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Bioethanol Production via Fermentation of Waste substrates using Yeast Isolates

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As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Getu Tsegu entitled: **Bioethanol Production via Fermentation of Waste substrates using Yeast Isolates** and recommend that it will be accepted to fulfill the thesis requirement for degree of Masters in **Biotechnology.**

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DECLARATION

I, the undersigned, declare that, this is an original work and has never been presented in any other university as well as research institutes and all the source material used for this thesis have been fully acknowledged.

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List of Abbreviations

EDGE	Enhanced Dry Grind Enzymatic
ELCAM	Energy Life Cycle Analysis Model
MEA	Malt Extract Agar
YPD	Yeast Peptone Dextrose
YEPX	Yeast Extract, Peptone and Xylose
WRF	White Rot Fungi
VHG	Very High Gravity

Abstract

Bioethanol is one biomass derived platform molecules which has a potential to be a sustainable feedstock for a variety of commodity chemicals. The aim of this study was to produce bioethanol from grain waste flour, *tella* spent, pineapple and papaya peels. Dilute sulfuric acid was used to hydrolyze polysaccharide in the raw materials. Starch content in grain waste flour and reducing sugar concentration in the hydrolysates of each raw material was determined. Indigenous yeast was isolated from *areki difdif* and *tej* samples. Indigenous yeast isolates with high fermentation efficiency and high ethanol producing ability were used to ferment hydrolysates in each of raw material to bioethanol. The results indicate that pretreatment of grain waste flour, papaya peels, pineapple peels and *tella* spent with 2% sulfuric acid at 110^{o}C for 90 minutes yielded 29.7–30.8, 28.2–28.9, 16.2–16.6 and 10.3–10.5% of reducing sugar, respectively. The yeast isolate 1T-10 from *tej* samples was efficient in converting the D-glucose to ethanol (75.58%) and produced highest yield of ethanol (7.72%) from 20% D-glucose compared to other yeast isolates. Fermentations of grain waste flour with 1T-10 produced the highest yield of ethanol 1.380% (w/v) from pretreatment of substrates with 2% sulfuric acid at 168 hours of fermentation time compared to other combinations or 0% at (72, 120 and 168 hours), 1% at (72, 120 and 168 hours), 1.5% at (72, 120 and 168 hours) and 2% at (72 and 120 hours). Similarly, fermentation of pineapple peels, papaya peels and *tella* spent produced the highest yield of ethanol; 7.883%, 6.400% and 4.813% (w/v) with pretreatment of 2% sulfuric acid at 120, 72 and 72 hours of fermentation time, respectively compared to other combinations when fermented with the same yeast isolate (1T-10). It was concluded from the present study that it is possible to produce economically viable bioethanol by digesting waste and cheap resources like grain waste flour, *tella* spent, pineapple and papaya peels using indigenous yeast isolates.

Keywords: Bioethanol, *Tella* spent, grain waste flour, *Tej*, *Areki difdif*, Indigenous yeast

1. INTRODUCTION

Today, the majority of countries in the world depends up on the use of fossil fuels as a source of energy. However, the use of fossil fuel as a source of energy has been encountering a number of challenges ranging from the cost of petroleum and transportation, growing demand owing to rapid increase in population size and industrialization, environmental problems like global warming and climate change resulting from the emission of greenhouse gases, which were together or alone share a role in the depletion of fossil fuel (Andualem and Gessesse, 2012; Keller, 2018; Liguori *et al.*, 2015). For this reason, another renewable source of energy with a characteristic of being sustainable, non-competitive with food, minimum production cost and environment friendly source of energy is required. One of the most renewable substrates to use as a source of energy is biomass, which mainly reduces the cost of production and environmental impact of petrol energy (Vallejos *et al*., 2017). Among the most promising biomass source of energy in the future include lignocelluloses and microalgae, which are getting great attention as a potential substrate for the production of alternative fuel like bioethanol for the replacement of petroleum based fuel (Singh *et al.*, 2015).

