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SCHOOL OF CHEMICAL AND BIO ENGINEERING

CHARACTERIZATION OF DAIRY WASTE WHEY AND ITS' UTILIZATION FOR THE PRODUCTION OF ETHANOL

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This is to certify Thesis prepared by Wubshet Alemu on Title; Characterization of Dairy Waste Whey and Its' Utilization for the Production of Bioethanol and Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Engineering Stream that conforms to the regulations of the university and encounters standard quality.

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I declare that this thesis for the M.Sc. Degree submitted by me to Addis Ababa University, is my original work and has not previously been submitted for the award of degree at this or any other university, and that all resources of materials used in this thesis have been duly acknowledged.

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LIST OF ACRONYMS

Adj - R^2	Adjusted regression coefficients.
ANOVA	Analysis of variance
BOD	Biological oxygen demand
CCD	Central composite design
CFU	Colony forming units
COD	Chemical oxygen demand
CW	Concentrated whey
FTIR	Fourier transmission infrared
GCMS	Gas chromatography mass spectroscopy
HPLC	High performance liquid chromatography
IR	Infrared
MIFC	Multispectral Imaging Flow Cytometer
MTBE	Methyl tertiary butyl ether
OH	Hydroxyl
PH	Hydrogen potential
R^2	Regression coefficient
RF	Response function
RSM	Response surface methodology

TDS	Total dissolved solid
TS	Total solid
TSS	Total suspended solid
WPC	Whey protein concentrates
WPI	Whey protein isolate
UV	ultraviolet

ABSTRACT

The objective of this study was characterization of dairy wastes (cheese whey) and its utilization for the production of bioethanol. The key to the utilization of this resource has been changing the perception of cheese whey from a 'waste material' to an 'opportunity' for further processing. Dairy cheese whey consisted of biological oxygen demand (BOD = 230mg/mL) and chemical oxygen demand (COD = 70mg/mL). It represents largely disaccharide sugar content lactose (0.036 – 0.048 mg/mL or 3.36 – 4.48 %) and thereby, it is managed by converting into bioethanol with the help of yeast strain. In this work, *kluyveromyces delphensis* yeast strain was used for bioethanol production from cheese whey in batch fermentation (Erlenmeyer flasks). The composition of cheese whey was determined and characterized to identify its impacts on environment. The experimental design was studied with central composite design (CCD) to investigate the effects of significant factors including initial lactose concentration (4.5, 6.15 & 7.8g/L), yeast cell concentration (5, 7.5 & 10g/L), temperature (27^{o}C, 31^{o}C & 35^{o}C) and pH (5, 5.5 & 7) on fermentation process. The response surface methodology (RSM) was applied to investigate the interaction effect of fermentation process variables and to achieve maximium yield of bioethanol from cheese whey. The best operating conditions were found to be initial lactose concentration 6.15g/L, yeast cell concentration 10g/L, temperature 27^{o}C and pH 5.5. Under this condition, 15.5 mL of bioethanol per 500mL of cheese whey was fermented as a final point. The maximum ethanol yield obtained from cheese whey was 20.4 %. The functional groups of cheese whey Bioethanol were determined by using FT-IR with the help of IR correlation charts and moreover, the characteristics of bioethanol was investigated. This ascertained that the product obtained from cheese whey is definitely ethanol due to the confirmation of regions. Consequently, cheese whey is a good resource for production of bioethanol.

Keywords: *Bioethanol, Cheese whey, Fermentation process & Kluyveromyces delphensis yeast.*

1. INTRODUCTION

1.1. Background

Milk is the only food designed for mammals by nature through evolution. Mammals have adapted to consume all other foods. Milk provides nutrition in the form of energy from the carbohydrate present in the form of lactose, nitrogen from the protein content and a rich source of calcium to build bones to name but a few. Milk also provides other important benefits. For example, there are many biologically activities associated with certain components in milk. Almost without exception, these biologically active components are exclusively to be found in the whey or serum fraction of milk. Whey is the watery and thin liquid, which is received during cheese making by coagulating and separating casein proteins from milk. In the case of sweet whey rennet type enzymes are used at a min pH of 5,6 to induce coagulum and in the case of acid whey coagulum is created when milk is acidified by lactobacillus culture or mineral acid at a max pH of 5.1 Whey's composition and sensory characteristics vary depending on the kind of the whey (acid or sweet), the source of the milk (cow, sheep, bovine milk etc.) and the feed of the animal which produced the milk, the cheese processing used, the time of the year and the stage of lactation (Tsakali et al., 2010).

Cheese whey is a yellowish liquid remaining after milk coagulates during cheese production. It is a by-product of the manufacture of cheese and has several commercial uses. Cheese whey is produced in huge amounts and is a significant environmental problem due to the high levels of organic matter content. Cheese whey represents a biochemical oxygen demand (BOD = 230mg/mL) and a chemical oxygen demand (COD = 70mg/mL). Lactose is largely responsible for the high BOD and COD, since protein recovery reduces only about 12% of the whey COD. On the other hand, whey retains much of the milk nutrients, including functional proteins and peptides, lipids, lactose, minerals and vitamins and therefore has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource (Ghanadzadeh et al., 2012).

Ethanol fermentation from different raw materials containing carbohydrates has to be studied extensively in the future. One such is the cheese whey. The disposal of whey is a worldwide problem in dairy industry. Large quantities of whey are produced as a byproduct during the

manufacture of cheese and casein; which must be processed in an environmentally acceptable form before disposal as they can quickly deplete oxygen level in natural water systems because of its high chemical oxygen demand (Diniz et al., 2014).

Whey may be defined broadly as the serum or watery part of milk remaining after separation of the curd, which results from the coagulation of milk proteins by acid or proteolytic enzymes. The type and composition of whey at dairy plants mainly depends upon the processing techniques used for casein removal from liquid milk. In addition the dairy industry suffers from an economic blow due to several treatment costs that are incurred in proper disposal of whey. Although several possibilities of cheese whey utilization have been explored, a major portion of the world cheese whey production is discarded as effluent. Its disposal as waste poses serious pollution problems for the surrounding environment (Macwan et al., 2016).

Whey is also one of the most typical examples of the product under processing raw material milk. This is partly due to a biochemical oxygen demand (BOD) and large sugar content. Containing carbohydrates lactose, salts, lactic acid, proteins and fat could be used as a substrate for fermentation biotechnology for the energies production. It should be added that the whey has already been tested to be exploited as raw material for the fermentation production of various metabolites, such as citric acid, ethanol, and the production of animal feed (Macwan et al., 2016).

Impacts of the irresistible use of fossil fuels to produce energy has been recognized as problematic with high environmental impact. These impacts are seen in the increase of greenhouse gases released to the atmosphere and in the decrease in the reserves of gas, carbon and other fossil fuels. Currently, more than 80% of the energy and nearly of the 90% of organic chemical compounds produced annually are derived from fossil sources. Although, the solution of this problem is the implementation of renewable process the investments required and the perception of high risk in using new technologies are decelerate its implementation. Nowadays, bioprocess play a major role in biomass transformation for the manifold generation of products and its recovery (Chandra et al., 2018).

Bioprocess integration is defined as the incorporation of two or more unit operations simultaneously in a single process. Sustainable bioprocess for the processing of high amounts of organic biomass and the concomitant generation of bio-based products and bioenergy takes place

in a defined biorefineries, which are analogue to classical petroleum refineries. Biorefinery has been subject of intensive research and significant advances have been obtained in order to improve production of bioenergy and valuable chemical compounds with low refining costs (Chandra et al., 2018).

Whey is a cheap, abundant feedstock produced year-wide and can be fermented to various processing forms e.g., whey permeate and whey powder. There are two main types of whey i.e., acid whey and sweet whey, according to the procedure used for casein precipitation during cheese manufacturing. Sweet whey is produced during the rennet types of hard cheese (cheddar or Swiss cheese) processing. On the other hand, acid whey is a byproduct during the processing of acid types of dairy products e.g. cottage cheese and strained yogurt. Lactose is the most abundant organic content in acid whey (4.5 - 5% w/v)(Parra-Saldivar et al., 2018).

Development of novel alternative technologies that require minimal preprocessing would not only improve overall economics for the ethanol industry, but would also create a new value-added market for this surplus dairy waste stream and reduce the environmental burden of whey permeate disposal(Parashar et al., 2016). It is known that the fermentation process performance is affected by operational conditions such as temperature, stirring rate, initial inoculum and substrate concentrations, dissolved oxygen, among others. A suitable control of these variables is great importance for a respectable process performance and procurement of high-quality products. The present study aimed to optimize the conditions for ethanol production from CW through RSM designed with central composite design(Almeida, 2011).

Ethanol is a source of energy and renewable energy sources with no emission of greenhouse gases. The production of ethanol provide a means of energy independence and it is being as potential substitutes for current pollutant fuels such as oil fuels and coal derived from conventional sources. Therefore, using ethanol for automobiles can significantly reduce non- renewable fuels (oil, coal) use and exhaust greenhouse gases emission. Ethanol is one of the most important energy fuels that produced from carbohydrate rich wastes like molasses and cheese whey. Production of ethanol from sugar containing materials is technically feasible as well as its use is environmentally friendly. Ethanol production from cellulosic materials is highly expensive because it needs separation technique to hydrolysis cellulosic materials to small sugar molecules. However, ethanol

production from dairy cheese whey is inexpensive since the process no requirement for further separation techniques. Additionally, cheese whey is highly available for ethanol production (Shrestha et al., 2012).

1.2. Statement of Problem

Dairy waste discharged by shola milk processing industry in Ethiopia under uncontrolled and unsuitable conditions is causing significant environmental problems. The importance of dairy wastewater treatment is undoubtedly the key factor to bring sustainable development and clear environment to the society. However, since this company has discharged the waste whey into water bodies and local environment without any treatment, it has a serious impact on natural water bodies, public health, environment and soil because the societies around that factory daily use this water and children are highly got cough as well as it creates crazy dogs and cats that transfer diseases to human. It also affects aesthetic merit, nuisance, and transparency because shola milk processing industry was found in city and affect human health. Therefore, cheese whey is the main byproduct of the dairy which is produced in large quantities and usually causing major environmental pollution due to its high organic load. Cheese whey discharged from milk processing industries is highly contaminated with organic compounds such as chemical oxygen demand (COD = 70mg/mL) and biological oxygen demand (BOD = 230mg/mL) which severely affect the environment, soil and water bodies. The whey contains protein, nutrient and carbohydrate that affect the water bodies through eutrophication process. It reduces dissolved oxygen of water and soil and thereby, affects aquatic life. The dairy industry in Ethiopia was largely found in rural areas and released whey to the environment without any treatment. This cause soil pollution and thereby, affects farmer's agricultural production because whey is a constituent of nutrients and acids. This changes the soil properties and fertility. In addition, it affects animal feeding such as grass and changes its contents. Generally it has an effect on economic destruction and losses of soil fertility. In other hand, there is high energy demand in Ethiopia. It is important to convert this dairy waste into ethanol to satisfy energy demand in our country. Its' utilization for ethanol production offer special advantages for Ethiopia;

- By providing cheap raw materials
- Simultaneous waste treatment with ethanol production.

1.3. Objective

1.3.1. General objective

The general objective of this study was to characterize dairy cheese whey and to utilize it for the production of ethanol.

1.3.2. Specific objectives

- To characterize physiochemical parameters of dairy cheese whey.
- To concentrate and hydrolyze lactose contents of cheese whey for increasing yield.
- To determine reducing and total sugars (both reducing and non – reducing sugars) for cheese whey.
- To prepare yeast strains as culture media and study its cell viability.
- To ferment lactose sugars of cheese whey into ethanol for minimizing organic load.
- To study effects of lactose concentration, yeast cell concentration, pH and temperature on fermentation process with central composite design (CCD).
- To characterize the ethanol physical properties, its quality and to determine its impurities.

1.4. Significance of the Study

The ethanol produced from dairy cheese whey is important to be used as renewable and sustainable energy clean fuel. As a result, ethanol product is a source of energy and it can substitute non-renewable energy such as oil and coal energy source as alternative source of energy to reduce greenhouse gasses emission. This study is significant in different ways;

1. Ethanol fermented and distilled has no impacts on environment and it is a source of energy that can be used for households and cooking as well as for motors. It is nontoxic product and has no risk on environment as well as on ground water and aquatic life.
2. Other significance of this study is utilization of dairy cheese whey for ethanol production as well as recycling and recovering the dairy wastes for developing ethanol product. This is one of method to mitigate waste released to environment and it is a waste minimization method.
3. For academic researcher: this study provides good information to practice on dairy cheese whey from milk processing industries. Therefore, it could be an initial idea for academic researchers in the future. Generally, bio ethanol is a source of energy used with no greenhouse gases. Its production provides energy independence and is being substitutes for current high

pollutant oil and coal derived from conventional sources. Consequently, utilizing bioethanol as energy source can significantly reduce oil or coal use and exhaust greenhouse gas emission.

1.5. Scope of study:

To achieve the objective of this research, the following scopes have been identified.

1. To characterize the physiochemical properties of dairy waste and recovered product using analytical instrument analyses such as;

i. Milk analyzers (Lactoscan)
ii. Titration method
iii. Density meter
iv. Spectrophotometry
v. FTIR

2. Environmental impacts of dairy waste (cheese whey) was studied in terms of biological oxygen demands (BOD) and chemical oxygen demand (COD)
3. The impacts of dairy waste on human health was minimized by converting into bioethanol.
4. To study the effect of initial lactose concentration, yeast cell concentration, pH and temperature on fermentation process.

2. LITERATURE REVIEW

Bozanic et al, 214 have studied the economical possibilities of whey utilization, primarily how to unwanted by-product converted into a valuable raw material. From this overview of literature, cheese whey is utilized for different purposes. Cheese whey is dried traditionally into powder. However, processing method is used to improve the economic value such by-product, whey could be utilized, for example, in fermentation, production of soft drinks, production of Whey Protein Concentrate (WPC) and whey protein isolate (WPI), fractionation of certain protein components, such as isolation and purification α-lactalbumin (α-la) including specific peptides, and production lactose, lactic acid and bioethanol. This review provides most recent development (Bozanic et al., 2014).

Agustriyanto et al, 2009 also study the utilization of cheese whey as a fermentation substrate to produce bio-ethanol is an effort to supply bio-ethanol demand as a renewable energy. Like other process systems, modeling is also required for fermentation process design, optimization and plant operation. In case, fermentation process of cheese whey was investigated by applying mathematics and fundamental concept in chemical engineering. Kinetics model of bio-ethanol production from cheese whey will enhance the understanding of what really happen in the fermentation process (Agustriyanto et al., 2009).

Whey is an abundant waste stream generated during cheese production. After cheese curdling, about 10% of the used milk is converted in cheese, while the remaining liquid is a by-product called whey, which still contains about 55% of the milk nutritional load. In particular, although whey composition depends on several factors (e.g., milk quality, animal breed and feed), a high lactose concentration (about 45 g/l) is usually present. It corresponds to the total amount of milk lactose content and about 20% of milk proteins, respectively (Pasotti et al., 2017).

Generally, there is a gap among the literature mentioned above and they only focuses on the development of ethanol from dairy waste whey and try to point out kinetics model of fermentation process variables. However, it is possible to control fermentation temperature and to test ethanol quality to ensure its environmental feasibility. Additionally, it is possible to optimize effects of lactose concentration, yeast concentration, pH and temperature on the ethanol production as well

as to control fermentation temperature to enhance microbial activities and conversion of lactose into ethanol.

2.1. Bioethanol

Bioethanol is the fuel alcohol produced by the fermentation process from agricultural wastes, agro-industrial wastes and by products. Mostly the world supply of ethanol was produced by fermentation of sugarcane/bagasse/molasses, starch containing crops, cellulosic materials or byproducts from industries. Because of high demand for ethanol, there is a need to search for high yielding strains and less expensive technology for production of ethanol to satisfy human demand. Ethanol fermentation from different raw materials containing carbohydrates has to be studied extensively in the future. One such is the cheese whey. The disposal of whey is a worldwide problem in dairy industry. Large quantities of whey are produced as a byproduct during the manufacture of cheese and casein; which must be processed in an environmentally acceptable form before disposal as they can quickly deplete oxygen level in natural water systems because of its high biological oxygen demand.

Ethanol is a high-octane fuel which is used primarily as a gasoline additive and extender. The reduction in use of methyl tertiary butyl ether (MTBE) due to its environmental problems caused by groundwater contamination and surging prices for petroleum-based fuels are dramatically increasing the demand for ethanol and the interest in ethanol production. Ethanol can be produced from carbohydrates such as sugar, starch, cheese whey and cellulose by fermentation using yeast or other organisms(Shapouri et al., 2006).

Bio-ethanol is most commonly blended with gasoline in concentrations of 10% bio-ethanol to 90% gasoline, known as E10 and nicknamed ‘‘gasohol”. The use of ethanol as an alternative motor fuel has been steadily increasing around the world for a number of reasons. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural areas, reduce air pollution and reduce global climate change carbon dioxide buildup. Also, Ethanol considered both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity. It is nowadays an important product in the fuel market(Zohri et al., 2014).

