\text{density of water}} \ldots\ldots\ldots (3.13)$$

ii) pH test

The sample pH measurements were carried out using a pH meter. The pH meter was kept in a standard buffer solution and calibrated against a buffer solution of pH of 7 before use. Then, a pH was for the sample from optimum point

iii) Viscosity

Firstly, distilled water was allowed to flow in a narrow tube of viscometer use pipetting, avoid air bubbles and note down time of water flow through a tube. Next a 50 ml distilled bioethanol was applied in a same manner as that of distilled water and a sucker was used to lift the sample to the arm of the capillary to the marked point. A stop watch was used to regulate the time it took the bioethanol to return (flow) to the mark under time noted.

iv) Functional group

FTIR was used to know the ethanol functional group. The compositions were known from data of wavelength and corresponding ethanol percentage in the distillates.

v) Boling point

Boiling point is the temperature at which the vapor pressure is equal to atmospheric pressure and a rotary flash evaporator was used through temperatures in the range of (75 – 80) ^{0}C which were applied for the determination of exact boiling point of produced bioethanol.

vi) Flash Point and Fire Point

This test was carried out using flash point tester apparatus. The cup in the apparatus was dried. 50 ml of each sample (ethanol produced) was transferred into the position in the apparatus assembled with thermometer and the apparatus was switched on, the heat was controlled by a steady stirrer to maintain a uniform temperature while passing a small flame across the material every five seconds.

The temperature at which the vapor first flashes with the blue flame was recorded as the flash point of the samples after each test cup was washed and dried before the subsequent test. The point at which application of an ignition source causes the vapors of on a test specimen of the sample was taken as a fire point. The fire point determination was done through continued application of the ignition source until it causes the test specimen to ignite and sustain burning for about 5 seconds (Maina et al., 2017).

vii) Appearance and Color

This was known by looking into the final product and checked through sense organ.

4. RESULTS AND DISCUSSION

The findings recorded on various experiments during the thesis lab work have been presented in this chapter along with tables, graphs and photographs.

4.1 The proximate analysis of waste potato

In this part, various experiments were conducted to know the chemical composition of waste potato (substrate) to ensure its suitability in the production of bioethanol using Saccharomyces cerevisae.

4.1.1 Moisture Content

Table 4.1: Results of moisture content

A pre-weighed aluminum foil (W_1)	0.9876 g
The weights of waste potato sample before drying plus aluminum foil (W_2)	10.9876 g
Weight of dry sample plus aluminum foil (W_3)	7.516 g

% Moisture = 34.71%

The above moisture content result is in between the results of previous literature which implies a reasonable amount of water content. While moisture determination may seem simplistic, it is often one of the most difficult assays in obtaining accurate and precise results. Moisture analysis data may be needed quickly for quality control purposes, in which high accuracy may not be necessary. Therefore, the above result can be taken as agreeable for further work in the production of bioethanol.

4.1.2 Total Ash Content

Table 4.2: Results of ash content

mass of crucible (W_1)	67.9011 g
mass of crucible plus waste potato sample before dried in oven (W_2)	83.8877 g

mass of crucible plus dried waste potato sample (W_3)	68.8320 g

% Total ash = 5.823%

The ash content result refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. Conventional dry ashing was used based upon incineration at high temperatures in a muffle furnace. Except for certain elements, the residue may be used for further specific mineral analyses. The above result of ash content comparable with most fresh foods rarely which ash is greater than 5% (Nielsen, 2010).

4.1.3 Total Reducing Sugars

Data analysis for the fitting of graph can be stated as:

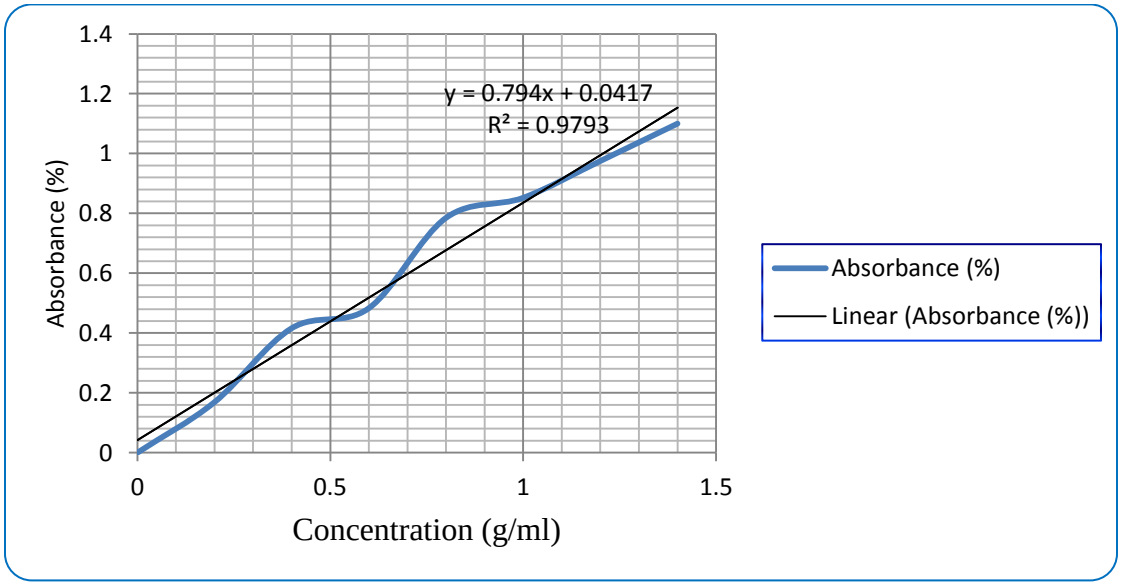

Figure 4.1 Graph of glucose concentration versus absorbance for hydrolysate

From the linear regression of absorbance and glucose concentration the value of R-squared shows the presence of sugar inside the prepared mixture of waste potato.

Table 4.3: Linear fitting results of absorbance versus concentration

Equation	Y = a + b*x
Plot	Absorbance
Intercept (a)	0.0417
Slope (b)	0.794
X	Concentration of unknown sample (g/ml)
R-square (COD)	0.9793

The TRS concentration of unknown sample became -0.05, 0.12, 0.36, 0.43, 0.73, 0.79, 0.92 and 1.04 (g/ml). The value of R- Squared was 0.9793 which indicates the model nearly explains the accurate variability in absorbance. This implies the model must pass closely through every measurement of (x_i, y_i) and the values larger than 0.5 is usually considered a significant relationship (Vaughn et al., 2010).

4.1.4 Protein Content

Table 4.4: Result of protein determination

volume (ml) of 0.2 N HCL used sample titration (A)	2.5 ml
volume (ml) of 0.2 N HCL used in blank titration (B)	1.959 ml
Normality of HCL (N)	0.2 N
Weight (g) of sample (W)	4.0 g

Protein content = 9.46 %

Proteins have unique conformations that could be altered by denaturants such as heat, acid, alkali, urea, organic solvents, and detergents. Similarly, the obtained value is a reasonable result as compared to other composition content. Therefore, it is required to be conducted since heat and acid are among denaturants added to potato sample in hydrolysis step.

4.1.5 Fat Content

= 7.33 %

Fats generally refer to those lipids that are solid at room temperature and oils generally refer to those lipids that are liquid at room temperature.

4.1.6 Fiber Content

Table 4.5: Result of crude fiber determination

Weight of crucible before ashing (W_1)	72.9011 g
Weight of crucible with sample after ashing (W_2)	91.0271 g
Weight of sample (W_3)	6 g

= 3.021%

4.1.7 Carbohydrate content determination

Carbohydrates = 39.65%

Table 4.6: Comparison summary of proximate analysis with theoretical results

Characteristics	Amount (%)		
	This work	Other literature	
		(Ghosal et al. 2013)	(Nielsen 2010)
Moisture	34.71	37.2	72.4
Ash	5.823	2	1.6
Starch	39.65	34.26	12.4
Protein	9.46	19.87	2.0
Fat	7.33	6.3	10

The above result of composition analysis for the waste potato indicates it has high amount of starch which could be able to be converted into bioethanol. The results obtained in proximate conduct for protein and fat content implies tolerable when compared to previous potato properties from which bioethanol was produced. So, it can withstand the effects of denaturants like acids, alkalis and heat which can be used during production.

4.2 Optimization of Fermentation Condition

Response surface methodology (RSM), which is a collection of statistical and mathematical techniques, was applied to optimize conditions for fermentation process. By analyzing the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical processes within the experimental range. The application of response surface methodology offers on the basis of parameter estimate, an empirical relationship between the response variable (yield) and the test variables (T, pH and t). Box-Behnken experimental Design with three numeric factors and three levels was used. Based on BBD a total of 15 experimental runs were performed including three central point that measures process stability and inherent variability.