The main factor in the production of bioethanol, however, is its cost and many of the substrates that are obtained from agricultural crops. This in turn results in competition for food source and then contribute to food insecurity (Wilkinson *et al*., 2017). Therefore, we must search for alternative cheap and sustainable raw materials for production of bioethanol from available agricultural or industrial wastes. Lignocellulosic waste substrates mainly molasses, paper waste (Byadgi and Kalburgi, 2016), agricultural waste (Cutzu and Bardi, 2017) and fruit waste (Borah and Mishra, 2011; Kandari and Gupta, 2017; Pandey *et al.*, 2014) are non-edible sources that can be used as raw materials for bioethanol production in recent times. Among waste materials, waste flour from milling house, *tella* spent and peels of fruits (pineapple and papaya) can also serve as raw materials for bioethanol production (Rafiq *et al*., 2018).

It is well known that bioethanol is one of the most valuable organic chemicals in the world trade (Mussatto *et al*., 2011). This is due to its application in different sectors like chemical, pharmaceutical and other industries. For this reason, world bioethanol production is highly increased from 63, 906 billion metric tons in 2010 to 84, 121 billion metric tons in 2017 (World statistic, 2019). Bioethanol is widely produced and used in both Brazil and the United States

(Soccol *et al*., 2010). These countries were responsible for 85% of world ethanol production in 2016. United States produced approximately 15,250 million gallons in the year 2016 and exported to nearly 50 different countries (RFA, 2017).

Recently many studies were conducted on production of bioethanol from peels of different fruit wastes like banana, apple and grape (Janani *et al*., 2013; Tiwari *et al*., 2014). But there are little or no scientific reports on the production of bioethanol from local wastes such as peels of fruits (like pineapple and papaya), grain waste flour from milling house and *tella* spent which could serve as a good raw material for biofuel production.

In Ethiopia, ethanol production linked with two sugar factories (Fincha and Metehara) with an annual production capacity of 11.1 million liters (Mebrhit , 2016). The government of Ethiopia also suggests 5% (E5) of ethanol with gasoline blend starting from the year 2008 and 10% (E10) of ethanol blend were started from the year 2011 GC (Shete and Rutten, 2014), creating different strategies like expansion of land for sugar factory and building new sugar factory, but the strategies E15 for the year 2015 did not look successful due to various factors and E20 for 2020 is also creating distrust among many individuals (Wendimu, 2016).

Bioethanol fermentation from edible and lignocellulosic feedstocks using enzymatic hydrolysis has been carried out with success; but due to the tough structure of cellulose, lignin and other cell wall component of lignocellulosic feedstocks, its conversion to useful product is still challenging. Currently, the cost of enzymes for pretreatment and their stability is another issue (Alvira *et al*., 2010). But compared to enzyme, acid is cheap, easily available and appropriate for the pretreatment and release of reducing sugar from waste substrates fermented to produce bioethanol using yeast. Additionally, bioethanol fermentation from lignocellulosic material can only be achieved by adequate pretreating lignocellulosic material. Pretreatment would remove the lignin and make the cellulose and hemicellulose accessible for conversion to xylose, pentose and hexose sugars. However, industrial yeast used today in bioethanol production or *Saccharomyces cerevisiae* converts only hexose sugars in to bioethanol under anaerobic condition; in other words, they are unable to ferment starch, pentose and xylose (Byadgi and Kalburgi, 2016).

1.1. Statement of the problem

In Ethiopia, a collection of solid wastes, for example, solid wastes like fruit peels and *tella* spent are disposed off in a drainage system or on open land and are posing serious environmental problems. Disposal of waste to drainage system in addition clogging the pipe lines, it threatens both open and ground water quality and serves as a breeding site for insects that include vectors of diseases (Wondale, 2012).

On the other hand, burning waste to clean environment emits different gases to the atmosphere, resulting in air pollution. Air pollution is not the result of only burning waste materials; instead, it is a combination of different gases that are generated from combustion of fossil fuels from vehicles, generators and different industries. To minimize such problems, conversion of waste material to useful, economical and alternative energy source is required. Generating alternative energy from waste is characteristically renewable, sustainable, efficient, cost-effective, convenient and environmentally friendly. The process, in addition to generating safe product, it will reduce the damping of huge amount of solid waste from edible food crops to in the drainage system.