In terms of the exploitation of yeasts beyond the field of food and beverages, the most important biotechnological application is the production of bioethanol as a gasoline additive or even substitute(Prazeres et al., 2012).

2.2. Bioethanol properties

2.2.1. Chemical Properties of Ethanol

Ethanol's chemical formula is C_2H_5OH.

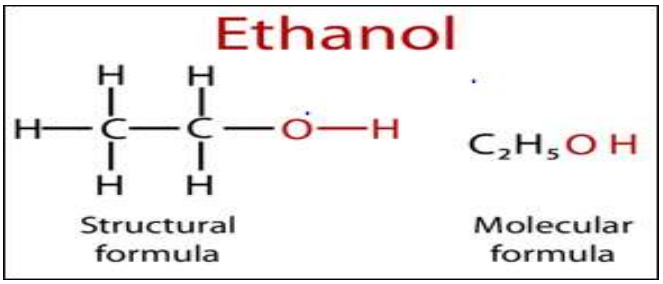


Fig 2. 1. Ethanol Structural and Molecular Formula.

2.2.2. Physical Properties of Ethanol

Appearance, Odor and Taste**:** At room temperature, ethanol is a clear, colorless, volatile liquid with a characteristic odor. When diluted, it is somewhat sweet, but concentrated alcohol has a strong, burning taste.

Solubility: Ethanol is highly soluble in water and organic solvents, but poorly soluble in fats and oils.

Density: Density of ethanol at 68 °F (20 °C) is 0.789 g/mL.

PH: Pure ethanol is neutral (pH ~7). Most alcoholic beverages are more or less acidic: table wine pH = 3.3-3.7, beer pH ~ 4.

Boiling Point: Boiling point of ethanol is 173.3 °F (78.5 °C)

2.2.3. Uses of ethanol

- Ethanol is present in alcoholic beverages such as beer, wine, whisky
- Ethanol is used as antiseptic for sterilizing wounds
- Ethanol is used in cough syrup, digestive syrup and tonics
- Ethanol is being mixed with petrol and used as motor fuel. This mixture is called power alcohol
- A mixture of ethanol and water has lower freezing point than water. This mixture is known as antifreeze and is used in radiators of vehicles in cold countries and at hill stations.
- Ethanol is used for preparation of chloroform, iodoform, ethanoic acid, ethanal, ethyl ethanoate etc.

2.3. Bioethanol production in world

While bioethanol and biodiesel are the most typical biofuel types, the two top producers of ethanol in the world by far are the United States and Brazil, collectively producing almost 83% of the 20,503 Million gallons world ethanol production in 2014 and 84 % of the 22,860 million gallons collectively in 2017. Generally, global ethanol production increase from time to time dramatically to satisfy global ethanol demand in the world.

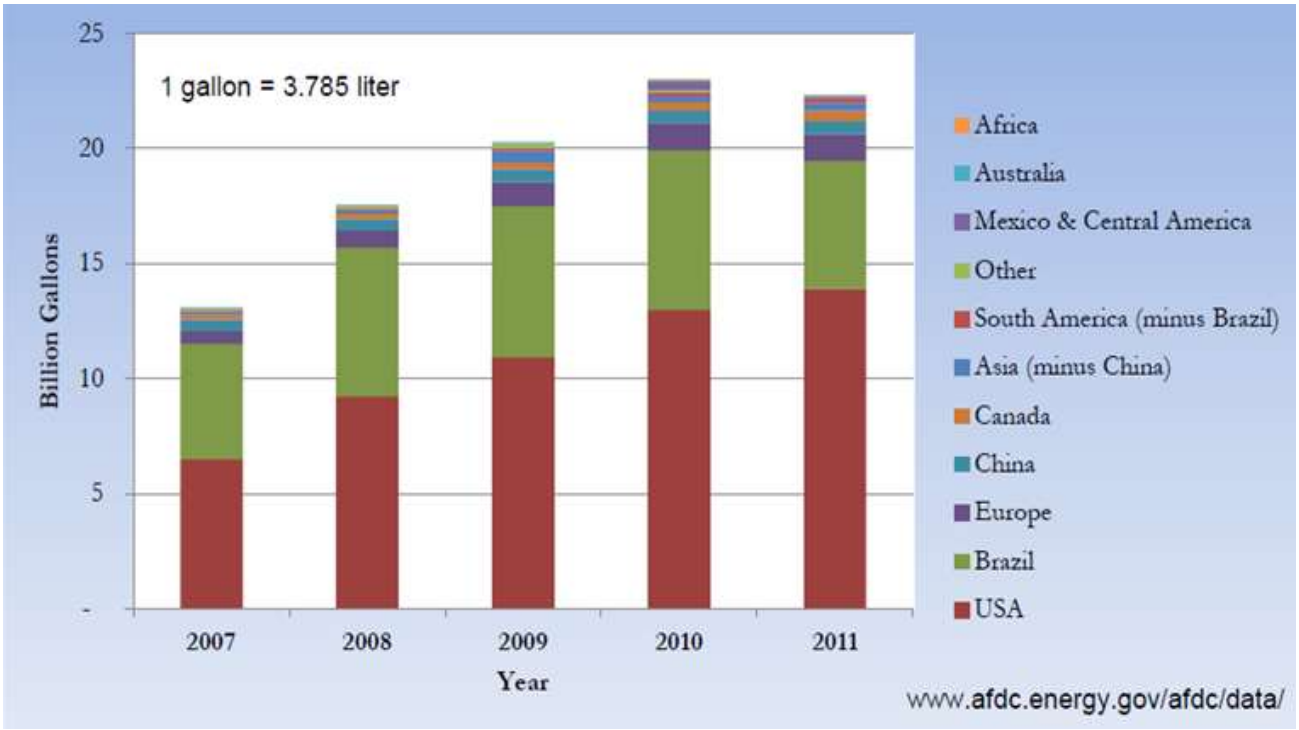
Biofuels, mainly produced from biomass, are a broad range of fuels in the form of solid biomass, liquid fuels (bioethanol, biodiesel, and vegetable oil), and various biogases (Demirbas, 2009). Biofuels have paid attention globally due to concerns on climate change, energy security, and dependency on import encumber of petroleum products.

They are increasingly premeditated by many countries as much as practicable to substitute the fossil fuel source in the transport sector. At present bioethanol and biodiesel are the main biofuels for transport as both can be used in blended or neat form although neat usage requires engine modification. Bioethanol, derived from starch crops like sugarcane, sugar beets, corn, wheat, cheese whey and sorghum is utilized blended with petroleum based gasoline, and biodiesel, derived from oil crops like rapeseed, palm oil, Jatropha, sunflower, and soy is utilized blended with petroleum based diesel(Johansson, 2008).

Table 2. 1. World Bioethanol Production

Region	2014		2015		2016		2017	
	Million gallons	% of world production	Million gallons	% of world production	Million gallons	% of world production	Million gallons	% of world production
USA	14,313	58	14,807	58	15,329	58	15,800	58
Brazils	6190	25	7093	28	7295	27	7060	26
EU	1445	6	1387	5	1377	5	1415	5
China	635	3	813	3	845	3	875	3
Canada	510	2	436	2	436	2	450	2
Thailand	310	1	334	1	322	1	395	2
Argentina	160	1	211	1	264	1	310	1
India	155	1	211	11	225	1	280	1
Rest world	865	4	391	2	490	2	465	2

Source: RFA analysis of public and private data sources



Source: (F.O.Licht, 2007-2011)

Fig 2. 2. Global Ethanol Production by Country/Region and Year

Regarding the socio-economic and environmental issues, ethanol production contribute to mitigate the price rise of fossil fuels and energy security as well as it reduces greenhouse gases effects on environment. For instance, in Brazil the prices of sugar are linked with the price of ethanol (Gebreyohannes, 2013).

2.4. Bioethanol production in Ethiopia

Ethanol production in Ethiopia has been started since 1998/99 in Fincha sugar factory with the capacity of 1,907,000 Liters per year. In 2010/11 Ethanol production started in Metehara sugar factory with the capacity of 6,373,000 Liters per year. Both factories produce 19,805,000 Liters ethanol per year. In sugar factories ethanol is produced mainly from molasses. Molasses consists of sucrose that could be decomposed into simple glucose that finally fermented into ethanol. However, to satisfy ethanol demand it could be important to look for other raw materials such as starch corn and cheese whey.

The worldwide recent awareness for the use of ethanol to replace petroleum and generation of power along with sugar mill plants should have led to setting up of number of ethanol plants and co-generations. Ethiopia has several sugar real estate's such as Fincha, Metehara and WonjiShoa are among the industries which are run and administered by Sugar Development Agency. Among molasses derived products ethanol takes the largest part, but its utilization must attract the attention of the government policy makers in order to utilize as a bioethanol. Bioethanol or biofuel is ethanol based products that can process into liquid fuels for transport purposes (ESDA, 2015).

However, ethanol demand in the country is high than ethanol produced in the country. Therefore, to satisfy ethanol need, it is important to look the source of ethanol. Bioethanol could be produced from molasses, spent grain, cheese whey, waste paper, cellulosic materials, starch containing crops, municipal solid wastes etc. in our country there is no bioethanol produced at industrial level. Additionally, bioethanol production from dairy wastewater (cheese whey) is not practiced in our country.

2.5. Cheese whey

Cheese whey is the main by-product formed during the coagulation of milk casein in cheese making. Whey is produced in large amounts and has a high polluting load, therefore representing a significant environmental problem. On the other hand, whey retains much of the milk nutrients, including functional proteins and peptides, fats, lactose, minerals and vitamins, challenging the industry to face whey surplus as a resource and not only as a waste problem. Cheese whey is byproduct represents about 85-95% of the milk volume and retains 55% of milk nutrients.

Dairy cheese whey is a byproducts of milk processing industries and it contains lactose (4- 5) %, protein and fats. Cheese whey is used for different purpose. It is utilized for production of lactic acid, protein cell, methanol, Butanol, glucose and ethanol. As a result, this study deals with the production of ethanol. Dairy cheese whey give appropriate yield and bioethanol formation is possible because it is composed of disaccharide. The dairy cheese whey and other byproduct are suitable for the production of bioethanol because it contains organic matter content. Ethanol is biofuel used for household as energy source, for dairy plant and for different purpose. Production of ethanol from dairy whey by using bioreactor and yeast need appropriate control of fermentation pH, temperature and contact time.

Lactose is one of the main constituents of whey which is excellent substrates for bioethanol production as it is cheap and widely available in large volumes. This product is mainly used as a biofuel additive to gasoline, and most commonly produced from sugar cane or sugar beet, different crops or from cellulosic resources (wooden hydrolysates, agricultural by-products).

The production process of bioethanol can be divided into three main stages:- (a) preliminary processing of the substrate, i.e. preparation of the raw material, (b) alcoholic fermentation, and (c) separation of the end-product (distillation, rectification and dehydration of bioethanol). For purposes of alcoholic fermentation yeasts (i.e. *Saccharomyces cerevisiae*) are usually used since they have a fast fermentation capacity and tolerate high concentrations of ethanol (up to 20% v/v). Since S. cerevisiae cannot ferment lactose, whey has to be enzymatically hydrolyzed prior to the alcoholic fermentation. The hydrolysis step is not required if *Kluyveromyces spp*. are employed as they have the ability to catabolise lactose. In the commercial production of bioethanol from whey,

K. marxianus var. marxianus and *Kluyveromyces fragilis var. marxianus* are commonly used(Ghanadzadeh et al., 2012).

2.6. Availability of whey for bioethanol production

Ethiopia is developing its investment in dairy production and export market to increase foreign currency earnings. Milk production has increased linearly in the last decade, which flickers a gleaming hopes that the nation is on the right track to utilize its dairy potential. This is mainly attributed to the increase in market-oriented dairy system stimulated by interactive elements such as dairy investment reforms, expansion of milk processing industries, and rapid urbanization. As a result, huge amount of cheese whey produced in our country discharged to environment by milk processing factories. The total volume of milk produced in Ethiopia increased over the last 15 years from 1 billion liters to 3.06 billion liters in 2015/16. Currently, there are over 22 medium- and large-scale dairy processing companies in Ethiopia with nine of them operating in Addis Ababa and the rest in other major regional cities(Fombad, 2011).

Table 2. 2. Major private dairy enterprises operating in different parts of Ethiopia

Ser. No.	Dairy enterprise	Location	Year of establishment	Daily processing capacity, (liters)
1	Sebeta Agro Industry (Mama Dairy)	Sebeta	1998	35 000
2	Lame Dairy Processing (former DDE)	Addis Ababa	2008	60 000
3	Dire Dawa Dairy Processing Enterprise	Dire Dawa	1972	20 000
4	MB PLC (Family Milk)	Addis Ababa	2003	15 000
5	Yadeni Dairy Farm (Bora Milk)	Addis Ababa	2008	15 000
6	Ada'a Dairy Cooperative	Debre Zeit	1998	15 000
7	Lema Dairy	Debre Zeit	2004	10 000
8	Berta and Family plc.	Addis Ababa	2000	9 000
9	Genesis Farm	Debre Zeit	2001	4 000
10	Holland Dairy	Debre Zeit		4 000
11	Almi Tiku Wetet (Almi Fresh Milk)	Hawassa		4000
12	Ruth and Hirut Dairy Farm	Addis Ababa	2008	4 000

13	Abay fana Awash Agro-Industry	Adama		3 500
14	Chuye Milk and Milk Products Processing	Addis baba		3 000
15	Fantu and Family Dairy Farm	Addis Ababa		2 500
16	Zemen Milk	Mekelle		2 000
17	Penguin International Business plc. (cheese world)	Addis Ababa		1 800
18	Life Milk Processing Enterprise	Sululta		1 500
19	Semit Agro Industry/Enat Milk	Mojjo		
20	Beral Milk	Addis Ababa	1991	
21	Harmonius Agro Industry	Adama		
22	Jantekel Dairy Union (Facil Milk)	Gonder		1 200

Source: (Current study survey result; Land O'Lakes, 2011)

It is technically feasible to make bioethanol from cheese whey. Fuel ethanol could be made from whey contain lactose. Lactose is a disaccharide sugar that could be used for the production of bioethanol.

2.7. Environmental effect of cheese whey

In Ethiopia, dairy industries have sold whey as animal feed or discharged it to the sewerage system, and consequently have caused ecological disturbance through the pollution of water bodies. Furthermore, there is no utilization of whey in the forms of value-added products. The disposal of whey is a worldwide problem. Large quantities of whey are produced as a by-product during the manufacture of cheese and casein, and this must be disposed of or processed in an environmentally acceptable way. Since most of the components are of small molecular weight and soluble, they can quickly deplete oxygen levels in natural water systems: the BOD (Biological Oxygen Demand) and COD (Chemical Oxygen Demand) of raw whey are about 230 and 70 mg/mL respectively. The key to the utilization of this resource has been changing the perception of whey from a 'waste material' to an 'opportunity' for further processing.

Butterfat is traditionally of high value, and most plants separate it for use as an ingredient for further processing. The remaining whey may be made into various products by using an array of

processes and technologies, or is otherwise disposed of. Continuous land disposal of cheese whey can endanger the physical and chemical structure of the soil, decrease the crop yield, and lead to serious water pollution problems. Treating whey for BOD reduction before dis-charging it is costly(Ling, 2008).

Cheese whey is a green-yellowish liquid resulting from the precipitation and removal of milk casein in cheese making processes. The yellowish color of whey is caused by the presence of riboflavin (vitamin B2). The majority of the whey content is lactose and the remains in the cheese whey, constituting the main fraction (90%) of the organic load. Fat and protein contents are also partially responsible of organic contamination. BOD and COD values range 230 mg/mL and 70 mg/mL, respectively. The BOD5/COD ratio is commonly higher than 0.5. Hence, this substrate is suitable to be treated by biological processes. The inorganic contamination of cheese whey is attributable to mineral salts presence. Inorganic contamination is the consequence of NaCl addition during cheese production. High sodium contents can cause problems when operating biological digesters and low alkalinity also affect the biological conversion efficiency. From the previous statements, it is obvious that cheese whey cannot be directly discharged to the environment without an adequate treatment(Prazeres et al., 2012).

Continuous high discharging of cheese whey on land may pose serious health problems and environmental pollution. Excess application of cheese whey also has the potential of degrading soils. Cheese whey may contain 230 mg/mL of BOD and at high application (50mL), the soil remained wet for 24 h and caused wheat kills and severe crop damage to potatoes, alfalfa and barley. High application of cheese whey due to rapid consumption of oxygen for the oxidation of readily decomposable milk sugars and proteins can cause a serious environmental problem. It also reduce soil redox potential as a result of high whey released which cause solubilization of Fe & Mn, and contamination of domestic drinking water wells(Ghaly et al., 2007).

2.8. Bioethanol production process from cheese whey

The diary cheese whey contain lactose which easily hydrolysis into galactose and glucose. These sugar contents of carbohydrates are organic matter and support microorganism growth. First, lactose is decomposed into galactose and glucose and then, both sugars are fermented into ethanol.