Table 4.7: BBD with actual and predicted values of bioethanol yield (%R)

Order		Independent factors			Response	
Std	Run	Temperature (^oC)	pH	Fermentation time (hr.)	Experimental Yield (%v/v)	Predicted Yield (%v/v)
5	1	25	4.5	96	13.25	13.65
6	2	31	4.5	84	30.75	32.58
8	3	37	5	84	20.25	21.15
3	4	25	4.5	72	13.75	15.09
4	5	31	4	96	29.75	30.25
13	6	37	4.5	72	26.25	25.84
12	7	31	5	72	33	32.5
11	8	37	4.5	96	22.75	21.40
14	9	25	4	84	13.5	12.59
1	10	37	4	84	21.75	22.59
15	11	31	4	72	34.5	34.06
7	12	25	5	84	13.5	12.65
9	13	31	4.5	84	34	32.58
2	14	31	4.5	84	33	32.58
10	15	31	5	96	30	30.43

4.2.1 Model Fitting and Response Surface Analysis

Data were modeled by linear regression analysis and the statistical significance of the terms was examined by analysis of variance. The statistical analysis of the data and three dimensional plotting were performed using design expert software (V11.0.0.) trial version. The adequacy of regression model was checked by R^2, Adjusted R^2, Predicted R^2, Adequate precision and F-test. The significance of F value was judged at 95% confidence level. The regression coefficients were used to make statistical calculation to generate three dimensional plots from the regression model.

All statistical analysis including ANOVA test, Post ANOVA statistics, lack of fit test, normal plot of residuals etc. were done for the fermentation condition data, as shown in above Table 4.7.

Table 4.8: Analysis of variance (ANOVA) of Reduced Quadratic model for %Yield

Source	Sum of Squares	Df	Mean Square	F-value	p-value	Remark
Model	924.21	5	184.84	72.72	< 0.0001	Significant
A-A	171.12	1	171.12	67.32	< 0.0001	
B-B	0.9453	1	0.9453	0.3719	0.5570	
C-C	17.26	1	17.26	6.79	0.0285	
A^2	725.50	1	725.50	285.43	< 0.0001	
C^2	1.28	1	1.28	0.5027	0.4963	
Residual	22.88	9	2.54			
Lack of fit	17.33	7	2.48	0.8937	0.6213	not significant
Pure error	5.54	2	2.77			
Cor total	947.08	14				

The Model F-value of 72.72 implies the model is significant. There is only a 0.01% chance of large F-value could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, A^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), the model reduction helps to may improve the model.

The Lack of Fit F-value of 0.89 implies the Lack of Fit is not significant relative to the pure error. There is a 62.13% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good when the model is required to fit.

Table 4.9: The regression coefficients of the model

Std. Dev	1.59	R^2	0.9758
Mean	24.67	Adjusted R^2	0.9624
C.V %	6.46	Predicted R^2	0.9342
		Adeq Precision	21.7015

R-squared (R^2) is a statistic that explains the amount of variance accounted for in the relationship between two (or more) variables. Sometime R^2 is called the coefficient of determination, and it is given as the square of a correlation coefficient. Adjusted R^2 is used to the compare the goodness of fit for regression model that contain differing numbers of independent variables. Its value decreases when the term doesn't improve the model fit by a sufficient amount. Predicted R^2 value determines how well a regression model makes prediction.

This static helps to identify cases where the model provides a good fit for the existing data but isn't as good at making predictions.

The Predicted R^2 of 0.9342 is in reasonable agreement with the Adjusted R^2 of 0.9624; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 21.702 indicates an adequate signal. This model can be used to navigate the design space.

Based on regression analysis the quadratic model was suggested by the design program. The test of responses adequacy and description of its variation with independent variables can be written by considering the significant terms as follows by omitting insignificant model terms:

Final Equation in Terms of Coded Factors

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor.

By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

$$R(\%) = 31.81 + 4.63 * A - 0.3438 * B - 1.47 * C - 13.98 * A^2 + 0.5865 * C^2 \ldots\ldots\ldots (4.2)$$

Where A = Temperature, B = pH and C = Fermentation time

According to particularly high value coefficient of multiple determinations (R^2) for %R (0.9758) (Table 4.9) second-order polynomial model equation provides good representation of experimental values.

Moreover, for the response, mathematical model was statistically acceptable due to significant regression for the model ($p < 0.05$) (Table 4.8). Lack of fit testing confirmed adequacy of fitting experimental data to a second-order polynomial model, where p-value for lack of fit was insignificant ($p > 0.05$) (Table 4.8). Therefore, as suggested by ANOVA, Equation (4.3) would be able to adequately describe behavior of %R.

From acquired experimental data and developed model the three dimensional response surface were constructed to illustrate the interactive effect of independent variables on the response and shown in figure 4.1.

These figures reflect the relative effects of two variables while keeping the third variable constant. The interaction effects and optimal levels of temperature, pH and fermentation time were determined by plotting the response surface curves.

Table 4.10: Sequential model fitting for %Yield

Source	Sum of Squares	Df	Mean Square	F-value	p-value	Remark
Linear	752.21	9	83.58	30.16	0.0325	
2FI	748.64	6	124.77	45.03	0.0219	

Quadratic	7.89	3	2.63	0.9492	0.5498	Suggested
Cubic	0.0000	0				Aliased
Pure Error	5.54	2	2.77			

4.2.2 Individual Effect of Parameters

The effect of operating condition on the ethanol yield was investigated and the optimal values were identified in this study.

i) Effect of Temperature on Ethanol Yield

The plot of temperature versus the ethanol yield, when pH and fermentation time were actual factors, was depicted in figure 4.2 From the plot as temperature increases from 25℃ to 37 ℃, the maximum ethanol yield about (34%) volume by volume was obtained at a temperature of 31 ℃. Beyond 31 °C, a decrease in the ethanol yield was observed which is due to further conversion of other byproduct.

Therefore, the optimum temperature at which systems works accurately and gets a higher yield was found to be 31 ℃ and the yield at this temperature was 34%. During fermentation of sugar by yeasts it is required to use temperature at tolerant strain to manage an increment in temperature caused due to liberated heat, cooling of fermenter and to sustain a safe growth rate of microorganisms.

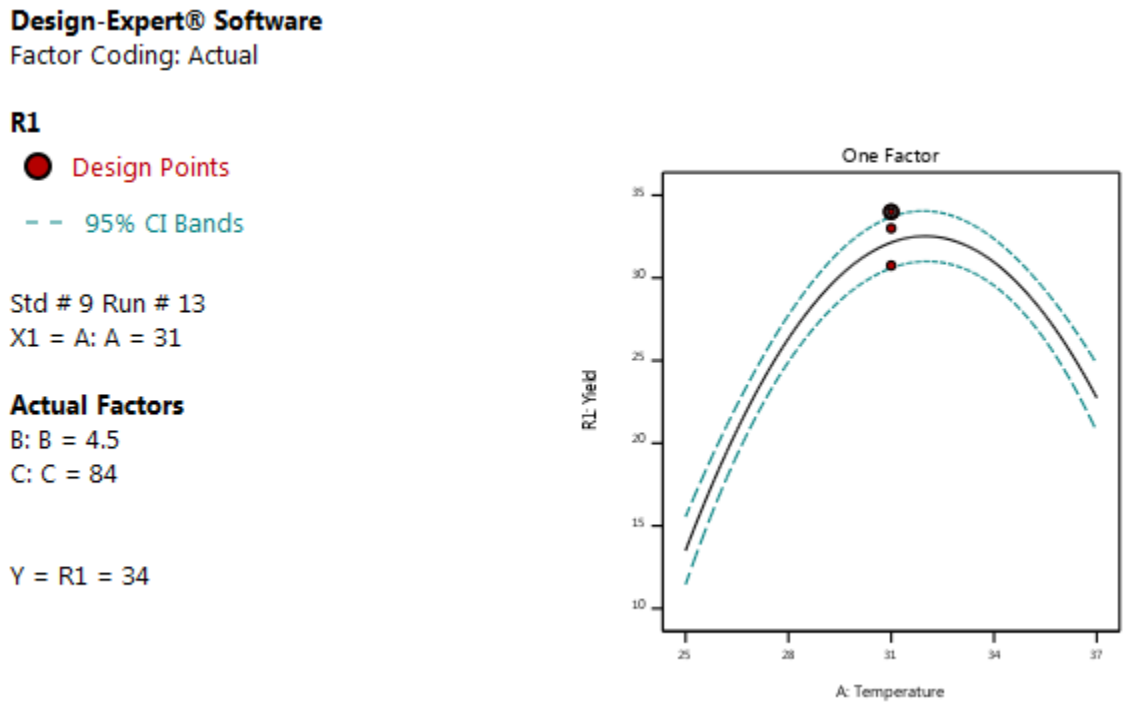

Figure 4.2 Effect of Temperature on the Ethanol Yield

ii) Effect of pH on Ethanol Yield

The plot of pH versus the ethanol yield, when temperature and fermentation time were actual factors, was depicted in figure 4.3 from the plot the maximum ethanol yield (34%) volume by volume was obtained at a pH of 4.5. The 4.5 pH values is in slight acidic range 3 to 8 which enhances the growth of yeast cultures; create balance among cellular growth and physio-chemical effect which helps for optimum ethanol production.