Bioethanol production is growing very slowly in developing countries like Ethiopia. This has its own disadvantage. To overcome such critical problems, alternative source from non-edible waste material must be investigated. Peels of fruits, waste flour from milling house and *tella* spent are important raw materials that can be utilized for ethanol production in Ethiopia. In line with this, peels of fruit were reported to contain about 14.6–15.2% (w/v) of fermentable sugar (Kandari and Gupta, 2017). These wastes are still discarded to the environment; on the other hand, fruit wastes are the least expensive and easily available raw materials that can be utilized for production of bioethanol (Vishwakarma *et al*., 2014). Therefore, the main objective of this study was producing bioethanol by acid treating cheaper waste substrates (grain waste flour, *tella* spent and selected peels of fruit) and fermenting the hydrolysates with wild native yeasts isolated from traditional beverages.

1.2. Objectives

1.2.1. General objective

The current study aimed at isolating yeasts from traditional beverages and using them for fermentation of different waste resources for bioethanol production, through cost effective acid hydrolysis under batch fermentation condition.

1.2.2. Specific objectives

The specific objectives of the study were to

- Isolate yeasts from traditional beverages (*areki difdif* and *tej*),
- Screen the yeast isolate for ethanol production,
- Examine the potential of yeast isolates in the production of bioethanol,
- Examine the impact of acid hydrolysis on different wastes (grain waste flour, *tella* spent, pineapple and papaya peels) resources for ethanol production.

2. REVIEW LITERATURE

2.1. Properties of bioethanol

Bioethanol is also called ethanol (a commonly known name); ethyl alcohol; grain alcohol; methylcarbinol; or simply alcohol having a molecular formula of CH_3CH_2OH or C_2H_6O (Danmaliki *et al*., 2016; Gasmalla *et al*., 2012). It is a clear, flammable, colorless liquid with a characteristic of vinous odor and burning taste. Other properties of ethanol are given in Table 1. Bioethanol is prepared through fermentation of certain carbohydrates or sugar rich substrates either through direct fermentation process of the substrate or by passing the substrate through different pretreatment steps.

Table 1: Properties of bioethanol

Properties	Values
Density	0.7894g/cm^3 at 20^{o}C
Boiling point	173^{o}F or 78.3^{o}C at 760mmHg
Melting point	-114.5^{o}C
Refractive index	1.3614 at λ = 589.3nm and 18.35^{o}C
Flash point	8^{o}C
Formula weight	46.069g/mol

Source: Gokel (2004)

Furthermore, ethanol is miscible with many organic solvents like acetic acid, acetone, benzene, carbon tetrachloride, chloroform, diethyl ether, ethylene glycol, glycerol, nitro methane, pyridine toluene, etc. (Gokel, 2004). Burning ethanol gives a smokeless blue flame, which is not easily seen in normal light. The properties of ethanol stem from its group (-OH group) and the shortness of its carbon chain. Its hydroxyl group participates in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight, such as propane (McMurry, 2000).

2.2. Potential applications of bioethanol

Bioethanol has many applications in different sectors like pharmaceutical, chemical and fuel industries. It is a good solvent for many organic compounds that are insoluble in water, such as paints, dyes, perfumes, etc. Today, bioethanol plays an important role in the fuel industries by being replacing the fossil fuel. Potential biofuel application areas are clearly stated as follows.

2.2.1. Pharmaceutical and chemical sector

Ethanol acts as a central nervous system depressant in medical centers. Ethanol is one of the most widely used legal drugs in the world. It is metabolized by alcohol dehydrogenase enzyme in our body. Drugs that inhibit alcohol dehydrogenase are the most likely theoretical compounds that would lead to a clinically significant pharmacokinetic interaction with ethanol, which may only include a small number of drugs. Acute ethanol primarily alters the pharmacokinetic of other drugs by changing the rate and extent of absorption, with more limited effects on clearance (Chan and Anderson, 2014).

Ethanol is a widely used solvent in blood plasma fractionation at industrial scale. Blood is very complex and has thousands of plasma proteins, among which, four (albumin, IgG, factor VIII and Factor IX) are commercially important for production due to significant need for patients (Burnouf, 2007; Hosseini and Wahid, 2016).

The starting material to produce biological drugs from plasma is natural which is different from synthetic starting material. So, the quality of plasma as starting material plays an important role in the quality of final product. Introducing new techniques for preparation of the biological drugs from human plasma has resulted in the improvements in purity of products, higher safety and yield noticeably (Hosseini and Wahid, 2016; Moure *et al*., 2003).