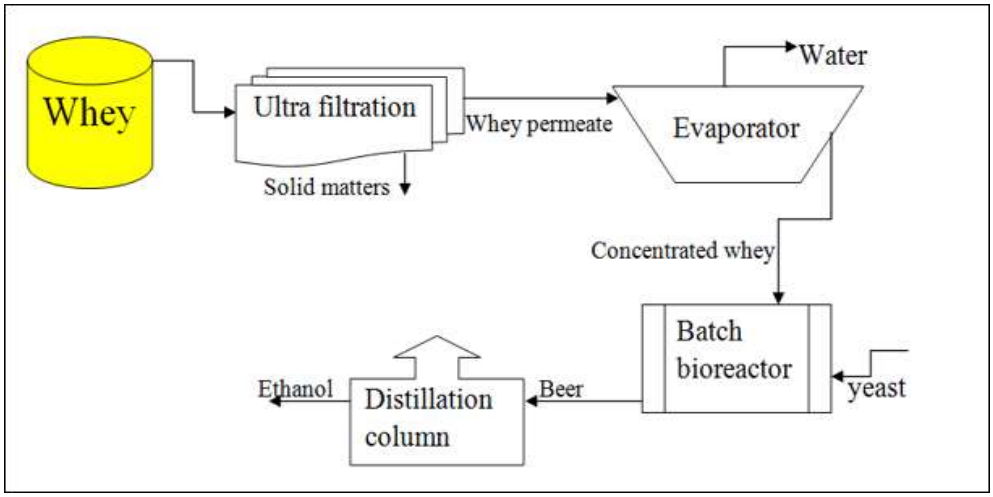


Fig 2. 3. Ethanol Production Process Flow Diagram

2.9. Process Description

The dairy cheese whey from the storage tank is filtered by ultra-filtration to remove solid particles and the whey permeate from filtration unit operation is entered into vacuum evaporator. The common choice of microorganisms for bioethanol production from cheese whey feed fermentation, are various strains of the yeasts. While strains of Kluyveromyces fragilis species are capable of directly metabolizing lactose, Saccharomyces cerevisiae cannot metabolize lactose, which must be hydrolyzed before the resultant glucose and galactose can be utilized. This process is however hampered by high concentrations of extracellular glucose, which causes catabolize repression of galactose utilization. Reaction shows ethanol formation from lactose. Then, the concentrated whey is going to distillation column units to be separated from liquid.

The whey from acid casein manufacture is unsuitable to be processed to lactose powder due to the presence of lactic acid and sulphate ions which interfere with the crystallization process. However, although the whey is only 4 -5% lactose, this is still enough to cause a significant environmental impact.

Step 1 - Cooling the serum

The serum must be cooled (the temperature at which it is transported to prevent bacterial contamination) to a temperature at which the yeast can function.

Step 2 – Fermentation

Yeast is grown up (or propagated) in separate vessels called 'Donas'. In the Donas, air is used to promote the growth of yeast biomass growing on the serum. After the serum is cooled, yeast from the Dona vessels is used to inoculate the cool serum.

The yeast is added to ferment the lactose in two reactions: firstly splitting the lactose into the two sugars of which it is composed and secondly fermenting these sugars to ethanol. The yeast used is not the one used to make bread or beer but a special lactose fermenting organism called *Kluyveromyces delphensis*. This yeast produces $-galactosidase, the enzyme required to split lactose (a disaccharide) into its component sugars which are glucose and galactose.

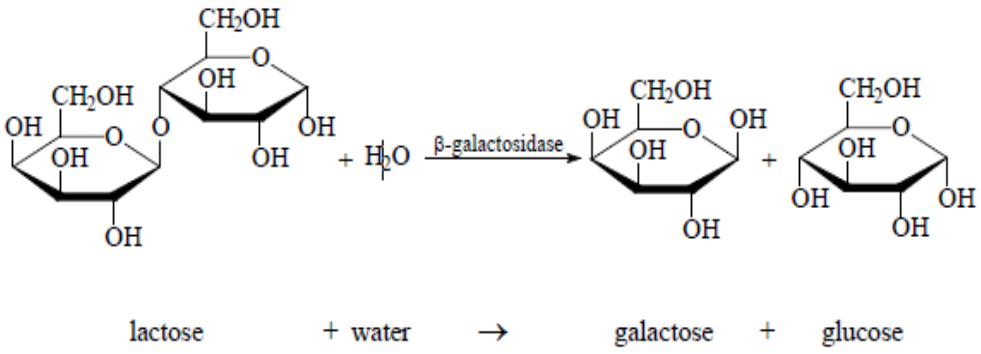


Fig 2. 4. Chemical Chain Bonding of Lactose and Its Decomposition Enzymatically.

The fermentation temperature is determined by the processing speed required, but it is kept as low as possible to minimize bacterial contamination of the process. The maximum conversion of reactants to products is 51%, and this percentage is used as the basis of the fermentation efficiency calculation. Serum has been found to have everything required by the yeast to grow so no further additions to the fermentation are required(Hamilton, 2011).

The first studies on alcohol production (ethanol) from CW fermentation date from the 1940s. Pollution reduction and lactose conversion to ethanol are achieved simultaneously. Consequently, the treatment of cheese whey and the simultaneous ethanol production has received a wide attention. In this sense several studies have been reported by using raw CW; CW powder solution; CW permeate from ultrafiltration and even deproteinized CW. This treatment requires a specific group of microorganisms such as; *Kluyveromyces fragilis, Kluyveromyces delphensis* and

Saccharomyces cerevisiae, etc. The reaction describing the bio-conversion of lactose to ethanol reveals a theoretical maximum value of 0.538 kg of ethanol per kg of lactose consumed. Ethanol yield efficiency was calculated as follows:

Ethanol yield efficiency = [(amount of ethanol/amount of fermentable sugar)/0.51].100..... (**2.1**)

Fermentable sugar refers to glucose and galactose. The value 0.51 represents 100% theoretical ethanol yield (0.51 g of ethanol/g of fermentable sugar)(Parashar et al., 2016).

Step 3 - Distillation

The beer enters the distillery flask and heated. Ethanol vapor from the top of this column enters the rectifier column from which the primary product is recovered. The product is then fed to the anhydrous columns (center) or to the extractive distillation column. The low boiling point impurities are vented to the atmosphere and the recovered ethanol returned to the surge tanks. The ethanol is produced depending on the customer requirements for the product. The grade of the product is determined by the number and type of distillation processes involved in its manufacture. High grade potable spirit (i.e. beverage grade) is made from a product using an extractive distillation process. The process involves diluting white spirit grade product with water and redistilling the diluted fluid. The impurities remain in the water phase. Some of the ethanol is further purified to make an anhydrous product. This is done using one of two processes. The yeast is then removed from the liquid and the ethanol separated out by distillation to produce different grades of ethanol for a variety of industrial uses (Hamilton, 2011).

2.10. Deproteinization of cheese Whey

Deproteinized whey is normally manufactured by heating whey to temperatures in the range of 70-90°C to denature the proteins, followed by acidification, and removal of the flocculants by decantation, filtration or centrifugation. Whey permeate is the major by-product of the Filtration of whey for manufacturing whey protein concentrate. These solids components, mainly lactose, minerals and non-protein nitrogen, represent a substantial pollution problem for the industry if not utilized (Hobman, 1984).

This whey fraction is most often derived as the Filtration permeate by-product resulting from the manufacture of whey protein concentrate, or from the manufacture of heat-precipitated whey protein, e.g. lactalbumin(Macwan et al., 2016).

2.11. Cheese whey Hydrolysis

Fermentation of lactose in whey permeate directly into ethanol has had only limited commercial success, as the yields and alcohol tolerances of the organisms capable of directly fermenting lactose are low. Treating the whey permeate with acid to liberate monomeric sugars are readily fermented into ethanol. Both lactose solutions and commercial whey permeates were hydrolyzed using inorganic acids and carbonic acid. In all cases, more glucose was consumed by secondary reactions than galactose. Galactose was recovered in approximately stoichiometric proportions. The number of commercially available microorganisms capable of metabolizing glucose and galactose are significantly higher than the number of microorganisms able to directly use lactose describe the lactose hydrolysis as a low-cost cheese whey pre-treatment. Hydrolysis can be accomplished in two ways. Chemical hydrolysis is characterized by acid conditions (pH < 3) and high temperatures (up to 140^{o}C). Chemical hydrolysis can be carried out with acid addition, such as sulfuric acid or using a solid acid, as the acid form of a cationic exchange resin. Chemical hydrolysis has some advantages such as protein denaturation. As a consequence, the enzymatic hydrolysis is the preferential path for lactose hydrolysis. The enzymatic hydrolysis is carried out by means of the lactase enzyme (found in animals, plants, bacteria, fungi and yeasts) that converts the lactose disaccharide into its monosaccharide components, glucose and galactose. The main strains utilized in this process are *Aspergillus* and *Kluyveromyces*. Due to the impossibility of lactase reutilization, lactose hydrolysis conducted with the free enzyme(Prazeres et al., 2012).

2.12. Cheese whey concentration

Concentration of a liquid by evaporation under vacuum was introduced in 1913. The process was based on a British patent by E.C. Howard, which covered a steam-heated, double-bottomed vacuum pan with condenser and vacuum pump. The normal whey samples either sweet or acid were concentrated using vacuum scraper concentrator(Alsaed et al., 2013). Concentration of a cheese whey involves evaporation of a water to concentrate sugar (Lactose). Concentration is distinguished from drying in that the final product the concentrate is still cheese whey. The final

concentration of the whey is controlled by means of a Refractometer that, in function of its data, regulates the capacity of the extraction pump of the concentrated.

2.13. Fermentation

The fermentation takes about three days (72 hours), with the fermenting serum passing from one vessel to the next. In process control involves monitoring the fermenting rates and ethanol yield depending the selected parameters, which affect the fermentation progresses. The decline in specific gravity reflects the change of lactose to ethanol with the evolution of carbon dioxide. Yeast is grown up (or propagated) in separate vessels or Erlenmeyer flasks aerobically with the help of incubator shaker. As a result, air is used to promote the growth of yeast biomass growing on broth media. The yeast used is not the one used to make bread or beer but a special lactose fermenting organism called *Kluyveromyces delphensis*. This yeast produces $-galactosidase, the enzyme required to split lactose (a disaccharide) into its component sugars which are glucose and galactose.

2.14. Factors affecting fermentation

Kluyveromyces delphensis yeast for lactose decomposition or ethanol fermentation could be described in terms of its parameters and other factors such as processes, efficiency of equipment and technical feasibility of product. The main parameters that affect the performance of ethanol fermentation are initial lactose concentration (L), yeast cell densities (Y), temperature (T) and pH

2.14.1. Lactose concentrations

Concentration of lactose can influence the yeast growth rate and ethanol released during fermentation in different ways. Lactose concentration is one of the considered factor that affect ethanol yield and its effect is discussed in chapter four in details. Therefore, the amount of lactose added has inverse relation with ethanol yield. As lactose consumption increase ethanol production also increased. However, high concentration of lactose can cause yeast cellular osmotic pressure and low lactose concentration limits ethanol yield on fermentation.

2.14.2. Yeast cell concentrations

Yeast cell concentration also a significant factor that affect ethanol production rate during fermentation period. The growth and metabolic activities of micro-organisms are profoundly affected by the temperature at which they grow. In continuous culture the metabolic activities of

a micro-organism vary with growth rate. But the growth rate in batch culture varies with temperature and thus there is always doubt as to whether reported effects of temperature on metabolism are direct or arise from an alteration in growth rate. An assessment of the effect of culture temperature on the metabolism of a micro-organism, therefore, is best carried out under conditions where the growth rate is constant and independent of temperature. This is readily achieved with continuous culture(By, 2018).

2.14.3. Temperature

Another factor affecting fermentation process is temperature. Temperature is a significant factor because it affects highly the growth rate of yeast, yeast cultures and ethanol production rate. Yeast is sensitive to temperature and moreover, high temperature denatures the yeast cells. Although very low temperature retards the yeast activities and growth rate.

2.14.4. PH

The most important fermentation process factor is pH. The media condition is depend on the acidity and alkalinity of mixture and this is determined by pH. The yeast needs optimum conditions to be doubled in their numbers. Highly acidic and basic media affects growth rate of yeast.

2.15. Distillation

For the ethanol to be usable as a fuel, water must be removed. Most of the water is removed by distillation. The purity is limited to 95-96% due to the formation of a low-boiling water-ethanol azeotropic. This may be used as fuel alone but unlike anhydrous ethanol it is immiscible in Petrol meaning it cannot be mixed i.e. E85. The water fraction is typically removed in further treatment in order to burn with in combination with petrol in petrol engines.

2.16. Ethanol concentration/dehydration

Currently, the most widely used purification method is a physical absorption process using a molecular sieve, for example, ZEOCHEM Z3-03 (a special 3A molecular sieve for ethanol dehydration). Another method, azeotropic distillation, is achieved by adding the hydrocarbon benzene which also denatures the ethanol (to render it undrinkable for duty purposes).

3. METHODOLOGY

3.1. Materials and methods

3.1.1. Apparatus used:

Storage tank was used in order to store dairy cheese whey from milk processing unit operations. The temperature of this whey was decreased at room temperature and solid matters were filtered using centrifuge. Lactose contents of whey was concentrated by using vacuum evaporator to obtain high yield of ethanol. Also microorganism (yeast) used for fermentation was sterilized with autoclave and incubators. Erlenmeyer flasks were used to hydrolyze lactose to ethanol and the ethanol product was separated by distillation column. Mass concentration of sugar was measured by density meter and the brix or total solid soluble of cheese whey was measured by both density meter and Refractometer. Water bath and oil bath were also used as energy source. The spectrophotometer was used to measure the absorbance to know the concentration. Additionally, the cell density and viability were determined by Microscope.

3.1.2. Chemicals used:

Agar was used to count colony forming unit (CFU) of *Kluyveromyces delphensis* yeast and to support growth on solid with plate. However, nutrient broth was used to increase and grow yeast in liquid or suspension form. Some nutrient broth, dextrose, ammonium sulfate, peptone, KH_2PO_4 and $MgSO_4.7H_2O$ were added as supplement to prepare culture media. Sulphuric acid was used for hydrolysis of the cheese whey. NaOH and H_2SO_4 were also used to adjust the pH of whey as well as Benedict's solution and lactose standard solution were used to detect sugar concentration. Methylene blue solution also used as straining technique to observe both viable and dead cell with the help of Microscope.

3.1.3. Yeast used:

The yeast strain (*kluyveromyces delphensis*) used in this study was gifted from Ethiopian Biodiversity Institute in Addis Ababa, capital city of the country. The major carbohydrate in whey was lactose and it was a disaccharide sugar containing glucose and galactose. The *kluyveromyces delphensis* had ability to ferment lactose into ethanol as well as its activity facilitated lactose in the cheese whey to be hydrolyzed into glucose and galactose that finally fermented into ethanol.

3.1.4. Substrate used:

Cheese whey was collected from dairy factory shola milk processing and characterized by lactoscan milk analyzer in this factory. In all dairy milk processing industries and private enterprises the cheese whey was collected and stored in storage tank. The stored cheese whey was transported to ethanol production sites. Cheese whey served as substrate and helped microorganism to rely on it. Dairy cheese whey was a byproduct that containing lactose (0.036 – 0.048 g/mL) and was used as fermentation media to support microorganism growth during fermentation. Sugar was added to fermentation in order to obtain good yield of ethanol or to facilitate fermentation.

3.2. Methods

3.2.1. Characterization of Cheese Whey

Cheese whey was collected from dairy factory shola milk processing plant located in Addis Ababa capital city of Ethiopia and its components were characterized by analytical methods in laboratory before and after filtration. The main environmental quality determining parameters that had been characterized were chemical oxygen demand (COD), biological oxygen demand (BOD), total solids (TS), pH and the components of cheese whey that had been determined by milk analyzer (Lactoscan) at shola milk processing factory were lactose, protein, minerals, fats, and lactic acid. These compositions were determined in laboratory through analytical instruments and methods as following;

1. Fat content determination

Fat component of the cheese whey was determined by analytical instruments of Milkoscan or Lactoscan in shola milk processing laboratory.

2. Protein content determination

The total protein components of cheese whey also determined in laboratory at shola milk processing dairy factory with milk analyzer of Lactoscan.

3. Lactose determination

The sugar content of cheese whey was the main component of the whey that could be fermented into ethanol and it was determined by Lactoscan analytically in the laboratory at the factory. The cheese whey had high value of lactose which gave a good yield of ethanol by fermented with *kluyveromyces delphensis* yeast strain.

4. Mineral salts determination

Lactoscan analytical instrument was milk analyzer that could determine the salt components of cheese whey.

5. Total solid (Non - fat) determination

Milk analyzer was also had capacity to determine the solid matters in whey and it could be removed by centrifuge to avoid disturbance during fermentation.