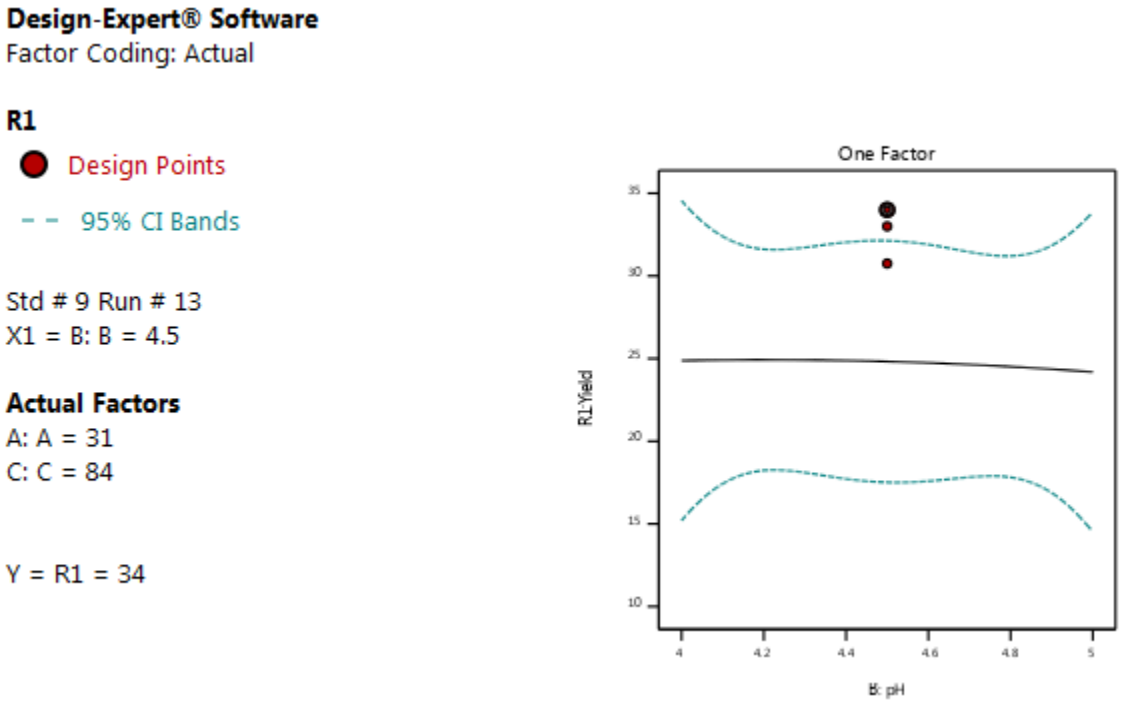

Figure 4.3 Effect of pH on the Ethanol Yield

iii) Effect of Fermentation Time on Ethanol Yield

The resulting plot of fermentation time versus ethanol yield, when pH and temperature were actual factors, was depicted in Figure 4.4 from the plot the maximum ethanol yield (34%) volume by volume was obtained at a fermentation time of 84 hr. This is time at which a fermentation process becomes economical and produces a required conversion of fermentable sugar into alcohol.

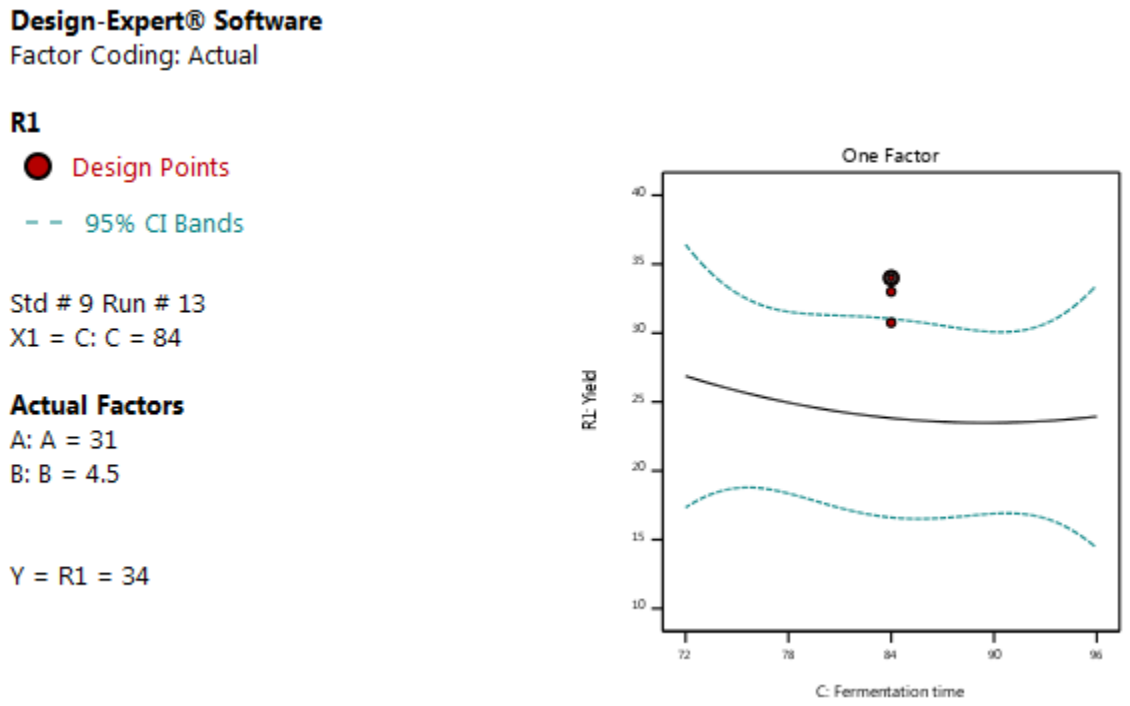

Figure 4.4 Effect of Fermentation time on the Ethanol Yield

4.2.3 Interaction Effect of Parameters

Response surface was generated by plotting the response (bioethanol production) on the y-axis against any two independent variables on the x-axis, while keeping the other independent variables at constant level. Therefore, three response surfaces were obtained by considering the possible combinations. In figure 4.5 the green shaded area implies a 34 percentage maximum yield was obtained at an interaction between 31 °C Temperature and pH of 4.5, whereas in Fig 4.6 the green shaded area implies a 34 percentage maximum yield was obtained at an interaction between 31 °C temperature and fermentation time of 84 hours. Similarly, in Fig 4.7 the green shaded area implies a 34 percentage maximum yield which was obtained at an interaction between fermentation time of 84 hours and pH of 4.5.