Still, the backbone of the modern plasma fractionation technique is mainly based on cold ethanol fractionation of the human plasma that is almost the same as fractionation of crude oil, breaking it down into its components. Nowadays, cold ethanol fractionation is followed by chromatographic methods, since they offer higher purity. It can be concluded that plasma fractionation with ethanol at low temperature for the preparation of the main human plasma biological components including albumin, IgG, coagulation factors VIII and IX is still the most widely used method at an industrial scale. This can be used in combination with different chromatographic techniques in order to achieve a higher quality and yield (Burnouf and Radosevich, 2001).

Ethanol is also used in medical wipes and it is the most commonly used antibacterial hand sanitizer (antiseptic) chemicals (Janani *et al*., 2013; Lachenmeier, 2008; Vishwakarma *et al*., 2014). Ethanol kills organisms by denaturing their proteins and dissolving their lipid and is effective against most bacteria, fungi and many viruses. However, ethanol is ineffective against

bacterial spores. Ethanol at 70% is the most effective concentration, particularly because of osmotic pressure. Absolute ethanol may inactivate microbes without destroying them because alcohol is unable to fully penetrate the microbes' membrane (Pena-Pereira *et al*., 2009).

Ethanol, often in high concentrations, is used to dissolve many water-insoluble medications and related compounds in pharmaceutical sectors. Liquid preparations of cough and cold remedies, pain medication and mouth washes may be dissolved in 1–25% concentrations of ethanol and may need to be avoided in individuals with adverse reactions to ethanol such as alcohol-induced respiratory reactions. Ethanol is present in over 700 liquid preparations of medicine (Lachenmeier, 2008; Zuccotti and Fabiano, 2011).

2.2.2. Fuel industry

The largest single use of ethanol is as an engine fuel and fuel additive (Cutzu and Bardi, 2017). Brazil in particular relies heavily upon the use of ethanol as an engine fuel, due in part to its role as the globe's leading producer of ethanol from sugarcane molasses. Gasoline sold in Brazil contains at least 25% anhydrous ethanol (E100) and hydrous ethanol (about 95% ethanol and 5% water) can be used as fuel in more than 90% of new gasoline fueled cars, sold in Brazil (Portner *et al*., 2014).

The US and many other countries primarily use E10 (10% ethanol, which is sometimes known as gasohol) and E85 (85% ethanol) ethanol to gasoline mixtures (Timilsina and Shrestha, 2010). According to the same workers, Australian law limits the use of pure ethanol from sugarcane waste to 10% in automobiles, older cars and vintage cars have to use a slower burning fuel or their engine valves should have to be upgraded or replaced.

According to an industry advocacy group, ethanol as a fuel reduces harmful tailpipe emissions of carbon monoxide, particulate matter, oxides of nitrogen and other ozone-forming pollutants (Harvey and Pilgrim, 2011; OECD, 2011). Argonne National Laboratory analyzed greenhouse gas emissions of many different engine and fuel combinations and found that biodiesel to petrol diesel blend of (E20) showed a reduction of 8%, conventional E85 ethanol blend showed a reduction of 17% and cellulosic ethanol showed a reduction of 64%, compared with pure gasoline (Oketch, 2014).

2.2.3. Other applications/alcoholic drinks

Ethiopia is one of the countries where a wide variety of traditional fermented beverages are prepared and consumed. The various traditional fermented beverages are produced on a fairly small scale and usually for local consumption. Among Ethiopian fermented beverages are varieties of *Tella, Tej, brode, areki, Keribo, korefe* are consumed in Ethiopia (Tafere, 2015).

Among fermented foods, alcoholic beverages have been widely consumed since prehistoric times by people around the world. It can improve the taste of food, enhance the digestibility of a food, preserve food from degradation by noxious organisms, and increase nutritional values. Further, it is used for medical reasons, recreational purposes, in marriages, in religious and nonreligious ceremonies (Yohannes *et al*., 2013).