6. Density determination

The density of cheese whey also determined by Lactoscan at 19.97^{o}C.

7. PH determination

The pH of cheese way was also determined by this instrument.

8. Ash content determination

The crucible was washed and dried in furnace at 550^{o}C for 15 minutes and taken out of furnace and put into desiccator. The mass of crucible was measured (w_1) and 5ml of whey was added into crucible and the sample mass was measured (w_3). The sample in crucible was preheated before it was taken into furnace for 20 minutes. The crucible contained sample was taken into furnace at 550^{o}C for 3 hours by using desiccator. Finally, the mass of crucible with sample was measure (w_2) and ash content was calculated.

3.2.2. Pretreatment of cheese whey

3.2.3. Filtration of cheese Whey

The cheese whey collected from dairy factory was filtered using vacuum pump and filter paper to remove suspended solids and residual lipids.

3.2.4. Deproteinization of cheese Whey

Hard cheese whey was normally deproteinized by heating the whey at 90^{o}C for fifteen minutes to denature the protein. The protein was removed in forms of flocculants during the sterilization of cheese whey at 90^{o}C with Autoclave. The protein coagulated was removed by filtration methods.

3.2.5. Hydrolysis of cheese Whey

Lactose hydrolysis was catalyzed enzymatically by heating in the presence of strong mineral acids, Sulphuric acid. For acid hydrolysis, it was necessary to lower the pH of the lactose solution to 2.3 and heated to approximately 140°C for fifteen minutes using Autoclave. This hydrolysis of the lactose made lactose sweetness and prevented lactose crystallization in concentrated whey syrups. Also, solubility of lactose was increased and viscosity of whey was decreased by Sulphuric acid hydrolysis.

3.2.6. Concentrating of cheese Whey

Cheese whey was concentrated by rotary vacuum evaporator at 105^{o}C. The initial concentration of lactose in whey was 4.5g/L. The first sample, 500ml of cheese whey containing lactose was concentrated 105^{o}C for 3 hours. The second 500ml of cheese whey sample was also concentrated at 105^{o}C for 5 hours.

3.2.7. Determination of sugar Concentration:

Total sugar determination included the determination of both reducing and non-reducing sugar concentration in different methods. The reducing sugar concentration was determined by titration method with benedict's solution.

3.2.8. Sugar concentration by Density meter

The total mass sugar concentration was determined by Refractometer and Density meter (Laboratory Density DMA 4100M Anton Paar GmbH, Anton – Paar str.20, A – 8054 Graz, AUSTRIA - EUROPE) in Bio – lab at AAiT.

The reducing sugar mass concentration was determined by titration method and the standard solution was prepared with different mass concentration of lactose and constant volume of distilled water. Five lactose standard solution was prepared with 5ml of distilled water. The mass of lactose (10, 20, 30, 40 & 50gm) was measured with electronic balance and 5ml distilled water was added into each test tubes (6-test tubes). The measured masses of lactose were added into each test tubes except one test tube (which was taken as control test tube) and the solutions were mixed by vortex mixer until the uniform solution was performed. Similar six test tubes were prepared and 5ml benedict's solution was added into each test tubes (6-test tubes) and 1ml of the prepared lactose standard solution was taken into test tubes contained benedicts solution respectively and mixed on vortex vibrator. The mixed solution was taken into water bath at 90 ^{o}C until color change was observed (for five minutes). The heated solution was taken out of water bath and cooled at room temperature for some minutes. Then, it was filtered by filter papers and its absorbance was measured at 600nm by Spectro UV-VIS Double Beam PC 8 Scanning Auto Cell UVD – 3200, Labomed, INC. Finally, the calibration curve was prepared and drawn.

3.2.9. Inoculums preparation:

There were different types of yeast used to ferment carbohydrates based on their sugar type. However, lactose was metabolized by *Kluyveromyces delphensis* yeast strain and it was maintained in 64g/l agar. 500ml Erlenmeyer flask was washed and sterilized with autoclave at 121^{o}C for 15 minutes. Then, 200ml distilled water was poured into 500ml empty Erlenmeyer flasks. Reagents(4gm of Nutrient broth , 3gm of Peptone and 2gm of Yeast extract) were measured and added into Erlenmeyer flask contained distilled water. The solution was shaken on heater to mix the reagents with distilled water and thus, it was sterilized at 121^{o}C for 15 minutes. The sterilized broth was cooled and the *kluyveromyces delphensis* yeast strain was transferred into Erlenmeyer flask contained broth under UV-visible light (cleaned Cabinet by Alcohol) to avoid contamination. Then, it was taken into incubator and maintained for three days in shaker at temperatures of 26^{o}C with velocity of 200rpm (for each flask). At complete growth, the *kluyveromyces delphensis* yeast strain cultured was preserved with microorganism conserver at 4^{o}C.

3.2.10. Ethanol fermentation from whey

The whey from dairy plant was cooled and filtered to remove solid matters by centrifuge. 1000mL Erlenmeyer flasks were washed and dried. 500mL of cheese whey substrate was poured into 1000mL Erlenmeyer flasks and supplements (1.5gm of Nutrient broth, 1.5gm of peptone, 1.5gm of NH_4Cl, 0.5gm of $MgSO_4.7H_2O$ and 1gm of KH_2PO_4) were added into flasks contained cheese whey substrate. The substrate mixture was preheated to mix properly and its pH was adjusted before sterilization. Then, it was sterilized at 121^oC temperature for fifteen minutes with Autoclave and the sterilized substrate was cooled and maintained under UV- light to avoid bacterial contamination. Inoculated kluyveromyces delphensis yeast strain was added into flask contained sterilized substrate under UV- light. Finally, it was taken into incubator for three days at different temperatures (27, 31 and 35 ^oC) and 200 rpm respectively. The initial lactose concentrations, yeast cell concentrations and ethanol concentrations were measured at 10 hours difference.

3.2.11. Yeast cell density Determination

The cell density was determined by counting cells colony forming units with microscope after the cell serial dilution and growth on plate were obtained. The colony forming units was counted by microscope for each broth (3 flask with different concentration).

3.2.12. Yeast cell viability determination

The *kluyveromyces delphensis* yeast strain health was determined by microscope with methylene blue standard solution. Both the live cell and dead cell were isolated.

3.2.13. Distillation

The distillation column setup was fixed. The beer from fermentation was added into flask and tied with condenser. The oil bath temperature was set to 78^oC and condenser temperature was set to 0^oC. The distillates was collected for one day and measured.

3.2.14. Ethanol characterization

The following characteristics of ethanol was determined:

1. Density: - ethanol density was determined by density meter in Bio- lab at AAiT.
2. Alcohol content at 20^oC & Color

3. Boiling point

4. Refractive Index

3.2.15. FT-lR determination of Cheese whey Bioethanol

The functional groups of cheese whey Bioethanol were determined by using prinks Elmer spectrum 65 FT-IR with the help of IR correlation charts in Addis Ababa University, 4kilo chemistry department. The IR spectrum was reported by % transmittance. The wave number region for the analysis was in the mid-infrared range.

3.2.16. Design of the Experiment

Data analysis was carried out by DESIGN EXPERT version 6.0.8 software (central composite design) to evaluate the effects of the process variables; Temperature (27^{o}C, 31^{o}C and 35^{o}C), Initial lactose concentration (4.5g/L, 6.15g/L and 7.8g/L), Yeast cell concentration(5 g/L, 7.5g/L and 10g/L) and pH (5, 5.5 and 7.5). Three points are used as center points and 24 points are not center points. A full factorial experimental design with 27 experiments were employed. The response variable was ethanol yield after fermentation. This design of the experiment helps us to optimize of process parameters using Response Surface Methodology (RSM).

Table 3. 1. Minimum, Center point and Maximum levels

Process variables	**Units**	**Minimum level**	**Central level**	**Maximum level**
Initial lactose conc.	g/L	4.5	6.15	7.8
Yeast cell conc.	g/L	5	7.5	10
Temperature	^{o}C	27	31	35
pH	-	5	5.5	7.5

4. RESULTS AND DISCUSSIONS

4.1. Evaluation of cheese whey Characterization

Biochemical oxygen demand (BOD) is the amount of dissolved oxygen (DO) consumed by microorganisms for the biochemical oxidation of organic (carbonaceous BOD) and inorganic matter (nitrogenous BOD) components of cheese whey. Cheese whey is consisted of lactose. Lactose is largely responsible for biochemical oxygen demand and chemical oxygen demand. Therefore, BOD was determined in order to investigate environmental polluting effects of cheese whey in laboratory. BOD was determined by BOD_5, $_{20oC}$ digester (incubator). Initial dissolved oxygen was recorded at the first day and final dissolved oxygen also measured at fifth day. Therefore, $BOD_{5,\ 20\ oC}$ = initial ($BOD_{1,\ 20\ oC}$) - final ($BOD_{5,\ 20\ oC}$) − − − − − − − − **(4.1**)

= 320 mg/mL – 90 mg/mL

= 230mg/mL

The determined BOD values of cheese whey indicates high contents of organic loading and thereby, cheese whey discharged to the environment can cause environmental problem.

Chemical oxygen demand (COD) was also determined by titration with ferrous ammonium sulfate reagents in laboratory and the obtained values of chemical oxygen demand (COD = 70 mg/mL) shows cheese whey discharged to environment before treatment has capability to pollute environment.

4.1.1. Cheese whey Composition

1. Fat content determination

Fat component of the cheese whey was determined by analytical instruments of Milkoscan or Lactoscan in shola milk processing laboratory and the fat measured was 0.026g in one milliliter of cheese whey.

2. Protein content determination

The total protein components of cheese whey also determined in laboratory at shola milk processing diary factory with milk analyzer of Lactoscan and the measured value was 0.024g in one milliliter of hard cheese whey.

3. Lactose determination

The sugar content of cheese whey was the main component of the whey that could be fermented into ethanol and it was determined by Lactoscan analytically in the laboratory at the factory. The cheese whey had high value of lactose which gave a good yield of ethanol by fermented with *kluyveromyces delphensis* yeast strain as well as the lactose determined was 0.036 – 0.048g per milliliter of cheese whey.

4. Mineral salts determination

Lactoscan analytical instrument was milk analyzer that could determine the salt components of cheese whey and the determined value of mineral salt was 0.005g in one milliliter of whey and it was less content of whey.

5. Total solid (Non - fat) determination

Milk analyzer was also had capacity to determine the solid matters in whey and the determined value was 0.0065g in the milliliter of whey and it could be removed by centrifuge to avoid disturbance during fermentation.

6. The density of cheese whey also determined by Lactoscan at 19.97^{o}C and it was 1001.9 Kg/m^{3}

7. The pH of cheese way was also determined by this instrument and the cheese whey pH was 4.24. It was shown acidic properties.

8. Ash content determination

Calculation of Ash content:

$$\text{Ash} = \frac{\text{mass of crucible with sample} - \text{mass of empty crucible}}{\text{mass of sample}} \; x \; 100\ \% - - - - - - - - - - (\mathbf{4.2})$$

$$Ash\ content = \frac{w2 - w1}{w3}\ x\ 100\ \% \quad ----------(\mathbf{4.3})$$

$$Ash\ content = \frac{29.3301 - 29.3253}{4.7486}\ x\ 100\ \%$$

Ash content = 0.101 %

Whereas; W_1 is mass of empty crucible

W_2 is mass of crucible with sample;

W_3 is mass of total sample

Generally the measured composition of unconcentrated cheese whey were listed in the table 4.1 below. The density of cheese whey determined at 19.97^{o}C was 1001.9 kg/m^3 and the pH of cheese whey was 4.24.

Table 4. 1. Composition of Cheese Whey

NO	**Constituents of shola milk cheese whey**	**Cheese whey mass in (gm)**	**Percentage composition of cheese whey (%)**
1	Water	0.962	90
2	Lactose	0.036 – 0.048	3.36 – 4.48
3	Protein	0.024	2.24
4	Fat	0.026	2.43
5	Total solid (Non - fat)	0.0065	0.61
6	Mineral salts	0.005	0.47
7	Ash	0.0048	0.101

Cheese whey was collected from shola milk processing factory in Addis Ababa and its physiochemical properties was determined analytically to estimate compositions of cheese whey as well as to know possibilities of product to be obtained from dairy wastes. Since the dairy waste cheese whey has been consisted of lactose (4.48%), protein (2.24%) and fats (2.43%), it is possible to produce ethanol, protein cell, glucose, lactic acid and liquid fertilizers. However, since the amounts of lactose is higher than protein cheese whey could good ethanol rather than other expected products. As shown in figure, the maximium components of cheese whey was water because in nature milk consisted of high water component. As a result, the amount of lactose could be concentrated by evaporating this water and hydrolyzing to increase the lactose sweetness or solubility.

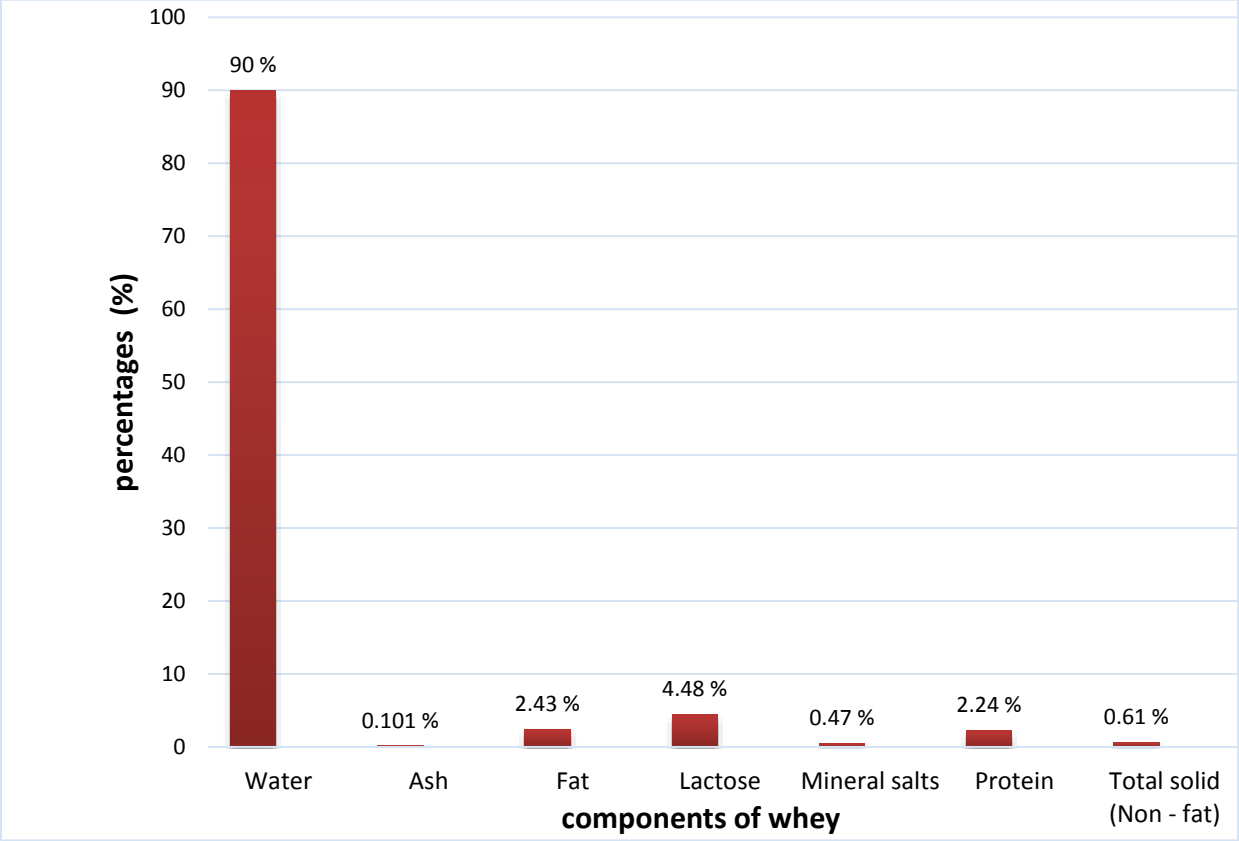


Fig 4. 1. Percentage Composition of Cheese Whey with Bar Graph

4.2. Deproteinized cheese Whey

The cheese whey collected from dairy factory was filtered using vacuum pump and filter paper to remove suspended solids and residual lipids. Hard cheese whey was normally deproteinized by heating the whey at 90^{o}C for fifteen minutes to denature the protein. The protein was removed in forms of flocculants during the sterilization of cheese whey at 90^{o}C with Autoclave. The protein coagulated was removed by filtration methods.

4.3. Hydrolyzed cheese Whey

Lactose hydrolysis was catalyzed enzymatically by heating in the presence of strong mineral acids, Sulphuric acid. For acid hydrolysis, it was necessary to lower the pH of the lactose solution to 2.3 and heated to approximately 140°C for fifteen minutes using Autoclave. This hydrolysis of the lactose made lactose sweetness and prevented lactose crystallization in concentrated whey syrups. Also, solubility of lactose was increased and viscosity of whey was decreased by Sulphuric acid hydrolysis.