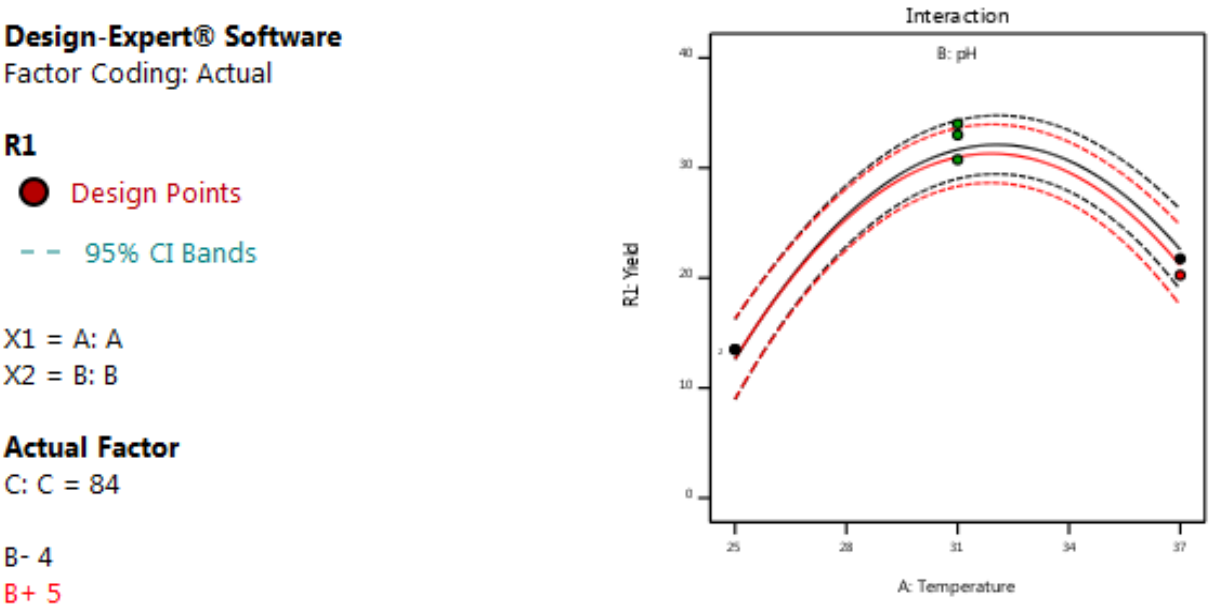

Figure 4.5 Interaction Effect of Temperature and pH interaction versus Yield

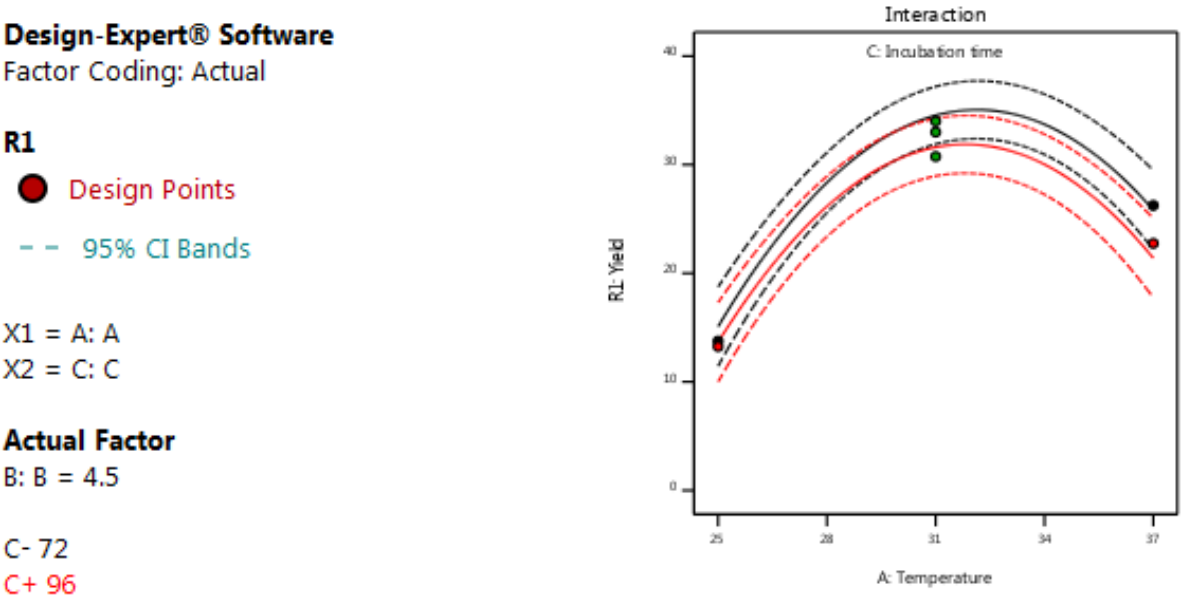

Figure 4.6 Interaction Effect of Temperature and Incubation time versus Yield

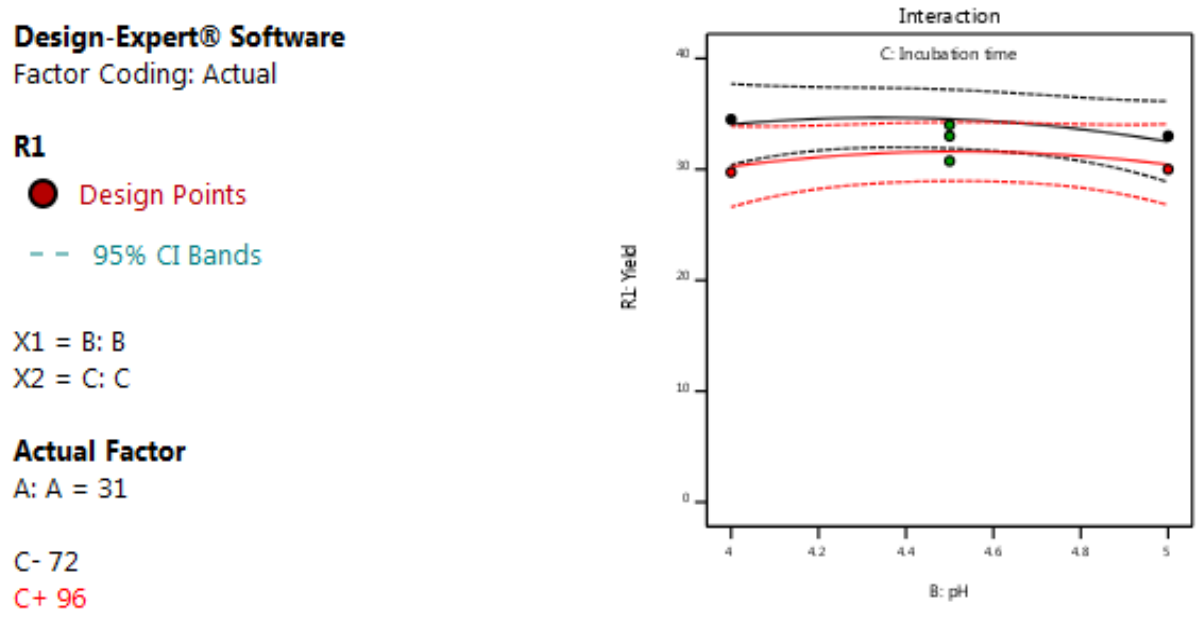

Figure 4.7 Interaction Effect of pH and Incubation time versus Yield

The response surface curves representing the interaction effects of two variables, i.e. Temperature versus pH, Temperature versus Incubation time and pH versus Incubation time on the yield maximization were plotted as shown in Figures 4.7. Figure 4.8 shows that a maximum yield was attained at medium temperature and relatively a low pH of 4. On the other hand, Figure 4.9 shows a maximum yield at 25 °C and high incubation time. Effect of incubation time and pH on yield of bioethanol from waste potato is presented in Figure 4.10.

This can explain the decrease of yield percentage of bioethanol which is well illustrated by the plateau line (red shaded area) after 34 (% v/v) of 31 °C Temperature and 4.5 pH.

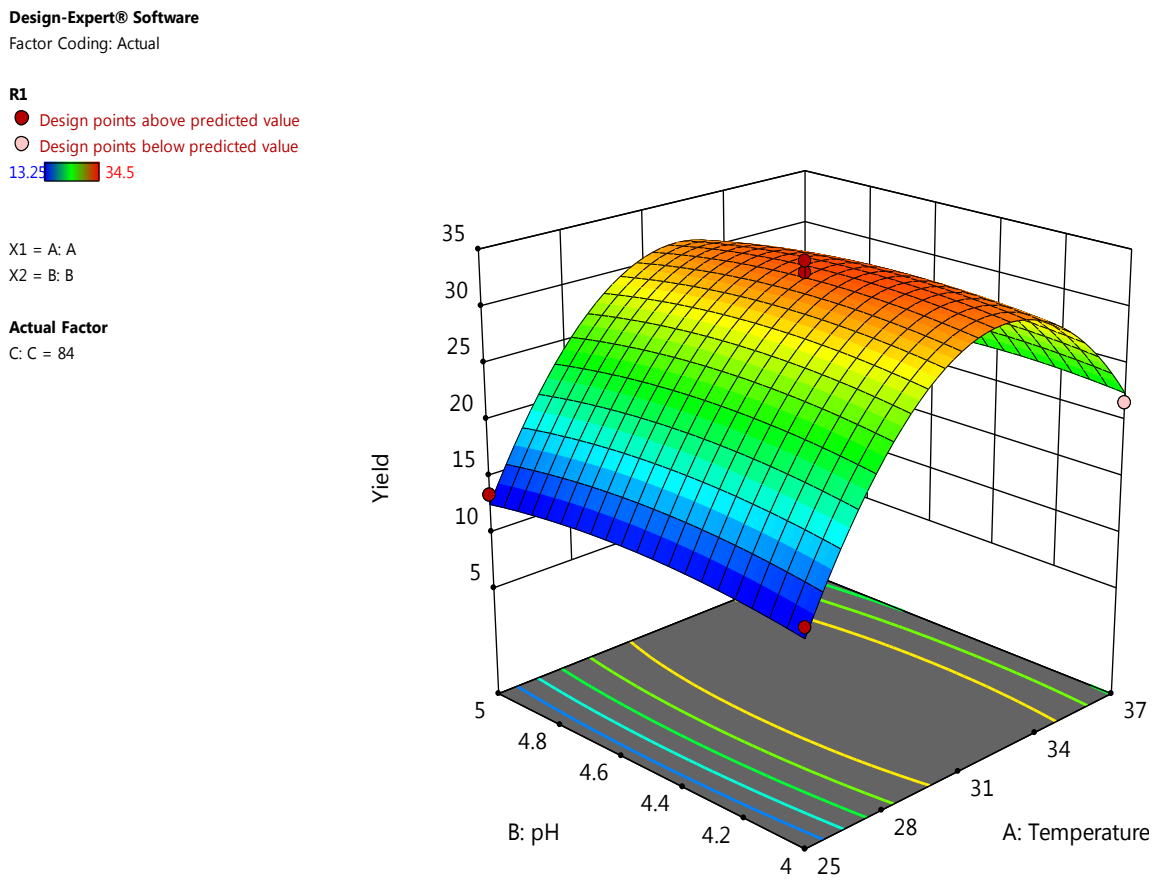

Figure 4.8 3D response surface plots representing interaction effects of Temperature and pH

This can explain the decrease of yield percentage of bioethanol which is well illustrated by the plateau line (red shaded area) after 33 (% v/v) of 31 ºC Temperature and 84 hours Incubation time.