4.4. Concentrated cheese Whey

Cheese whey was concentrated by rotary vacuum evaporator at 105^{o}C. The initial concentration of lactose in whey was 4.5g/L. The first sample, 500ml of cheese whey containing lactose concentrated to 6.15g/L at 105^{o}C for 3 hours. The second sample was also contained 500ml of cheese whey was concentrated to 7.8g/L at 105^{o}C for 5 hours.

4.5. Determination of sugar for cheese whey

Total sugar determination included the determination of both reducing and non-reducing sugar concentration in different methods. The reducing sugar concentration was determined by titration method with benedict's solution in AAiT in bio lab. Non – reducing sugar also determined by calculating total sugar with density meter in bio lab in AAiT.

4.5.1. Sugar concentration determination by Density meter

The total mass sugar concentration was determined by Refractometer and Density meter (Laboratory Density DMA 4100M Anton Paar GmbH, Anton – Paar str.20, A – 8054 Graz, AUSTRIA - EUROPE) in Bio – lab at AAiT.

Table 4. 2. Initial and Concentrated Sugar Concentration (at 105^{o}C for different time) by Density meter

No.	Types of Parameters determined	Initial Lactose concentration	Concentrated lactose @ T=105^{o}C	
			3 hours	5 hours
1	Mass sugar concentration	4.5g/L	6.15 g/L	7.8 g/L
2	Brix	2.37^{o}Brix	2.37^{o}Brix	2.37^{o}Brix
3	Density	1001.9 kg/m^3	1001.9 kg/m^3	1001.9 kg/m^3

4.5.2. Reducing sugar determination

The reducing sugar mass concentration was determined by titration method and the standard solution was prepared with different mass concentration of lactose and constant volume of distilled water. Five lactose standard solution (2, 4, 6, 8 & 10 g/L) was prepared with distilled water.

Table 4. 3. Concentration of Lactose Vs Absorbance

No	Concentration of lactose in g/L	Absorbance @ 600nm
1	2	0.250
2	4	0.501
3	6	0.595
4	8	0.770
5	10	0.785

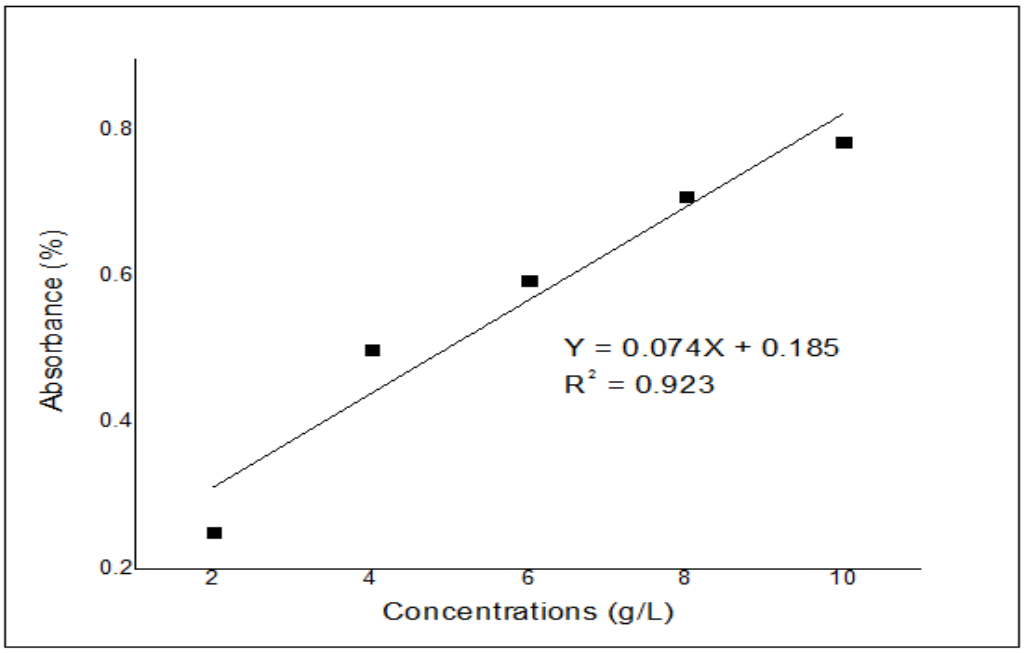


Fig 4. 2. Graph of Concentration of Lactose (in gram per liter) Vs Its Absorbance (in %)

The concentration of total reducing sugar in the hydrolyzed whey was obtained from the calibration plot in the above and expressed in gm/ml in table above. The maximium reducing sugar was 7.8g/L determined by calibration curve. The concentration of reducing sugar was calculated from calibration curve using line equation $y = mx + b$ ---------- (**4.4**)

Whereas; - y is absorbance

- x is concentration

- m is slope

- b is y-intercept

As a result the concentration of reducing sugar was determined by the following formula;

$$\text{Concentration} = \frac{\text{Absorbance} - \text{intercept}}{\text{slope}} \text{ ---------- } (\mathbf{4.5})$$

Note; slope (m) = 0.074 % / (g/L), intercept (b) = 0.185 %

Table 4. 4. Calculated Reducing Sugar Concentration from Calibration Curve.

Run	Volume of whey((ml)	Absorbance (%)	Concentration lactose (g/L)	Mass of fermentable lactose (gm)
1	500	0.5180	4.5	2.25
2	500	0.6401	6.15	3.075
3	500	0.7622	7.8	3.9

4.6. Evaluation of Inoculum

Preculture was started from single colonies obtained from Ethiopian biodiversity institutes at Addis Ababa and it was precultured in bio lab at AAiT. It was grown on nutrient broth in liquid form at different concentration with Erlenmeyer flasks (3 flasks).

The first flask was used to culture *kluyveromyces delphensis* yeast strain from 4gram of nutrient broth, 3gram of peptone, 2 gram of yeast extract, 200ml distilled water and 0.5ml *kluyveromyces delphensis* yeast strain in suspension form. It was resulted 9.98 gm/L yeast cell suspension.

The second flask also used to preculture the yeast strain from 3gram of nutrient broth, 2gram of peptone, 1 gram of yeast extract, 125ml distilled water and 0.2ml *kluyveromyces delphensis* yeast strain in suspension form. This flask resulted 7.51gm/L *kluyveromyces delphensis* yeast strain.

The third flask used to inoculate the yeast strain from 2gram of nutrient broth, 2gram of peptone, 1 gram of yeast extract, 125ml distilled water and 0.15ml *kluyveromyces delphensis* yeast strain in suspension form. 5.1gm/L concentration of *kluyveromyces* yeast strain suspension was obtained.

All flasks were covered with cotton to provide air for yeast strain and it was incubated at 26^{o}C in incubator shaker with 200rpm agitation for 72 hours.

4.6.1. Studied cell density and viability

Dry cell mass concentration was estimated by measuring the optical density of the sample at 600nm in spectrophotometer (Spectro UV-VIS Double Beam PC 8 Scanning Auto Cell UVD – 3200, Labomed, INC). The cell health was determined by methylene blue staining technique 0.2mL sterile solution of methylene blue (3.3mM in 68mM sodium citrate) was mixed with 0.2mL of *kluyveromyces delphensis* yeast cell suspension diluted to reach an optical density 600nm 0.4

to 0.8. The mixture was shaken and taken onto slide specimen to be counted after five minutes of incubation. The numbers of stained with methylene blue was dead cell (inactive) and the one that was unstained was live cell (active). The ratio of viable cell was the numbers of live cell divided by the total numbers of cells.

4.7. Fermentation of cheese whey

The batch fermentation was performed in 1000mL Erlenmeyer flasks covered with cotton and aluminum foil to prevent air. Cheese whey was used as substrate and thereby, kluyveromyces delphensis yeast decomposed lactose into simple sugars glucose and galactose and then after, into ethanol. Nutrients were initially added as supplements to enhance the yeast activities. However, the fermentation was carried out accordingly at 27^{o}C, 31^{o}C and 35^{o}C. Since the temperature affected the yeast cell growth, the fermentation of ethanol had been affected during decomposition of lactose into ethanol. As a result, the temperature was controlled and the yeast activities were high at 27^{o}C. The substrate conditions also affected the conversion of lactose into ethanol and the media condition considered were 5, 5.5 and 7.5. Consequently, *kluyveromyces delphensis* yeast cell activities were maximium at pH value of 5.5.

Therefore, the initial lactose concentration also affect the fermentation process. As initial lactose concentration increased ethanol production increased and yeast cell consume high lactose into their cell. This develop cellular osmotic pressure in the cell and the cell was be killed. Consequently, the amount of ethanol produced and the numbers of cell decreased as well. So that high amount of initial lactose concentration highly affected specific growth rate of cell, lactose consumption rate and ethanol production rate. In case, initial lactose concentrations was controlled to the rages of 4.5, 6.15 and 7.8 g/L. Not on this, yeast cell concentration also affect ethanol production rate because if the concentrations of cell is high the interaction of cells limits the access to the sugar and create less favorability to the conditions. It is also important to identify types of yeast strain used and why it is selected. In this study *kluyveromyces delphensis* yeast strain was used because it has the enzymes that decompose lactose into small sugar molecules and ferment it into ethanol.

The concentration of yeast cell obtained from inoculum cultured 5, 7.5 and 10 g/L were considered as significant factors and controlled to optimize ethanol produced.

4.8. Evaluated effect of fermentation process variables (Initial Lactose concentration, Yeast cell concentration, Temperature and pH) with Experimental design expert

The experimental design study consisted of the central composite design (CCD) aimed at determining the effects of 4 factors on the fermentation process and measuring the variation of ethanol, lactose and biomass concentration with time.

Four factors were considered to perform for response surface methodology of CCD: pH, initial lactose concentration (L), yeast cells concentration (Y) and temperature (T), with three different levels for each of the factors. The values of the chosen factors were 5 and 7.5 for pH, 4.5 and 7.8g/L for initial lactose concentration, 5 and 10 g/L for yeast cells concentration and, 27 and 35^{o}C for temperature. The range of these values was considered since it characterized the optimum range for the yeast activity and the expected range in which the process could be operated. In this study, the experimental design consisted of 27 runs and the independent variables were studied at three different levels. Table 4.5 shows the experimental design used for this study. All the experiments were done in duplicates and the average of ethanol production obtained was taken as the response function (RF). The Second degree polynomials, Equation (4.8), which contains all interaction terms, were used to calculate the predicted response:

Table 4. 5. Experimental Design and CCD Results of Response Surface Methodology.

Run	Factor-1 A: Lactose	Factor-2 B: yeast cell	Factor-3 C: Temperature	Factor-4 D: pH	Response Ethanol yield(mL)
1	6.15	10.00	31.00	7.50	5
2	4.50	10.00	27.00	7.50	9.11
3	7.80	10.00	35.00	5.50	5.5
4	4.50	10.00	35.00	5.50	3
5	7.80	5.00	27.00	5.50	15.3
6	6.15	7.50	31.00	5.00	9.5
7	4.50	5.00	35.00	5.50	7.8
8	4.50	5.00	35.00	7.50	10.5

9	6.15	7.50	31.00	5.00	9.21
10	7.80	5.00	35.00	7.50	12.5
11	7.80	5.00	27.00	5.00	12.5
12	6.15	7.50	31.00	5.00	7
13	7.80	10.00	27.00	5.50	15.5
14	6.15	7.50	31.00	5.50	8.75
15	7.80	10.00	35.00	7.50	8
16	7.80	7.50	31.00	5.00	7.5
17	6.15	7.50	27.00	5.00	11.5
18	6.15	5.00	31.00	5.00	6
19	7.80	5.00	35.00	5.50	12.5
20	4.50	5.00	27.00	7.50	9.4
21	4.50	10.00	35.00	7.50	7.9
22	4.50	5.00	27.00	5.50	10.2
23	7.80	10.00	27.00	7.50	11
24	4.50	10.00	27.00	5.50	10.5
25	4.50	7.50	31.00	5.00	6
26	6.15	7.50	31.00	5.00	4.5
27	6.15	7.50	35.00	7.50	15

4.9. Statistical analysis of the experimental results with ANOVA

The Model F-value of 7.67 implies the model is significant. There is only a 0.13% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, B^2, C^2, D^2, BC and CD are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Response: Response

ANOVA for Response Surface Quadratic Model

Table 4. 6. Analysis of Variance Table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	245.75	14	17.55	5.92	0.0019	significant
A	37.29	1	37.29	12.58	0.0040	
B	25.87	1	25.87	8.73	0.0121	
C	49.92	1	49.92	16.83	0.0015	
D	19.27	1	19.27	6.50	0.0255	
A2	0.086	1	0.086	0.029	0.8677	
B2	20.78	1	20.78	7.01	0.0213	
C2	20.69	1	20.69	6.98	0.0215	
D2	14.63	1	14.63	4.94	0.0463	
AB	0.91	1	0.91	0.31	0.5900	
AC	1.09	1	1.09	0.37	0.5563	
AD	3.30	1	3.30	1.11	0.3125	
BC	19.64	1	19.64	6.62	0.0244	
BD	0.48	1	0.48	0.16	0.6947	
CD	19.54	1	19.54	6.59	0.0247	
Residual	35.58	12	2.97			
Lack of Fit	19.42	9	2.16	0.40	0.8742	Not significant
Pure Error	16.16	3	5.39			
Cor Total	281.34	26				

The regression coefficients and the corresponding 95% CI (Confidence Interval) High and Low were presented in table 4.7 below. If zero was in the range High and Low 95% Confidence interval, the factors has no effect. From the 95% CI High and Low values of each model term, it could be concluded that the regression coefficients of initial lactose concentration, yeast cell concentration, temperature, pH, the interaction terms of yeast cell concentration and temperature, and the interaction terms of temperature and pH have highly significant effect in fermentation process for ethanol production.

Table 4.7. Regression Coefficients Estimates and Analysis of Variance (ANOVA) for the Response Surface Quadratic Model.

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	11.87	1	1.90	7.74	16.01	
A-Lactose	1.51	1	0.43	0.58	2.44	1.10
B-Yeast cell	-1.37	1	0.46	-2.38	-0.36	1.30
C-Temp	-1.90	1	0.46	-2.91	-0.89	1.30
D-pH	2.45	1	0.96	0.36	4.54	9.80
B^2	-3.26	1	1.23	-5.94	-0.58	3.06
C^2	3.25	1	1.23	0.57	5.93	3.06
D^2	-2.35	1	1.06	-4.65	-0.045	6.92
BC	-1.18	1	0.46	-2.19	-0.18	1.14
CD	1.18	1	0.46	0.18	2.18	1.50

The response function (RF) of the yield was determined by the formula derived from the regression coefficients. **General Model Equation was**;

RF= $11.87 + 1.51*A - 1.37*B - 1.90*C + 2.45*D - 3.26*B^2 + 3.25*C^2 - 2.35*D^2 - 1.18*BC + 1.18*CD$ ———————————————————————————————————(**4.6**)

The significant terms, as shown in Table 4.7, are A, B, C, D, B^2, C^2, D^2, BC and CD. Then, by eliminating the other terms from the model the regression equations was obtained as a function of the significant factors.

Table 4. 8. Evaluation of Model Adequacy

Std. Dev.	1.72	R-Squared	0.8735
Mean	9.30	Adj R-Squared	0.8735
C.V.	18.51	Pred R-Squared	0.4866
PRESS	144.44	Adeq Precision	8.856

Regression coefficient (R^2) quantitatively estimates the correlation between experimental data and predicted surface response. Results of R^2 = 0.8735 and Adj-R^2 = 0.8735 acquired explicates that the predicted values were found in respectable agreement with experimental values. Since the regression coefficient R^2 is closed to 1.0 it implies that the regression line perfectly fits the data. This indicates the achievement of response surface methodology. The adjusted regression coefficient (Adj-R^2) was also satisfactory for approving the importance of the model. Pred R-Squared indicating that the model will probably explain a high value yield of the variability in new data. "Adeq precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this study 8.856 indicates an adequate signal.

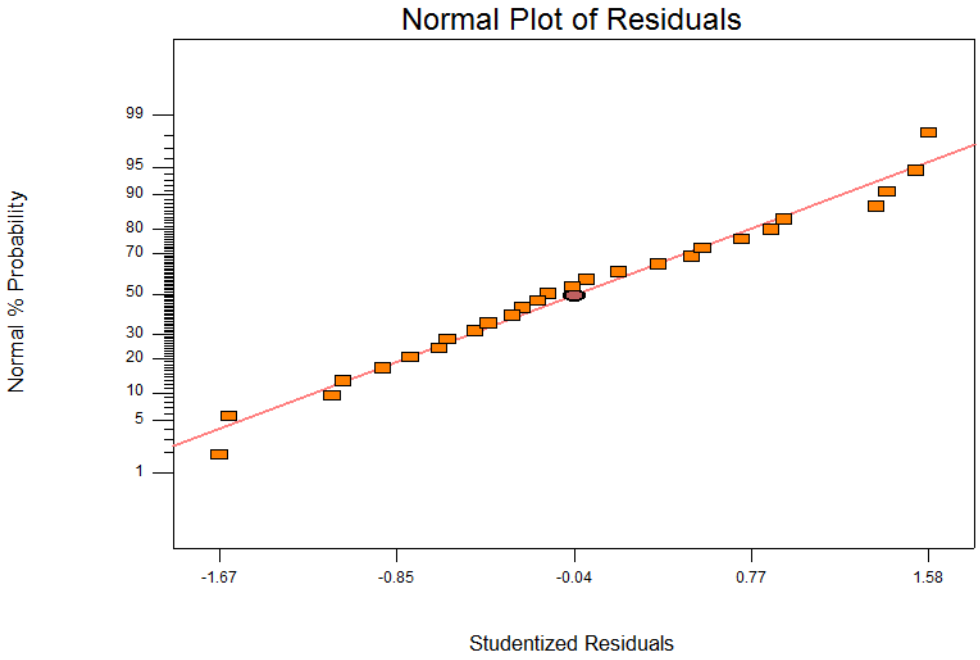


Fig 4. 3. Normal Plots of Residuals

The above plot of residuals implies the normal probability plot. In this experiment, the experimental data points in plots fits the straight line equation. As a result, the quadratic polynomial model satisfies the assumption of analysis of variance (ANOVA) which means the distribution of error was approximately normal.

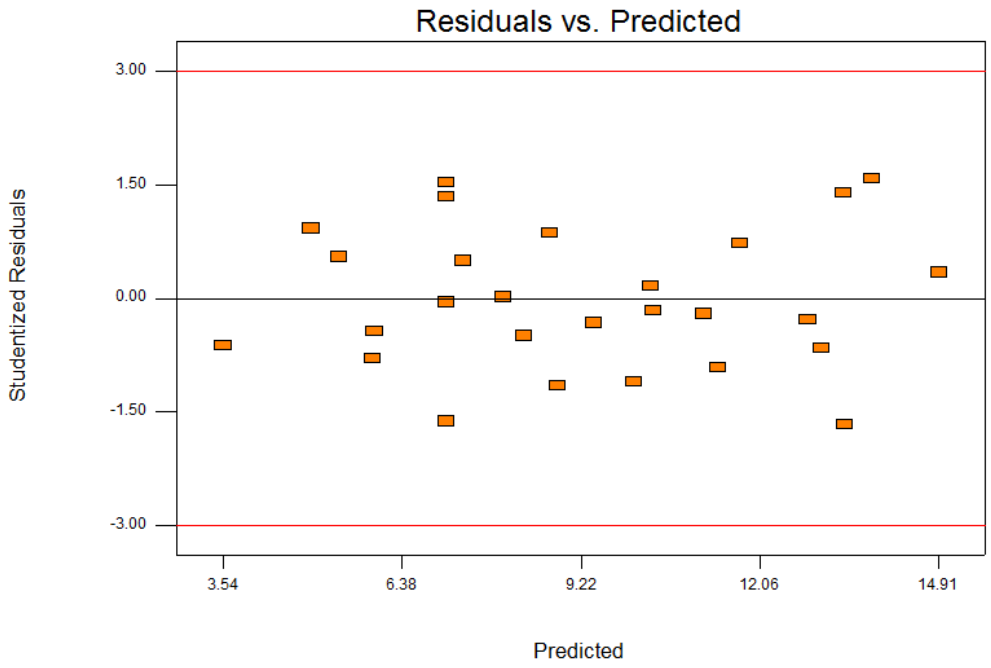


Fig 4. 4. Residuals Vs Predicted Values

The residuals Vs predicted values were indicated in the above figure and their assumptions were checked. If the model is corrected and the assumed values are satisfied, the residual could be structured less and accordingly, it could be unrelated to any others variance including the predicted values. The plots of residuals Vs predicted values implies the assumption of constant variance.

4.9.1. Response Surface Methodology (RSM) and Counter Plots

In order to evaluate the regression model equation, two dimension counter plots and 3D surface were attained by scheming the response on the Z axis against two variables while maintaining other variables at zero level. Some plots were selected to analyze the change in response surface. Half cylindrical shape response surface plots indicate the optimum operating conditions.

The optimized response value for bioethanol production from cheese whey during fermentation process was based on four process variables showed on response surface methodology (RSM). The response function was investigated in figures 4.5 – 4.6 and it shows the effect and interaction of the factors on the on response function. The response surface performance as a function of both yeast cell concentration and initial lactose concentration. A relatively weak effect of initial lactose concentration and a stronger effect of yeast cell concentration could be noted. The best conditions were achieved at yeast cell concentration 10g/L and lactose concentration 6.15g/L. As lactose

concentration increased beyond level 7.8g/L, the response function was decreased as a result of cellular osmotic pressure limiting. The response surface versus temperature and initial lactose concentration. It strengthens the convictions that cheese whey fermentation process is enhanced by relatively low temperature values. Factor initial lactose concentration has weak effect on the response functions even though better results were achieved with 6.15g/L value of lactose, about 15.5mL. It shows the response surface function as a function of both pH and initial lactose concentration slightly weak effect of initial lactose concentration and a stronger effect of pH can be investigated. The best conditions were achieved at pH 5.5 and initial lactose concentration 6.15g/L.

Figure 4.5 indicates that strong response surface dependence on both yeast cell concentration and temperature. Moreover, a good system behavior corresponding to a response function of 15.5mL is obtained at yeast cell concentration 10g/L and temperature 27^{o}C. Temperature is a key factor that mainly affect the growth rate of yeast strain and the interaction of both factors is high. It also exhibits the effects of pH and yeast cell concentration on ethanol fermentation from cheese whey and their response function was noted well. The interaction of the factors was also investigated in a good manner. The pH of the substrate highly affected the growth rate of kluyveromyces delphensis yeast strain.

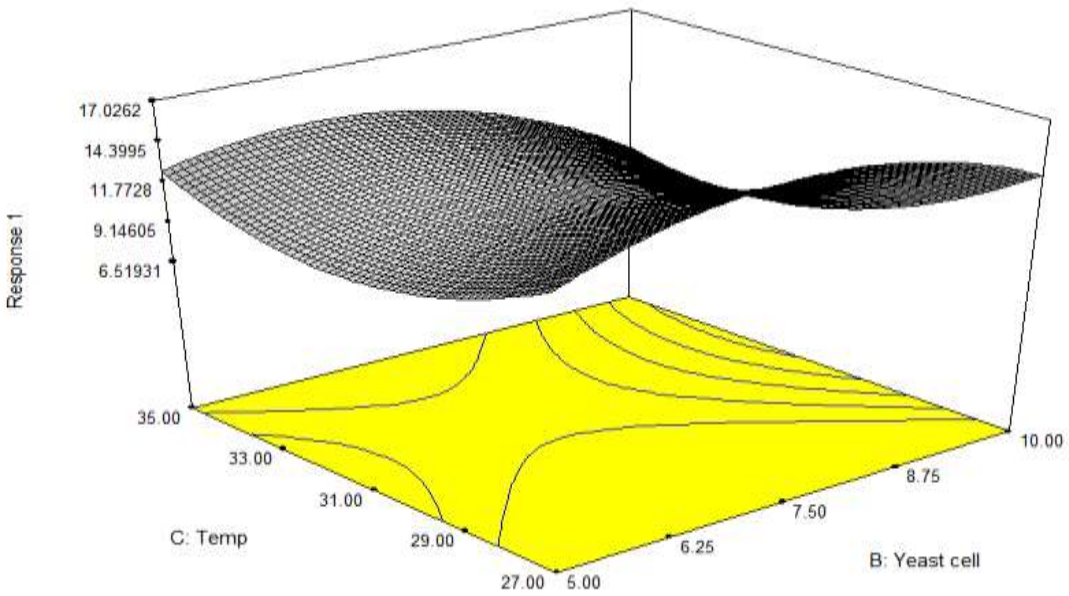


Fig 4. 5. Three D and Counter Plot Temperature Vs Yeast Cell Concentration.

Figure 4.6 shows the effect of pH and temperature on ethanol yield. The maximium ethanol yield was achieved at pH 5.5 and temperature 27ºC. Both factors are considered due to their effects on the growth rate of kluyveromyces yeast strain. However, the interaction of both factors on each other is nothing.

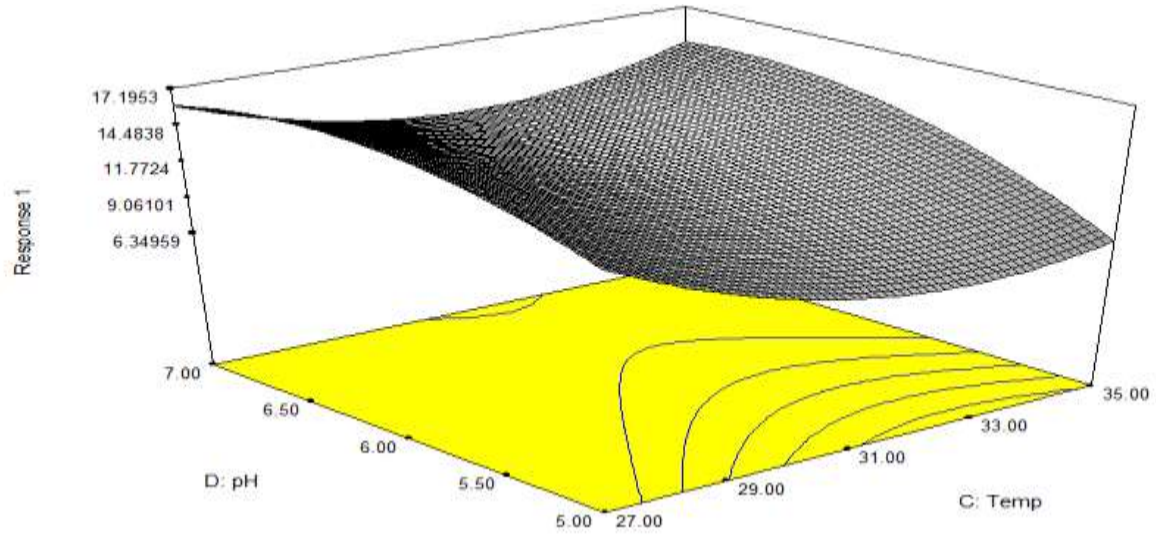


Fig 4. 6. Three D and Counter Plot pH Vs Temperature.

Generally, the cumulative effect of the factors considered in design was high and ethanol yield was mainly depend on their interactions. Specially, interaction effects of temperature and yeast cell is high. The temperature affect highly the specific growth of yeast cell which definitely influence the ethanol production rate. The media condition also affect specific growth of yeast cell and ethanol production rate.

4.9.2. Individual effect of fermentation process variables

4.9.2.1. Effect of Initial Lactose concentration

The lactose is a substrate for the yeast and as initial lactose concentration increased, the response was incredibly increased. The initial lactose concentration was varied at 4.5g/L, 6.15g/L and 7.8g/L. As a result, the effect of lactose concentration on growth of yeast and ethanol production was investigated as follows in figure 4.7. The yield of ethanol was highly increased as initial lactose concentration increased from 4.5g/L to 7.8g/L. However, high concentration of lactose had an inhibitory effect on the specific growth rate of yeast, lactose utilization rate and ethanol production rate. Inhibition of ethanol yield was pronounced at higher initial lactose concentrations

because cellular osmotic pressure was developed in the cell. In this study, minimum cell concentration was influenced by initial lactose concentration as well as by ethanol concentration. In spite of good initial lactose concentration variation was selected for this study.

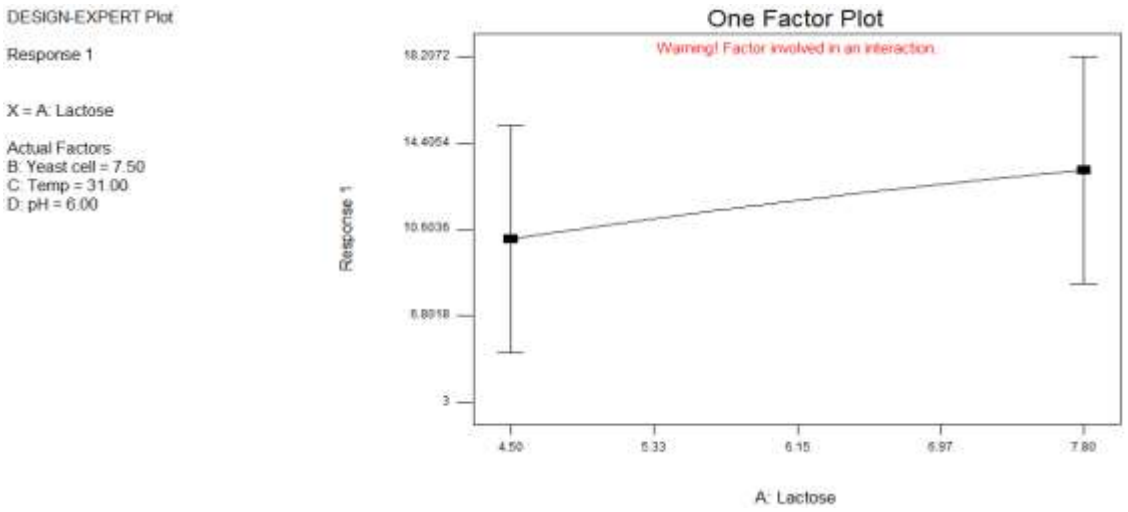


Fig 4. 7. Effect of Initial Lactose Concentration on Ethanol Yield during Fermentation

4.9.2.2. Effect of yeast cell concentration

Figure 4.8 represented effect of yeast cell concentration on ethanol yield at constant initial lactose concentration, temperature and pH. The yeast cell concentration variables considered in this study were 5g/L, 7.5g/L and 10g/L. As yeast cell concentration increased, the ethanol yield released was symmetrically decreased because at very high yeast cell concentrations, conditions for growth and metabolism are less favorable due to hindered access to nutrients, space limitations, and cells interactions.

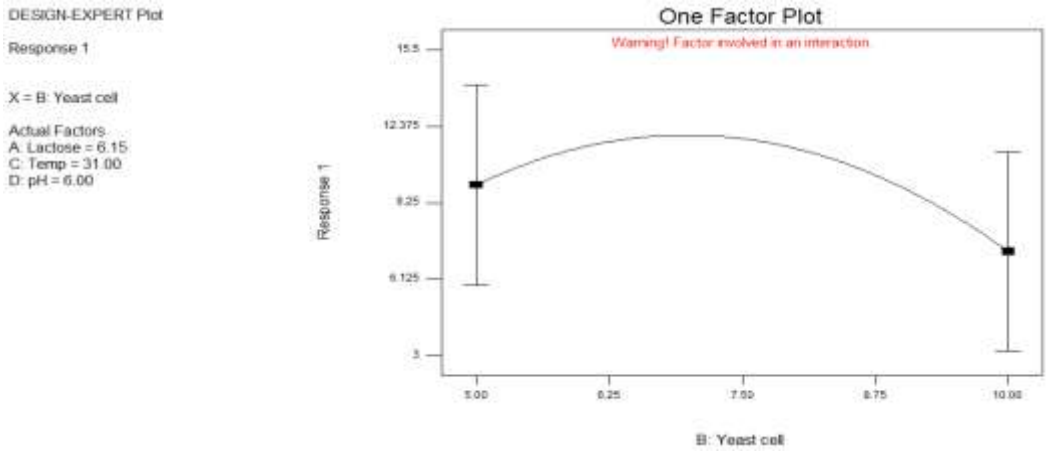


Fig 4. 8. Effect of Yeast Cell Concentration on Fermentation Process

4.9.2.3. Effect of Temperature

The effect of temperature on ethanol yield and yeast cell growth rate was explored and showed in figure 4.9. The temperature effect was incredibly investigated in this study because it affected mainly the growth rate of yeast which indirectly influence ethanol released from fermentation process. The variable temperature was kept between 27^{o}C and 35^{o}C for this study. *Kluyveromyces delphensis* yeast strain was sensitive to temperature ranges and its' growth rate was optimum to the temperature 27^{o}C. As temperature range increased from 27^{o}C to 35^{o}C, the ethanol yield released from fermentation process was suddenly decreased because high temperature range affect the growth rate of yeast. As a result, the temperature was considered as process variable to optimize ethanol yield.