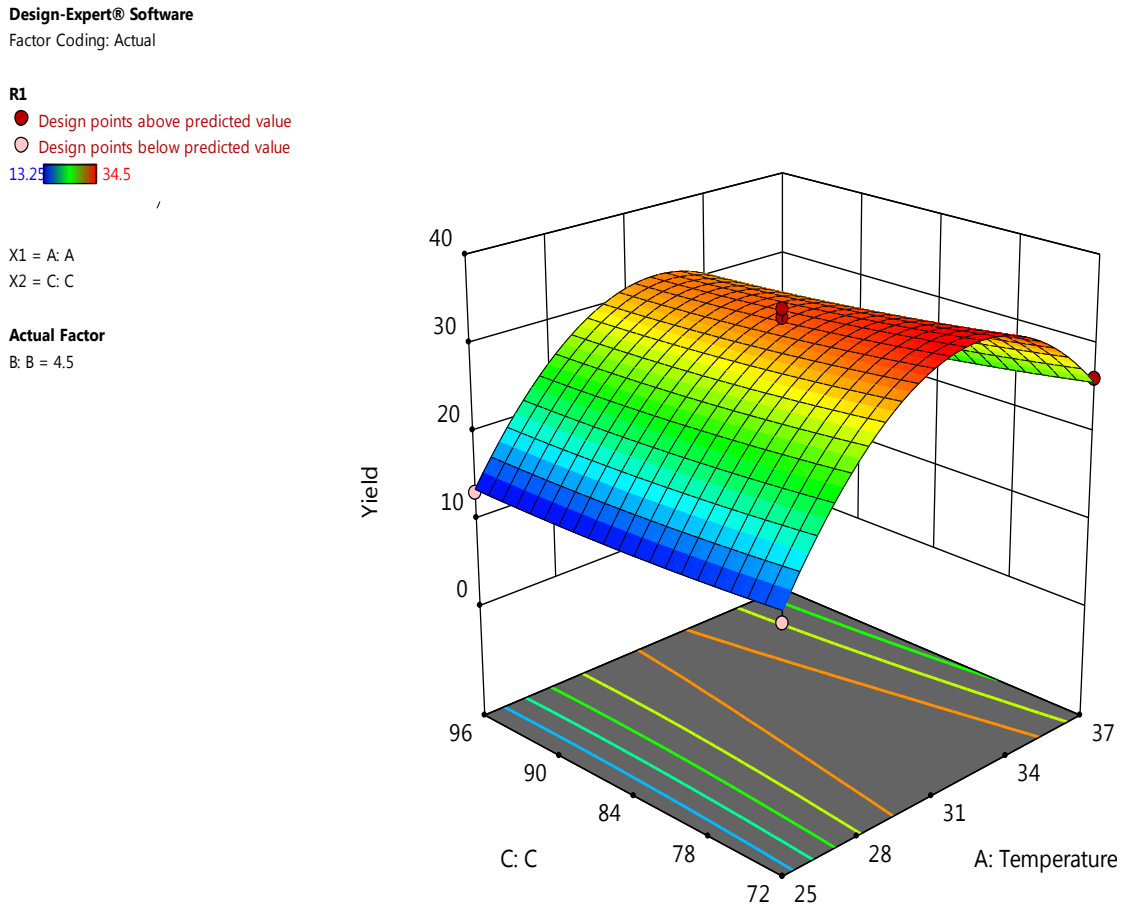

Figure 4.9 3D response surface plots representing interaction effects of temperature and Incubation time

This can explain the percentage yield of bioethanol which is located below and above predicted values and well-illustrated by the plateau line (red shaded area) after 34 (% v/v) of 4.5 pH and 84 hours Incubation time.

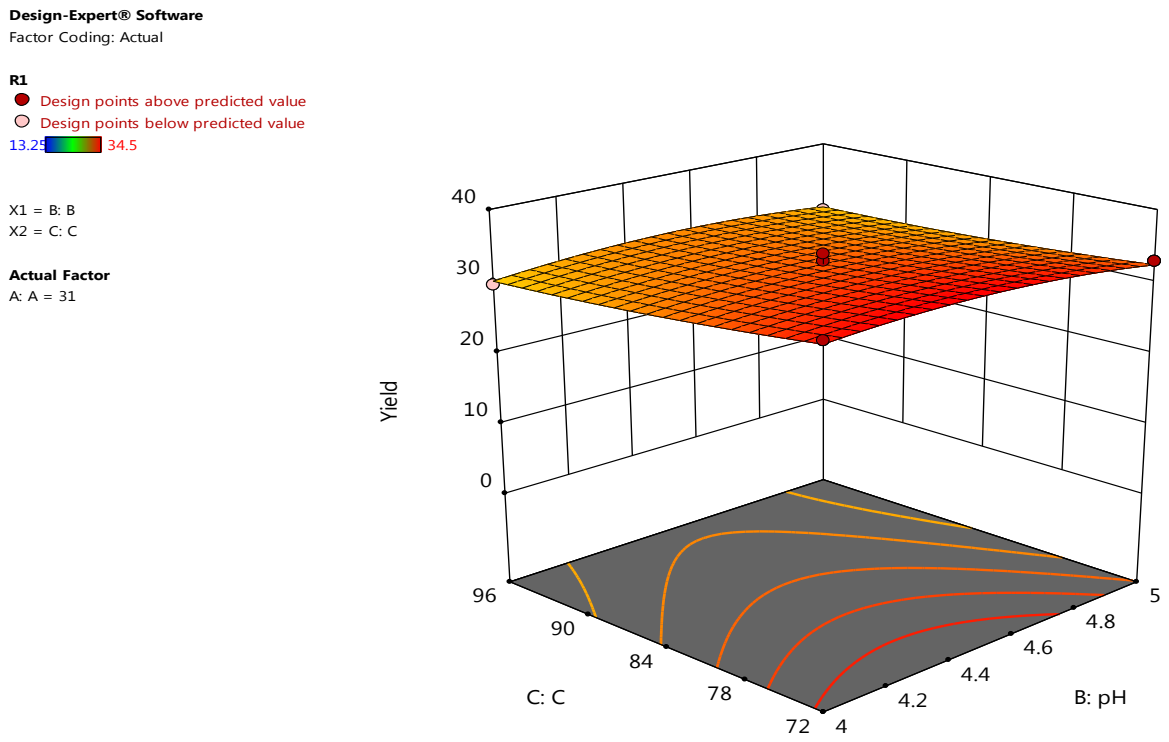

Figure 4.10 3D response surface plots representing interaction effects of pH and incubation time

4.2.4 Diagnostics of Model Adequacy

The adequacy of the model was checked by constructing different diagnostic plots shown in Fig 4.3. The normal % probability plot of residuals for response was normally distributed, as they lie reasonably close to the straight line and shows no deviation of the variance Figure 4.11. The analysis of residuals and difference between the predicted and experimental responses is another important diagnostic tool for judging adequacy of the fitted model for predicting the response. Thus, internally studentized residuals plot was constructed to facilitate the satisfactory fit of the developed model and the plot (Figure 4.12) shows that, all the data points lie within the limits (±3). This indicates that the plot of amount percentage of yield predicted versus residual did not show any outliers, all the points were found to fall in the range of +3 to -3, which indicated that the model presents a minimal deviation of the fitted value from the observed. In another word, there was close correlation between the experimental and predicted values.

The predicted values obtained from the developed model were quite close to the experimental values and lie reasonably close to the straight line and the indicated the adequate agreement with real data (Fig. 4.13).

The correlations between the theoretical and experimental responses, calculated by the model, are satisfactory. Therefore, the "R^2" are in reasonable agreement with the "R^2", Adj". It can be seen that, more than 93% of the response can be well predicted by the models, indicating that the terms which were considered in the proposed models are significant enough to make acceptable predictions.