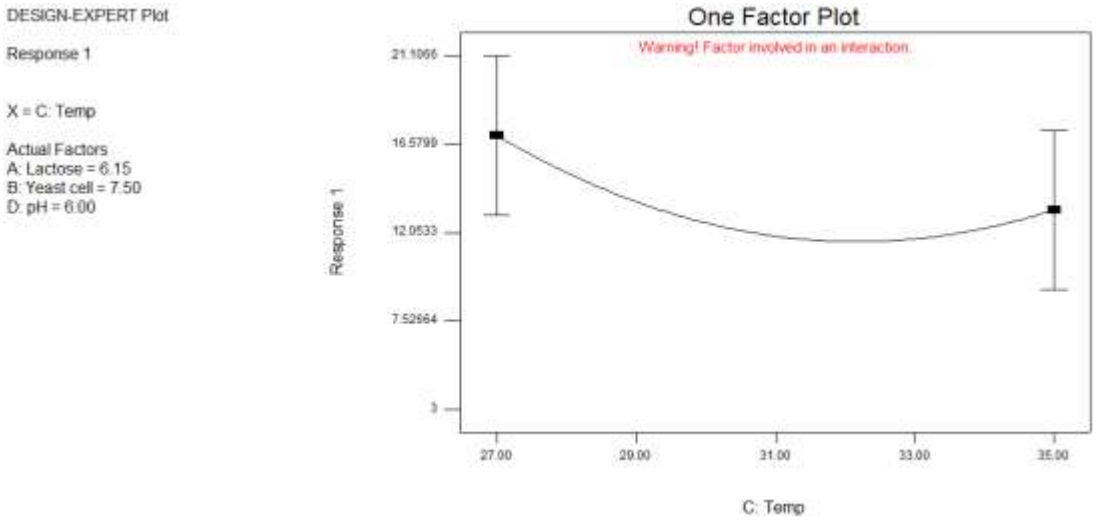


Fig 4. 9. Effect of Fermentation Temperature on Ethanol Yield

4.9.2.4. Effect of pH

For the regulated operation of bioreactors using immobilized yeast cells it is very important to know the precise effect of pH of the fermentation medium on cell growth and ethanol production. As shown in figure 4.10 below the yield of ethanol was affected slightly by pH. As the pH values increased from 5 to 7 the ethanol yield was somewhat increased. Beyond pH value 6.5, the yield of ethanol was duly decreased because high pH affect the condition of media and producing inhibitor molecules for the fermentation process.

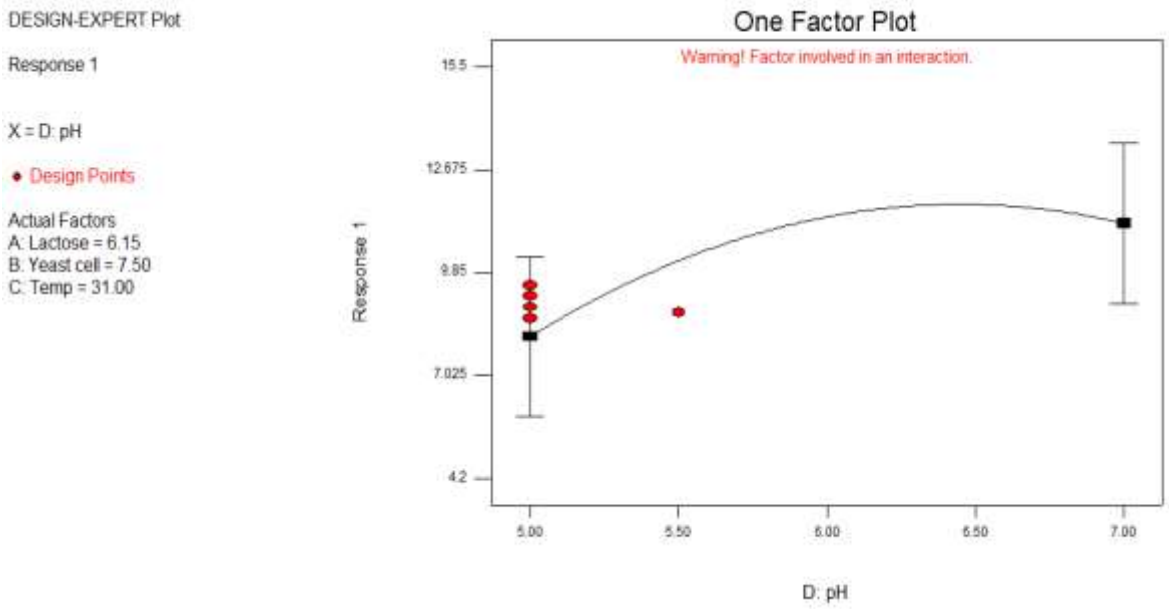


Fig 4. 10. Effect of pH on Ethanol Yield.

4.9.3. Optimizing of process variables for response surface methodology

Optimization of fermentation variable was performed at significant factors. The response surface methodology (RSM) showed that the best set of operating conditions as following; pH = 5.5, lactose = 6.15g/L, yeast = 10g/L and temp = 27^{o}C, with predicted values of the RF of 15.5mL (the optimization was strictly performed in the considered range of the factors). At this optimum conditions maximum ethanol was produced and high ethanol yield was 20.4% in terms of significant factors. Consequently, it is important check desirability from experimental design and thereby, the obtained desirability is 1 (desirability = 1). It means that the values of significant factors selected for the optimization has no effect on ethanol yield. Therefore, at this optimum point, initial lactose concentration, yeast cell concentration, temperature and pH have no effect on specific growth rate of yeast cell, lactose consumption rate and ethanol production rate.

From figure 4.11, the initial lactose concentration added was decreased as consumption rate increased and number of cell increased. The fermentation was carried over 72 hours and lactose was consumed during its decomposition. In the first ten hours, less consumption of initial lactose concentrations was occurred because the cells do not adapt the new condition of media.

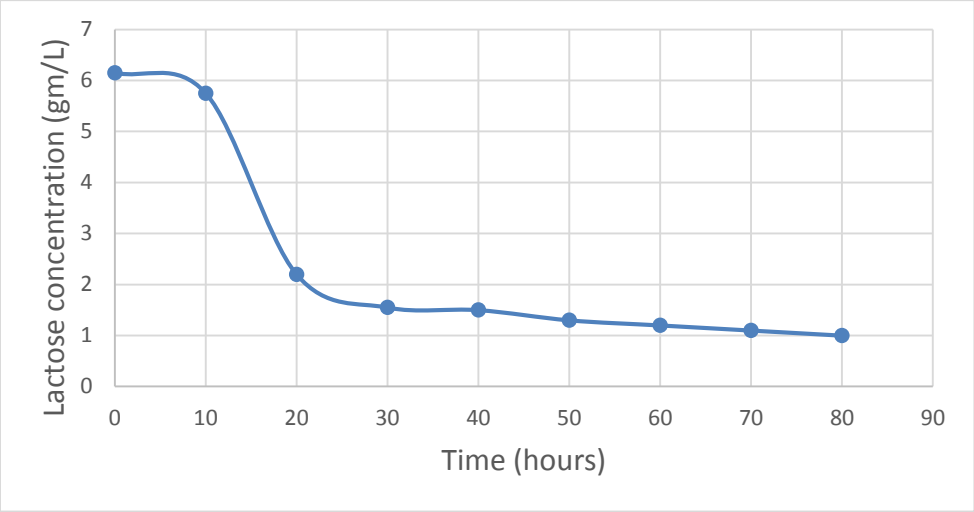


Fig 4. 11. Initial Lactose Concentration Consumption Rate

The specific growth rate of the *kluyveromyces delphensis* yeast strain had stages. In the first 20 hours the cell was grown slowly because the media conditions were new for the cell and it was adaptation period at this stage. The growth rate of this cell was almost linear from 20 – 45 hours and its specific growth rate was high at this stage. From 50 – 65 hours the specific growth rate of the cells was almost constant because of maximum specific growth rate of the cell was achieved. After 70 hours the growth rate decreased because of very low concentrations of sugar or no sugar.

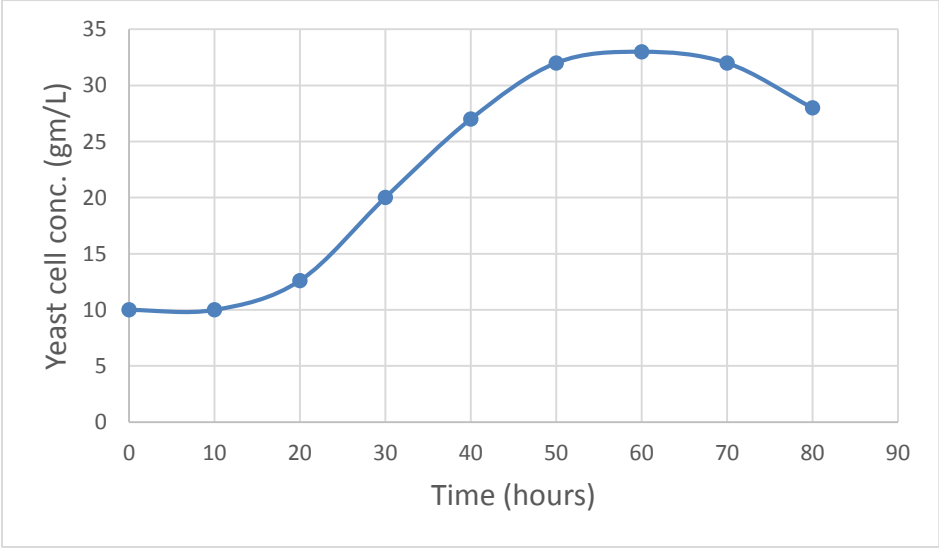


Fig 4. 12. Yeast Cell Growth Rate

The ethanol production rate was increased during the consumption rate of initial lactose concentrations and specific growth rate of the cell increased. However, after maximum specific growth rate was achieved, ethanol production rate was decreased because specific growth rate of the cells was decreased due to low sugar concentrations and maximum ethanol accumulations in the cells.

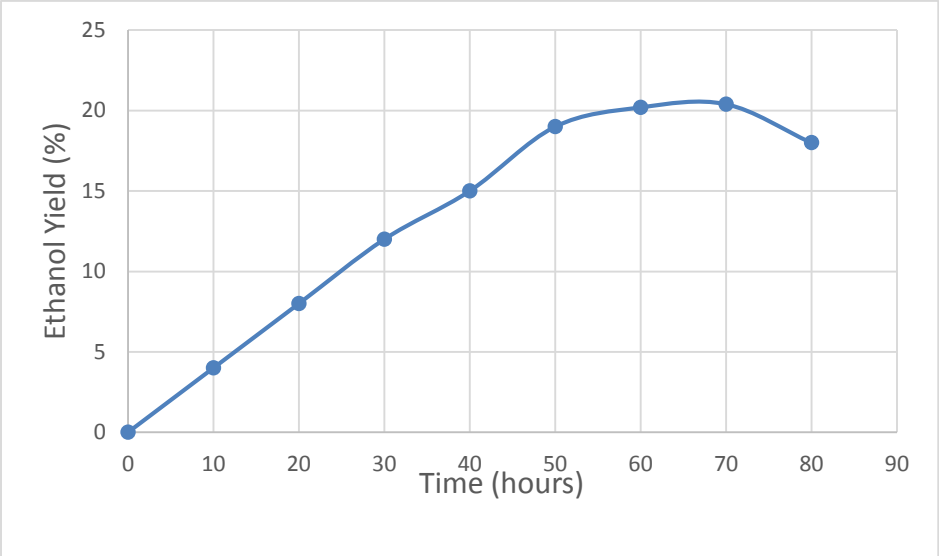


Fig 4. 13. Ethanol Production Rate

Due to large difference observed in the ethanol production, a statistical analysis was carried out to identify the variables that had the greatest influence on this fermentation process. Ethanol production mainly affected by initial lactose concentration, yeast cell concentration and temperature. Initial lactose concentration is a significant factor and it has an effect on ethanol yield. Higher ethanol production by yeast cell was obtained as the initial lactose concentration was increased. However, initial lactose concentration consumption increase as rate of ethanol production increase. Figure 4.11, shows that initial lactose concentration added was decreased as rate of its consumption increased and ethanol production rate increased. Yeast cell growth rate also increased as rate of lactose consumption increased. However, the growth rate of yeast at lag phase was activated with supplements in order to adapt the new environment. Maximium ethanol yield obtained was 20.4% (15.5 mL ethanol / 500 mL cheese whey substrate) at 6.15 gm/L initial lactose concentration and 10 gm/L yeast cell concentration.

4.10. Ethanol yield

Cheese whey is a good substrate to produce ethanol. Good ethanol yield was obtained from cheese whey fermentation by using *kluyveromyces delphensis* yeast strain. The ethanol yield is calculated by the following formula.

Ethanol yield (%) = [(amount of ethanol/amount of fermentable sugar)/0.51] *100.

Density of ethanol mixture distilled was determined by density meter in lab and it is 0.976 gm/mL. However, density of pure ethanol is 0.789gm/mL

4.11. Ethanol properties analyzed

Density of ethanol was determined by density meter in the lab and the obtained density of bioethanol is 0.976mg/mL. The boiling point of ethanol is also determined and it is 78^{o}C. Total brix was also determined by Refractometer in the lab and zero brix was recorded for this study. Ethanol is generally colorless. Alcohol contents of ethanol at 20^{o}C was determined by density meter in laboratory and thereby, the recorded average alcohol contents ethanol from cheese whey is 2.62 % (v/v).

From the above table 4.9, maximium ethanol yield determined was 20.4%. Cheese whey is a diary waste. Ethanol yield was calculated from the fermentable sugar and the amount of ethanol produced from cheese whey. Maximium theoretical ethanol yield was calculated from the chemical reaction and decomposition or metabolism reactions of lactose in the presence of yeast cell and in the absence of oxygen. The maximium theoretical ethanol yield was 0.51 g ethanol produced/ g of fermentable sugar. As a result, good ethanol yield was obtained and it could be maximized through process optimization and separation technique.

Table 4. 9. Calculated Ethanol Yields

Volume of ethanol mixture distilled (mL)	Mass of fermentable lactose (gm)	Concentration of ethanol (% v/v)	Volume of pure ethanol(mL)	Mass of pure ethanol (gm)	Ethanol Yield (%)
5	3.075	2.61	0.131	0.103	6.6
9.11	2.25	2.59	0.236	0.186	16.2
5.5	3.9	2.61	0.144	0.114	5.7
3	2.25	2.61	0.078	0.062	5.8
15.3	3.9	2.62	0.401	0.316	16
9.5	3.075	2.62	0.249	0.196	6.4
7.8	2.25	2.62	0.204	0.161	14
10.5	2.25	2.62	0.275	0.217	19
9.21	3.075	2.62	0.241	0.190	12
12.5	3.9	2.62	0.328	0.259	13
12.5	3.9	2.62	0.328	0.259	13
7	3.075	2.62	0.183	0.145	9.2
15.5	3.075	2.62	0.406	0.320	20.4
8.75	3.075	2.62	0.230	0.181	11.5
8	3.9	2.62	0.210	0.165	8.3
7.5	3.9	2.62	0.197	0.155	7.8
11.5	3.075	2.62	0.301	0.237	15.1
6	3.075	2.62	0.157	0.124	8
12.5	3.9	2.62	0.328	0.259	13
9.4	2.25	2.62	0.246	0.194	17
7.9	2.25	2.62	0.207	0.163	14.2
10.2	2.25	2.62	0.267	0.211	18.4
11	3.9	2.62	0.288	0.227	11.4
10.5	2.25	2.62	0.275	0.217	19
6	2.25	2.62	0.157	0.124	10.8
4.5	3.075	2.62	0.118	0.093	6
15	3.075	2.62	0.393	0.31	19.8

4.12. Analyzed FT-IR for Bioethanol Produced from cheese whey

Alcohols have characteristic IR absorptions associated with the O-H, C-O and the C-H stretching vibrations. When the region (3450 cm-1) run as a liquid film with a very intense and broad band indicated the O-H stretch of alcohols, while the region (1640 cm-1) confirms the C–O stretch. The bands at around 2060 cm-1 and 665 m^{-1} were assigned as the symmetric stretching modes of the $–CH_2$ and$–CH_3$ groups, respectively. This ascertains that the product obtained from cheese whey is definitely ethanol due to the confirmation of these regions.