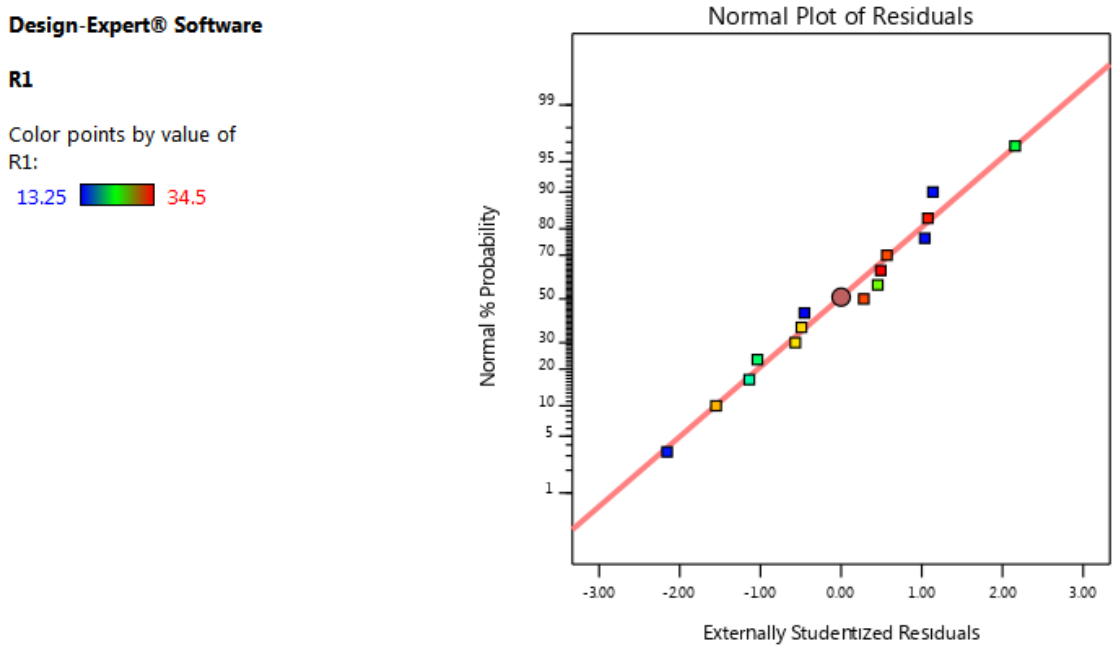

Figure 4.11 Diagnostic plots of Normal versus Residual

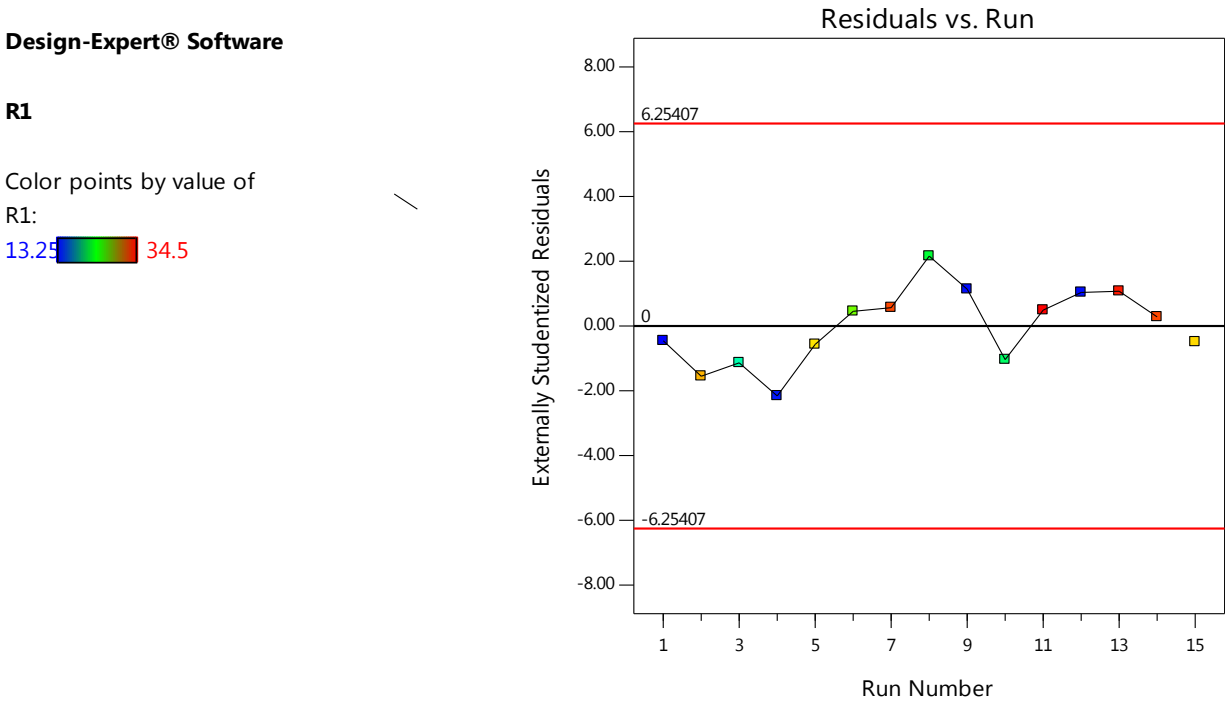

Figure 4.12 Diagnostic plots of Residual versus run

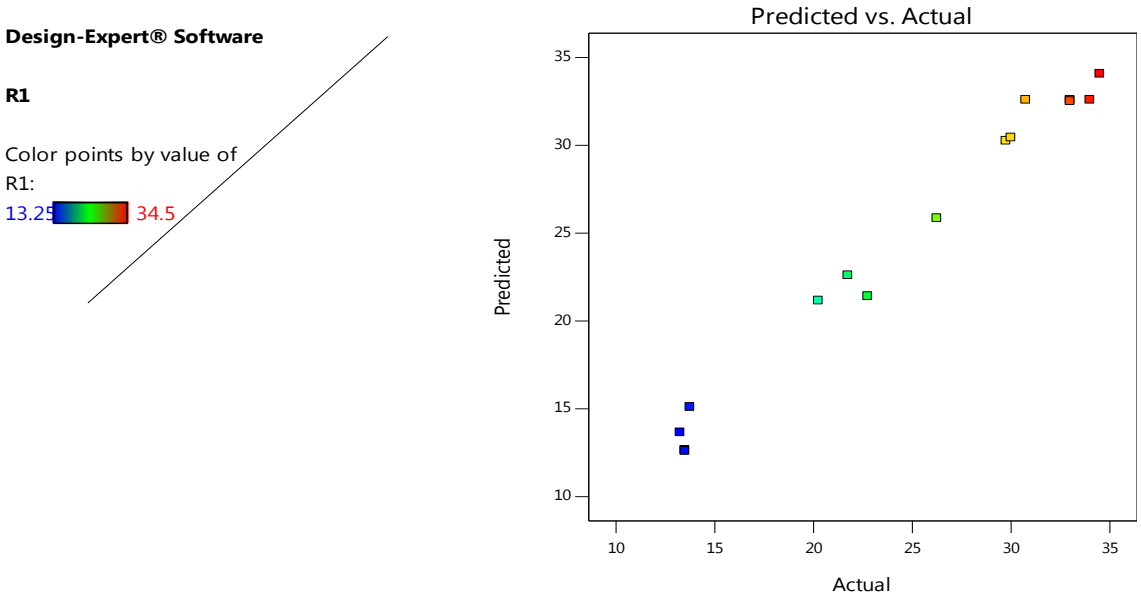

Figure 4.13 Diagnostic plots of Predicted versus actual

4.2.5 Verification of the Model

Optimization of ethanol 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 by increasing ethanol yield through minimization of process cost.

Table 4.11: Optimization criteria for optimum yield

Parameters	Purpose	Minimum value	Maximum value
Temperature (°C)	In range	25	37
pH	In range	4	5
Incubation time (hr.)	In range	72	96
Yield (% v/v)	Maximize	13.25	34.5

Table 4.12: An optimum possible 15 solution out of 100

No.	Temperature	pH	Incubation time	Yield	Desirability	Remark
1	32.2	4.066	72.082	34.509	1	Selected
2	32.042	4.027	72.201	34.526	1	
3	31.833	4.098	72.046	34.502	1	
4	31.842	4.004	72.176	34.539	1	
5	31.754	4.073	72.058	34.504	1	
6	31.99	4.093	72.088	34.506	1	
7	31.804	4.013	72.07	34.551	1	
8	32.333	4.04	72.072	34.501	1	
9	32.141	4.004	72.345	34.502	1	
10	31.972	4.004	72.165	34.55	1	
11	32.114	4.093	72.068	34.505	1	
12	32.312	4.057	72.022	34.506	1	
13	31.957	4.064	72.014	34.541	1	
14	31.896	4.114	72	34.507	1	
15	31.885	4.02	72.301	34.506	1	

The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If a response falls within 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 desirable ethanol yield 34.5 (% v/v) was found at Temperature 32.199 °C, pH 4.066 and Incubation time 72.082 hr. and the value of desirability obtained was 1.0.

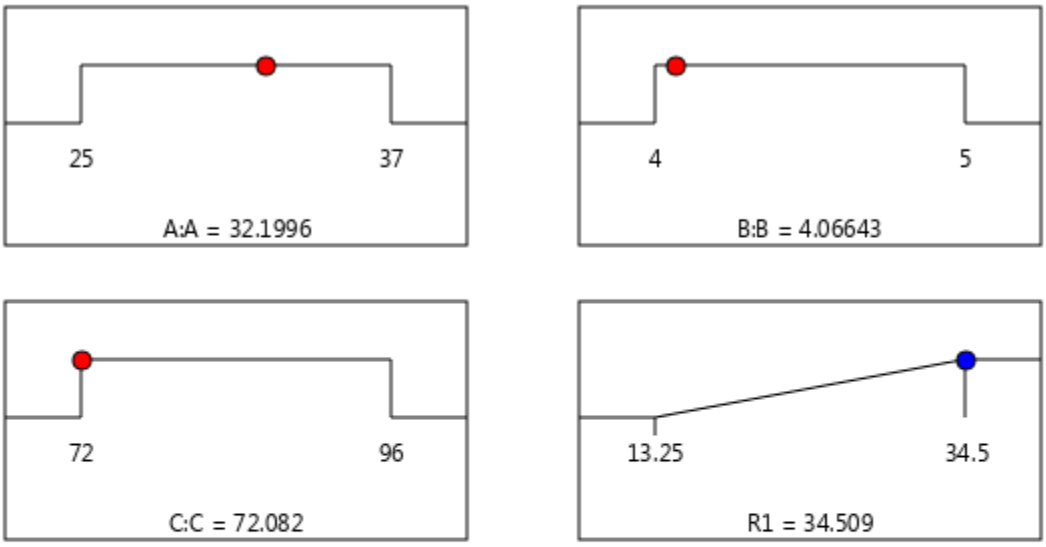

Figure 4.14 Ramp display of maximum yield and of corresponding optimum factors

Table 4.13: Factors sheet

Factors	Value	Axis Low	Axis High
Temperature (°C)	31.8963	25	37
pH	4.57011	4	5
Incubation time (hr.)	73.4423	72	96

4.3 Results of Bioethanol Characterization

In this investigation, various experiments on density, pH, viscosity, flash point, specific gravity, boiling point and functional group were conducted on bioethanol production by separate hydrolysis and fermentation using *Saccharomyces cerevisiae*.

4.3.1 Density

In this experiment, density of bioethanol, produced by separate hydrolysis and fermentation using yeast *Saccharomyces cerevisiae* was estimated. The observation recorded was showed that the density of bioethanol produced was 0.9987 g/ml.

4.3. 2 Specific Gravity

In this experiment, specific gravity of bioethanol, produced by separate hydrolysis and fermentation using the culture of *Saccharomyces cerevisiae* was estimated. The specific gravity of bioethanol produced was determined from the ratio of bioethanol density of water. The result was 0.9.

4.3.3 Viscosity

The observations recorded Appendix B-9 showed that the viscosity value of bioethanol produced by separate hydrolysis and fermentation method using yeast of *Saccharomyces cerevisiae* was 1.005. The viscosity value of the produced bioethanol is low compared to the set limit of 0.9.

4.3.4 Boiling Point

Firstly, the round bottom flask was removed from the base of the condenser and inspected directly above the hot bath to ensure it is clean. Then the distillate sample was loaded into the cleaned round bottom flask and round bottom flask was connected to the condenser.

Next the collection flask was located to the left of the hot bath, and below the condenser and the cabinet doors directly below the rotary evaporator was opened. Since the condenser was turned sideways, the back of the chiller was on the right side of the cabinet and the front of the chiller was located on the left side of the cabinet. Make sure the tubing is securely connected to the back of the chiller and the condenser. The chiller was normally set to 20 °C.

Then the rotary evaporator was turned on and the power button on the rotary evaporator control panel was pressed. After setting all the specifications, the presses dial on the rotary evaporator control was panel in. The rotary evaporator was begun to rotate and waited for about 2 hours. Finally, rotary evaporator was stop spinning then after successful separation was obtained near 79 ºC and that the boiling point of distillate substance collected in the original round bottom flask.

4.3.5 Flash point and Fire point

A more visible flame was occurred at 26 ^{0}C temperature which was recorded as the flash point. This implies a produced bioethanol requires a minimum temperature 26 ^{0}C to give off sufficient vapors which can be mixed with air and will ignite momentarily. The fire point was recorded when sample inflames across the cup of test flame and it was at higher temperature about 82 ºC.

4.3.6 pH

The results from pH meter for sample of optimum point was about 5.3. This implies inside a distillate there is an acidity value which is can be minimized by using a more diluted acid during hydrolysis. It is more acidic as compared to a standard pH value of ethanol that is for blend fuel purpose.

4.3.7 Functional Group

Fourier transforms infrared (FTIR) transmission spectra was obtained to characterize the functional group of the distillate as shown in Figure 4.15.

There was no percentage transmittance peaks in the O-H stretching region between 3000 and 3500 cm^{-1} and in the C-O stretching region from 1800 to 2500 cm^{-1}, while ethanol exhibits distinct bands in those regions.

The peak absorbance of the O-H stretching band was observed at (400, between 2000 – 2500 and 3900) cm^{-1}. Further characteristic features of ethanol can be found in the fingerprint region.

The dominant feature was a peak from (400 – 2500) cm^{-1}, which can be assigned to be C-O stretches. The C-H bending region is displayed enlarged in the stretching region of (1300 – 2000)

cm^{-1} and (3500 – 4000) cm^{-1} contributions from ethanol methylene groups can be observed (Corsetti et al., 2015).

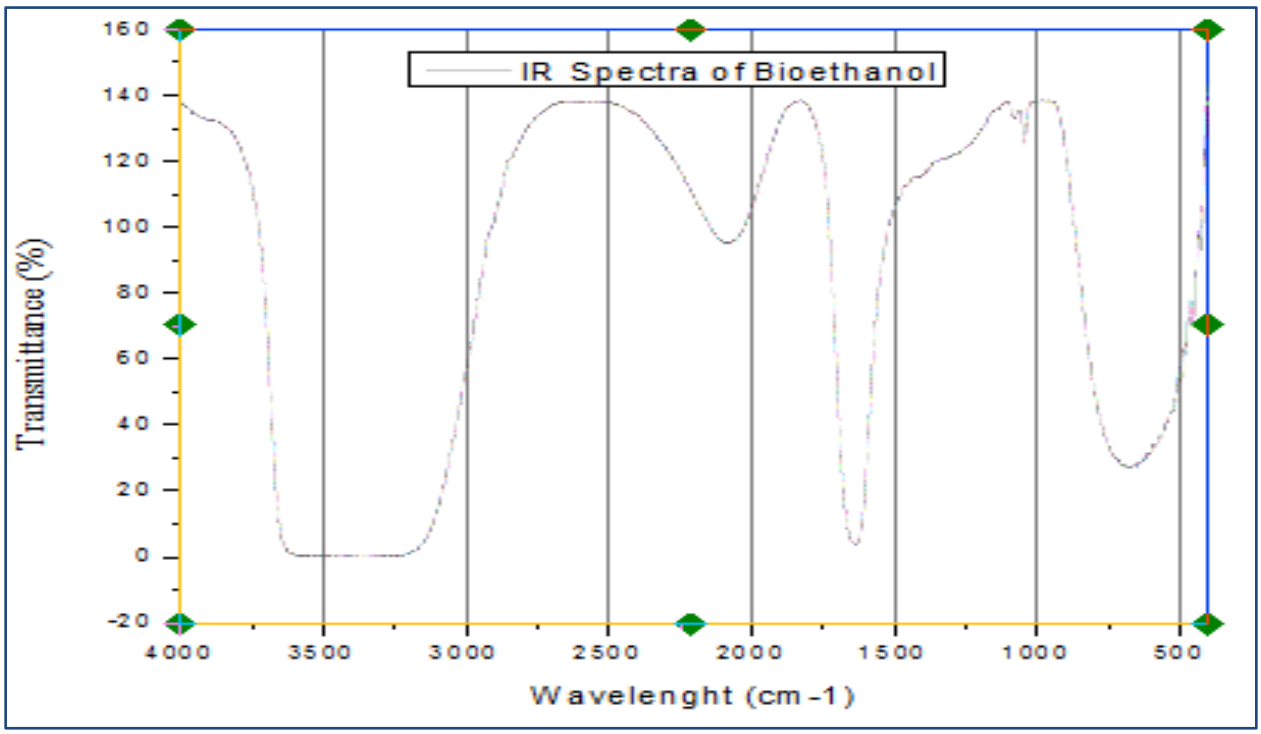

Figure 4.15 FTIR graph of bioethanol

Table 4.14: Properties comparison of produced bioethanol and theoretical ethanol

Properties	Produced bioethanol	Theoretical ethanol	
		(Rutz et al., 2006)	(Member et al., 2015)
Density, g/ml	0.9987	0.789	0.987
Viscosity	1.0	1.2	1.34
Flash point, °C	26	12.77	19.2
Fire point, °C	72	< 160	-
Boiling point, °C	79	78	78
Specific gravity	0.9	0.79	0.922
pH	5.3	6.5 – 9.9	-

Appearance and Color	Clear, without particles and colorless	Clear, without particles and colorless	-
Functional group	Contains alcohol molecules	Alcohol	Alcohol

Table 4.15: Comparison of bioethanol yield produced in optimization of fermentation condition

Optimized fermentation parameters	Maximum yield	References
Temperature, pH and substrate concentration	30 (g/l)	(Chniti 2018)
pH of 5.25-5.75, fermentation time range of (72-88) hr. and Temperature	33.9 (g/l)	(Flayeh 2017)
Temperature (°C) 30 - 60 Enzyme dose (mL) 0.2 - 0.6 and Incubation time (24 – 72) hr.	32.9 (g/l)	(Izmirlioglu et al. 2012)
Incubated at 30 ^{0}C for three days, and 10% of inoculum size	(42.5%)	(Bekele et al. 2015)
Temperature 25 - 37, pH 4 – 5 and Incubation time (72 – 96) hr.	34.5 % (v/v)	This work

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Agricultural residue is selected as an essential feedstock due to its higher availability, good starch content and economically cheap for biofuel production. The obtained result includes: 39.65 starch content, 34.71% of moisture content, 5.823% of ash content, 9.46% protein and 7.33% of fat content which is nearly similar with theoretical result of waste potatoes composition. In waste potato characterization of total reducing sugar, from R-squared value of 0.9793% it was observed that the presence of sugar inside the prepared mixture of waste potato. To determine the effects of various fermentation operating conditions (Temperature, pH and Incubation time) and their interactions on the yield of bioethanol, a Box-Behnken design was performed. ANOVA, F test showed that the three tested variables were statistically significant. However, yield was found to be highly enhanced by temperature and fermentation time. The predictive model was verified to generate yield response surfaces and contour plots, by the RSM method, which revealed the presence of high yield plateaus whose specifications will be useful in controlling industrial scale future units to ensure economically worthy yields at all times. A maximum desirable yield of ethanol was 34.5 (% v/v) with the optimum condition of 32.199 ºC temperatures, pH 4.066 and fermentation time of 72.082 hr. was suggested by the model. The regression model developed also shows that the operating parameters considered in this study have effect on the production of bioethanol from waste potato. From an experiment one can conclude the obtained value of density of bioethanol 0.9987 g/ml, specific gravity 1.0005, pH 5.1, Flash point 36 ºC, Viscosity 1.005 and boiling point 79 ºC are relatively similar with standard properties of bioethanol. The Fourier transforms infrared (FTIR) transmission spectra result of functional groups indicates the presence of alcohol component inside a produced distillate.

Therefore, to study and set optimum value of all significant condition in the production process were found to be essential to get a maximum yield of bioethanol.

5.2 Recommendations

Fermentation of starchy materials leading to the production of biofuel is economical and should be practiced in developing countries like Ethiopia. In this paper, it was found that the optimization of fermentation condition is an essential way to maximize the bioethanol yield. The following recommendations are made for further study.