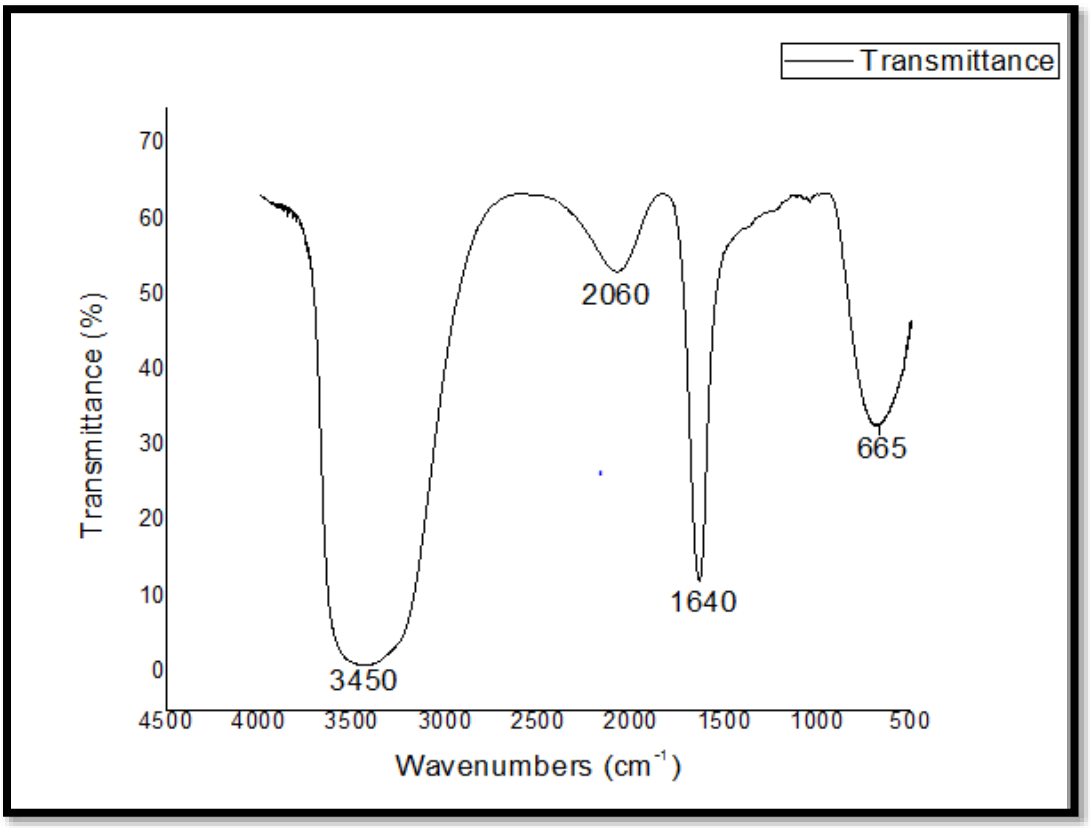


Fig 4. 14. FTIR Analysis Graph

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusion

Cheese whey is favorable dairy wastes for the production of bioethanol fuel. It is the most abundant by product generated from milk processing dairy industries. The majority of the cheese whey content is lactose and the remains in cheese whey constituting the 90% fraction of the organic load. Lactose is largely responsible of organic contamination and incase, consisted of biological oxygen demand (BOD = 230mg/mL) and chemical oxygen demand (COD = 70mg/mL) that cause environmental pollutions. Therefore, it is important to manage dairy cheese whey rather than discharging to the environment. Dairy cheese whey was a byproduct that containing lactose (0.036 – 0.048 g/mL) which can be fermented into bioethanol and was used as fermentation media to support microorganism growth during fermentation. Consequently, cheese whey from dairy industry is concentrated at 105^{o}C and hydrolyzed at 140^{o}C to increase amount of lactose by enhancing its solubility.

This lactose is not fermented by all saccharomyces yeast strain because this yeast has no enzymes that metabolize lactose into simple sugars glucose and galactose like sucrose. Therefore, another yeast strain is obtained in this study to deal with fermentation of cheese whey. The *kluyveromyces delphensis* had ability to ferment lactose into ethanol as well as its activity facilitated lactose in the cheese whey to be hydrolyzed into glucose and galactose that finally fermented into ethanol. The fermentation was carried out by Erlenmeyer flask anaerobically at 200rpm incubator shaker. Based on analysis of variance (ANOVA) Temperature (27^{o}C, 31^{o}C and 35^{o}C), Initial lactose concentration (4.5g/L, 6.15g/L and 7.8g/L), Yeast cell concentration(5 g/L, 7.5g/L and 10g/L) and pH (5, 5.5 and 7.5) have significant effect on the yield of ethanol. Their individual and interaction effects were studied by experimental design expert@ 6.0.8 version. As the result, RSM optimization at 6.15g/L lactose, 10g/L yeast cell, 27^{o}C temperature and 5.5 pH is achieved. Finally, the functional groups of produced ethanol was analyzed by FTIR and its general properties were also analyzed. Therefore, cheese whey is a good resource for production of bioethanol and another products.

5.2. Recommendation

Based on the contemporary investigation the subsequent recommendations are advanced;

- Supplementary researches have to be applied to increase the yield of bioethanol from diary cheese whey using other microorganism (in single and combine form) which have ability to ferment disaccharide and monosaccharide sugars of cheese whey into ethanol.
- Optimization of fermentation process variables were investigated in this study. However, further optimization of concentration, hydrolysis and distillation are recommended to maximize the yield of ethanol from cheese whey.
- Analysis of ethanol from cheese whey was investigated by FTIR and Density meter. Although further analysis by HPLC and GCMS are recommended to identify desired and undesired (side) products.

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APPENDIXES

Appendix – A: General Composition of Cheese Whey

Diary cheese whey waste is composed of different components. Cheese whey constitutes an inexpensive and nutritionally rich raw material for production of ethanol by fermentation. Typical cheese whey contains 4–5% lactose, 0.6–0.65% protein, and 0.1% citric acid. Production of ethanol from cheese whey is relevant because of its high carbohydrate content and availability. The remaining components of sweet and acid whey are mentioned as following in the table below.

The whey compositions

No	Constituent	Sweet whey (w %)	Acid whey (w %)
1	Water	93-94	94-95
2	Dry matter	6-6.5	5-6
3	Lactose	4.5-5	3.8-4.3
4	Lactic acid	traces	Up to 0.8
5	Protein	0.6-0.65	0.6-0.65
6	Citric acid	0.1	0.1
7	Minerals	0.5-0.7	0.5-0.7

Source: www.diaryforall.com

Appendix – B: Procedure for ash content determination.

Equipment used: crucible, furnace, heater, desiccator, measuring cylinder and electronic balance

1. The crucible was washed and dried in furnace at 550oC for 15 minutes

2. The crucible was taken out of furnace and put into desiccator

3. The mass of crucible was measured (w1)

4. 5ml of whey was added into crucible and the sample mass was measured (w3)

5. The sample in crucible was preheated before it was taken into furnace for 20 minutes.

6. The crucible contained sample was taken into furnace at 550oC for 3 hours by using desiccator.

7. Finally, the mass of crucible with sample was measure (w2) and ash content was calculated as follows.

Appendix – C: Procedure to prepare calibration curve

Equipment used: electronic balance, water bath, test- tubes, vortex mixer, pipettes, beakers, test-tubes holder, spoon, paper, cuvettes, computer and spectrophotometer

Chemicals used: Lactose, distilled water and Benedicts solutions

1. The mass of lactose (2, 4, 6, 8 & 10 gm) was measured with electronic balance

2. Distilled water was added into each test tubes (6-test tubes).

3. The measured masses of lactose were added into each test tubes except one test tube (which was taken as control test tube)

4. The solutions were mixed by vortex mixer until the uniform solution was performed.

5. Similar six test tubes were prepared and 5ml benedict's solution was added into each test tubes (6-test tubes)

6. 1ml of the prepared lactose standard solution was taken into test tubes contained benedicts solution respectively and mixed on vortex vibrator.

7. The mixed solution was taken into water bath at 90 oC until color change was observed (for five minutes).

8. The heated solution was taken out of water bath and cooled at room temperature for some minutes.

9. Then, it was filtered by filter papers and its absorbance was measured by Spectro UV-VIS Double Beam PC 8 Scanning Auto Cell UVD – 3200, Labomed, INC.

10. Finally, the calibration curve was prepared and drawn.

Appendix – D: Procedure to culture yeast strain on Broth

Equipment used: Erlenmeyer flask, autoclave, shaker, heater, stirrer, cabinet, pipettes, beakers, microorganism preserver, electronic balance and pH-meter

Chemicals used: Nutrient broth, Peptone, Yeast extract, distilled water, alcohol, NaOH and H_2SO_4

Yeast strain used: *Kluyveromyces delphensis*

1. 500ml Erlenmeyer flask was washed and sterilized with autoclave at 121^oC for 15 minutes.

2. 200ml distilled water was poured into 500ml empty Erlenmeyer flasks.

3. Reagents(4gm of Nutrient broth , 3gm of Peptone and 2gm of Yeast extract) were measured and added into Erlenmeyer flask contained distilled water.

4. The solution was shaken to mix the reagents with distilled water and thus, it was sterilized at 121^oC for 15 minutes.

5. The sterilized broth was cooled and the *kluyveromyces delphensis* yeast strain was transferred into Erlenmeyer flask contained broth under UV-visible light (cleaned Cabinet by Alcohol) to avoid contamination.

6. Then, it was taken into incubator and maintained for three days in shaker at temperatures of 26^oC with velocity of 200rpm (for each flask).

7. The *kluyveromyces delphensis* yeast strain cultured was preserved with microorganism conserver at 4^oC.

Appendix – E: Procedure for Fermentation of cheese whey

Equipment used: Erlenmeyer flask, pipettes, autoclaves, beakers, electronic balances, heater, stirrer, UV-light source (cabinet) and incubator

Chemical used: Cheese whey, Nutrient broth, peptone, NH_4Cl, $MgSO_4.7H_2O$, KH_2PO_4, distilled water, alcohol, cotton, aluminum foil, NaOH and H_2SO_4

Procedure:

1. 1000mL Erlenmeyer flasks were washed and dried.

2. 500mL of cheese whey substrate was poured into 1000mL Erlenmeyer flasks.

3. Supplements (1.5gm of Nutrient broth, 1.5gm of peptone, 1.5gm of NH_4Cl, 0.5gm of $MgSO_4.7H_2O$ and 1gm of KH_2PO_4) were added into flasks contained cheese whey substrate.

4. The substrate mixture was preheated to mix properly and its pH was adjusted before sterilization.

5. Then, it was sterilized at 121^oC temperature for fifteen minutes with Autoclave.

6. The sterilized substrate was cooled and maintained under UV- light to avoid bacterial contamination.

7. Inoculated kluyveromyces delphensis yeast strain added into flask contained sterilized substrate under UV- light.

8. Finally, it was taken into incubator for three days at different temperature.

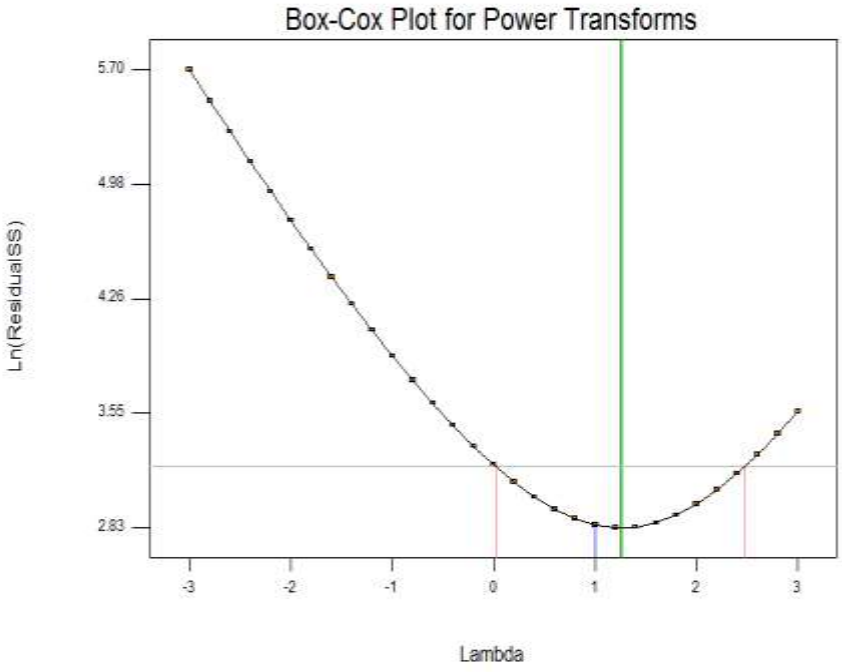
Appendix – F: Model Desirability

Appendix – G: Box – Cox plot for power transforms

DESIGN-EXPERT Plot
Response 1

Lambda
Current = 1
Best = 1.26
Low C.I. = 0.02
High C.I. = 2.47

Recommend transform:
None
(Lambda = 1)



Appendix – H: Laboratory Equipment's

Distillation setup

Density meter

Spectro photometer

Autoclave

Appendix – I: Determination of Reducing Sugar and Ethanol Charactering with Test Tubes.

No color change before heated

Color changed after heated

Filtered solutions

Ethanol distilled

Appendix – J: Substrate for Fermentation Process

Cheese Whey preheated

Nutrient Broth

Nutrient Broth preheated

Lactose scanning

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

%	10°C	15°C	20°C	25°C	30°C	35°C	40°C	%	10°C	15°C	20°C	25°C	30°C	35°C	40°C
0	0.99973	0.99913	0.99823	0.99708	0.99568	0.99406	0.99225	50	0.92126	0.91776	0.91384	0.90985	0.90580	0.90168	0.89750
1	785	725	636	520	379	217	034	51	.91943	555	160	760	353	.89940	519
2	602	542	453	336	194	031	.98846	52	723	333	.90936	534	125	710	288
3	426	365	275	157	014	.98849	663	53	502	110	711	307	.89896	479	056
4	258	195	103	.98984	.98839	672	485	54	279	.90885	485	079	667	248	.88823
5	098	032	.98938	817	670	501	311	55	055	659	258	.89850	437	016	589
6	.98946	.98877	780	656	507	335	142	56	.90831	433	031	621	206	.88784	356
7	801	729	627	500	347	172	.97975	57	607	207	.89803	392	.88975	552	122
8	660	584	478	346	189	009	808	58	381	.89980	574	162	744	319	.87888
9	524	442	331	193	031	.97846	641	59	154	752	344	.88931	512	085	653
10	393	304	187	043	.97875	685	475	60	.89927	523	113	699	278	.87851	417
11	267	171	047	.97897	723	527	312	61	698	293	.88882	446	044	615	180
12	145	041	.97910	753	573	371	150	62	468	062	650	233	.87809	379	.86943
13	026	.97914	775	611	424	216	.96989	63	237	.88830	417	.87998	574	142	705
14	.97911	790	643	472	278	063	829	64	006	597	183	763	337	.86905	466
15	800	669	514	334	133	.96911	670	65	.88774	364	.87948	527	100	667	227
16	692	552	387	199	.96990	760	512	66	541	130	713	291	.86863	429	.85987
17	583	433	259	062	844	607	352	67	308	.87895	477	054	625	190	747
18	473	313	129	.96923	697	452	189	68	074	660	241	.86817	387	.85950	407
19	363	191	.96997	782	547	294	023	69	.87839	424	004	579	148	710	266
20	252	068	864	639	395	134	.95856	70	602	187	.86766	340	.85908	470	025
21	139	.96944	729	495	242	.95973	687	71	365	.86949	527	100	667	228	.84783
22	024	818	592	348	087	809	516	72	127	710	287	.85859	426	.84986	540
23	.96907	689	453	199	.95929	643	343	73	.86888	470	047	618	184	743	297
24	787	558	312	048	769	476	168	74	648	229	.85806	376	.84941	500	053
25	665	424	168	.95895	607	306	.94991	75	408	.85988	564	134	698	257	.83809
26	539	287	020	738	442	133	810	76	168	747	322	.84891	455	013	564
27	406	144	.95867	576	272	.94955	625	77	.85927	505	079	647	211	.83768	319
28	268	.95996	710	410	098	774	438	78	685	262	.84835	403	.83966	523	074
29	125	844	548	241	.94922	590	248	79	442	018	590	158	720	277	.82827
30	.95977	686	382	067	741	403	055	80	197	.84772	344	.83911	473	029	578
31	823	524	212	.94890	557	214	.93860	81	.84950	525	096	664	224	.82780	329
32	665	357	038	709	370	021	662	82	702	277	.83848	415	.82974	530	079
33	502	186	.94860	525	180	.93825	461	83	453	028	599	164	724	279	.81828
34	334	011	679	337	.93986	626	257	84	203	.83777	348	.82913	473	027	576
35	162	.94832	494	146	790	425	051	85	.83951	525	095	660	220	.81774	322
36	.94986	650	306	.93952	591	221	.92843	86	697	271	.82840	405	.81965	519	067
37	805	464	114	756	390	016	634	87	441	014	583	148	708	262	.80811
38	620	273	.93919	556	186	.92808	422	88	181	.82754	323	.81888	448	003	552
39	431	079	720	353	.92979	597	208	89	.82919	492	062	626	186	.80742	291
40	238	.93882	518	148	770	385	.91992	90	654	227	.81797	362	.80922	478	028
41	042	682	314	.92940	558	170	774	91	386	.81959	529	094	655	211	.79761
42	.93842	478	107	729	344	.91952	554	92	114	688	257	.80823	384	.79941	491
43	639	271	.92897	516	128	733	332	93	.81839	413	.80983	549	111	669	220
44	433	062	685	301	.91910	513	108	94	561	134	705	272	.79835	393	.78947
45	226	.92852	472	085	692	291	.90884	95	278	.80852	424	.79991	555	114	670
46	017	640	257	.91868	472	069	660	96	.80991	566	138	706	271	.78831	388
47	.92806	426	041	649	250	.90845	434	97	698	274	.79846	415	.78981	542	100
48	593	211	.91823	429	028	621	207	98	399	.79975	547	117	684	247	.77806
49	379	.91995	604	208	.90805	396	.89979	99	094	670	243	.78814	382	.77946	507
								100	.79784	360	.78934	506	075	641	203

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