- The whole available waste potatoes were generally used as a raw material for the bioethanol production without further amount proportion of waste potato and separate potato peel in this paper. Since the starch content of separate potato waste and potato peel is different it is better if an appropriate mixing proportion for waste potato and potato peels are conducted for further study.
- Temperature, pH and incubation time are the three parameters that have been optimized in this work. However, there are parameters that have effects on the fermentation process such as substrate concentration, inoculum size and mixing rate. Further improvement of the process by taking these conditions into a consideration is recommended for further study.
- Work on culture media to improve yeast performance should be carried out by investigating all composition used for media preparation for maximum bioconversion of starch into bioethanol.
- Under the present study, only the availability of raw material and technical feasibilities were considered. Thus, the process of scaling up should be carried out for higher recovery at large scale production and detail of economic feasibility should be studied to show whether the process is economically feasible.
- Research collaboration should be undertaken with ethanol producing sugar factories regarding using ethanol as bio-fuel for motor engines.

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APPENDICES

APPENDIX A: List of Tables

APPENDIX A1: Properties of Ethanol

Chemical formula	C_2H_2OH
Molecular weight (g/moll)	46
Density at 100 ^{0}C (kg/m^3)	789
Calorific value	26.9
Lower flammability	2.06
Higher flammability	0.3
Temperature self-ignition (K)	665
Stoichiometric air fuel ratio (kg air/ kg fuel)	9
Heat of evaporation (KJ/Kg)	840
Molecular composition	
C (%)	52.2
H (%)	13
O (%)	34.8

APPENDIX A2: Comparison of Gasoline and Ethanol

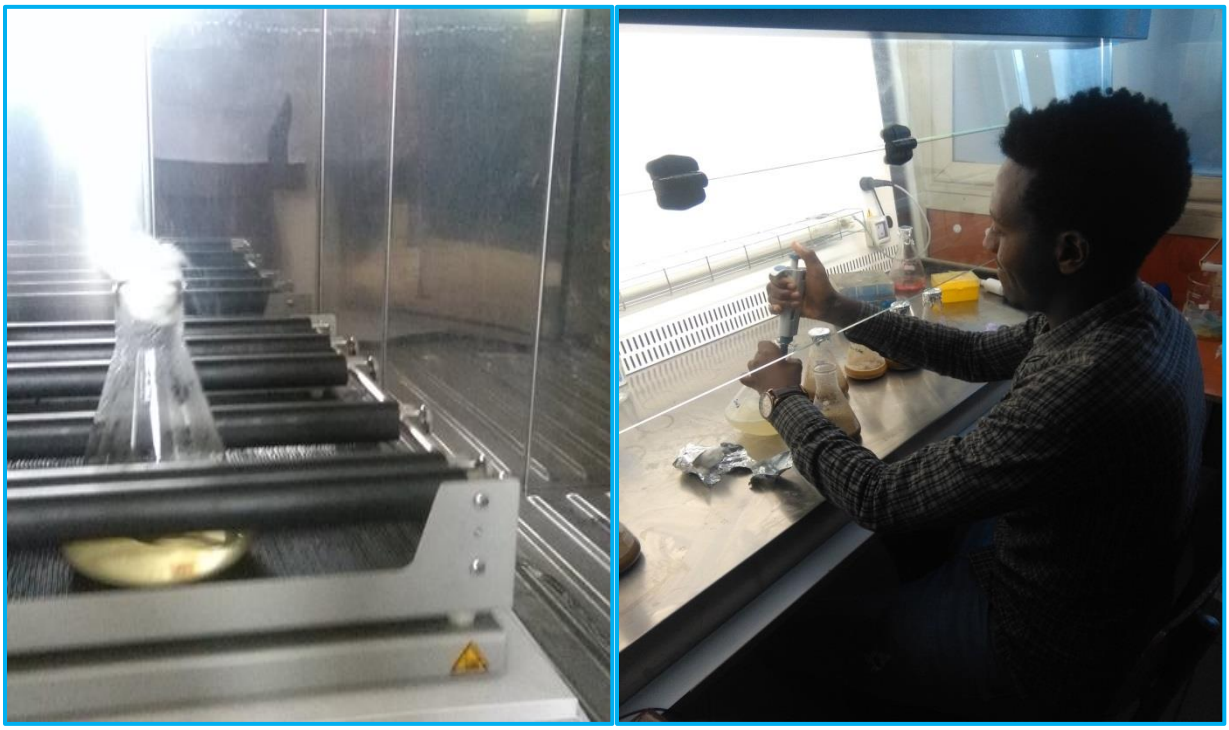
	Gasoline	Ethanol
Flash point	-45 ^{0}F	55 ^{0}F
Ignition temperature	530 – 853 °F	793 °F
Vapor density	3–4	1.49
Vapor pressure	38–300 mmHg	44 mmHg
Specific gravity	0.72–0.76	0.79
Boiling point	100–400 °F	173 °F
Toxicity		Lower than gasoline

APPENDIX B: Laboratory Work Pictures

B.1 Raw potato waste preparation

B.2 Prepared waste potato in oven and desiccators for moisture and ash content test

B.3 Mixture preparation and hydrolysis in autocloave



B.4 Media preparation and sterilization

B.5 Absorbance test for reducing sugar

B.6 Absorbance test result for standard stock solution

B.7 Setup for fermentation and distillation column

B.8 Setup for fermentation and distillation column

B.9 Recorded values of density and specific gravity

APPENDIX C: ANOVA Pictures

Design-Expert® Software
Factor Coding: Actual

R1

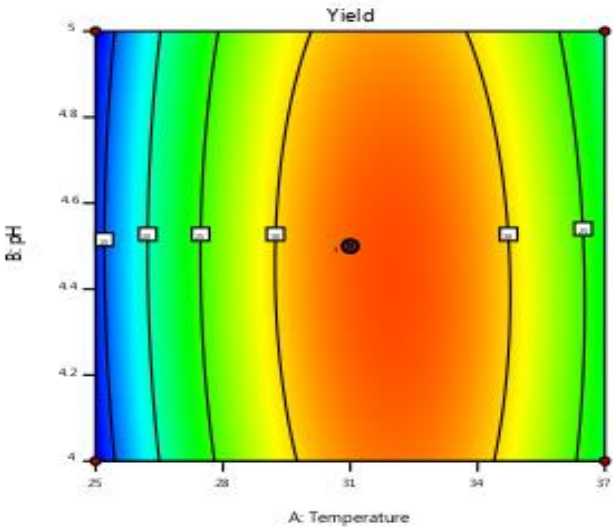
Design Points

13.25 34.5

R1 = 33
Std # 2 Run # 14

X1 = A: A = 31
X2 = B: B = 4.5

Actual Factor
C: C = 84



Design-Expert® Software
Factor Coding: Actual

R1

Design Points

13.25 34.5

R1 = 33
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X1 = A: A = 31
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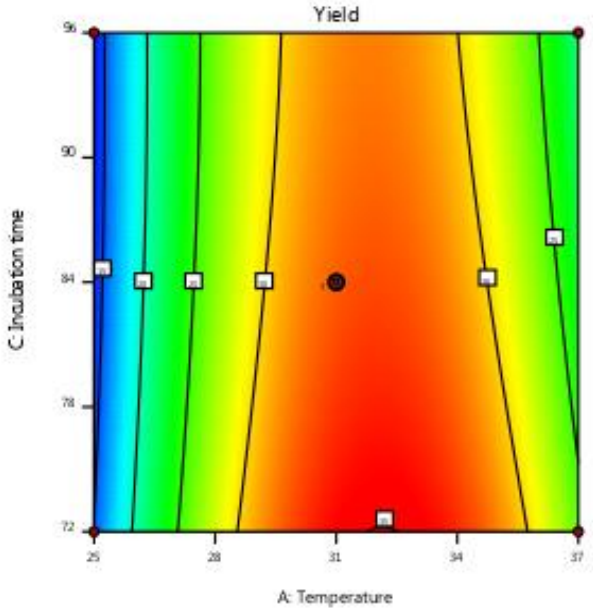
Actual Factor
B: B = 4.5



Design-Expert® Software
Factor Coding: Actual

R1

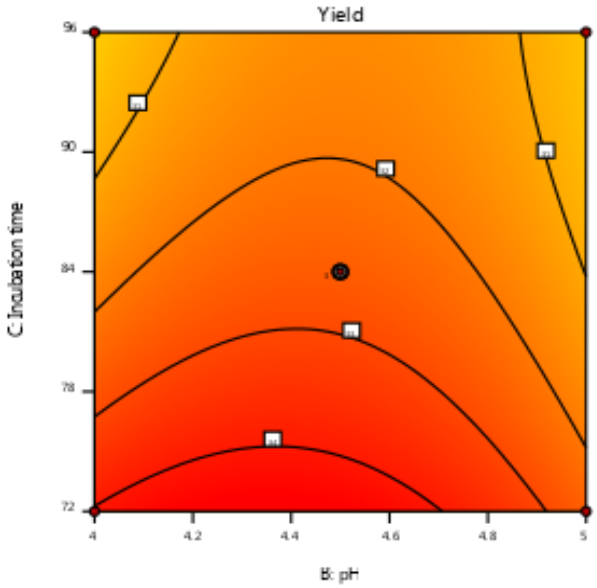
Design Points

13.25 34.5

R1 = 33
Std # 2 Run # 14

X1 = B: B = 4.5
X2 = C: C = 84

Actual Factor
A: A = 31



D.1 Interaction effects of (Temperatures and pH, Temperatures and Incubation time, pH and Incubation time) on ethanol yield innermost contour plot.