A standard drink is the amount of an alcoholic beverage that contains a fixed amount of pure alcohol (i.e., ethanol). Different countries have adopted a variety of standard drink sizes, ranging from a low of 8 grams (0.34 oz) of ethanol in the United Kingdom to a high of 19.75 grams (0.85 oz) of ethanol in Japan (International Center for Alcohol Policies 1998, cited from Dawson, 2003). Although the United States has no official definition of standard drink size, the two sets of drink sizes most commonly used are those volumes of various beverages that contain 0.6 oz (approximately 14 grams) and 0.5 oz (approximately 12 grams) of ethanol. The usefulness of the standard drink concept in measuring consumption, presenting meaningful risk curves, and developing low-risk drinking guidelines depends on all standard drinks containing the same amount of ethanol regardless of beverage type (Dawson, 2003).

2.3. Traditional fermented beverages of Ethiopia

Ethiopia is one of the countries where a wide variety of traditional fermented beverages are prepared and consumed. The various traditional fermented beverages consumed in Ethiopia consist of both high alcoholic and low alcoholic contents. The beverages are produced on a fairly small scale and usually for local consumption. Among Ethiopian fermented beverages are varieties of *tella, tej*, *Katikala* (*areki*), *korefe, borde, shamita, keribo*, etc. (Bacha *et al*., 1998).

2.3.1. *Tella*

Tella (a malt beverage like beer) is one of the several native fermented beverages, traditionally prepared and consumed in Ethiopia. It is brewed from various grains or cereals like barely, corn, wheat, sorghum, *teff*, millet and *gesho* (*Rhamnus prenoids*) (Debebe *et al*., 2016). The

preparation procedure for *tella* differs from place to place, but can easily made from a mixture of *enkuro* (a dark brown roasted flour of cereals), germinated wheat or barley grain (*bikil*), *gesho* (*Rhamnus prenoids*), *kitta* (pancake from different cereals) and water. After these ingredients are mixed sequentially and allowed to ferment, *tella* of different ages can be prepared. *Tella* in most parts of the country is indispensable at festivals, and in some areas, at social labor-pooling gatherings. *Tella* may have yellow, black or wine color, sweet or bitter taste, effervescent and it's cloudy. *Tella* is considered to be good quality, if the final ethanol content is in the range of 2-8% (v/v). The general experimental procedure for the production of *tella* is soaking of barley, roasting of barley, grinding of ingredients, preparation of *enkuro, yetella kitta* and fermenter tank (Getaye *et al*., 2018). After sloping the clear supernatant (*tella*) what was remained is *tella* spent which contains cellulose, hemicellulose and lignin.

2.3.2. *Areki*

Areki is a distilled homemade spirit made from fermented ingredients. It is a colorless and clear distilled traditional alcoholic beverage which is prepared in almost the same way as *tella* except that fermentation mass in this case is more concentrated and distilled extracted from the fermented ingredients with a higher alcoholic percentage (Bekele *et al*., 2005). A raw material used to produce *areki* is *Kitta* (a thin, 5–10 mm thick, pancake-like bread), *bikil* (germinated grain), and powdered *gesho* (*Rhamnus prenoids*). Additional ingredients such as *koso* (*Hagenia abyssinica*), *gibto* (*Lupinus albu* or *white lupin*), and *tenaadam* (*Ruta chalepensis*) are commonly added in order to produce special *areki* types and are known after the special ingredient added as *koso areki*, *gibto areki*, *tenaadam areki*, and some other types as well. These ingredients are mixed and allowed to ferment for 5-10 days and the fermented mash is locally known as *areki difdif* distilled to produce *areki*. The pH values of *tella* and *areki* are found between 3.07–5 and 4.30–4.51, respectively (Debebe *et al*., 2016).

2.3.3. *Tej*

Tej is a home processed fermented alcoholic beverages. It is also commercially available being as honey wine. *Tej* is prepared from honey, sugar, water and stalks of *gesho* (*Rmnus prenoids*). A *gesho stalk (Rmnus prenoids) is* boiled to extract ingredients. The extract and honey are mixed together, allowed to ferment for 5 days in warmer or for 15– 20 days in colder atmospheric condition (Yohannes *et al*., 2013) in order to produce *tej*.

2.3.4. Beer spent

Spent grains, the residues remaining after extraction of wort, which is a lignocellulosic rich biomass of brewing may provide a source of sugars for ethanol production. Brewers spent grain as the main byproduct of brewing industry. It represents approximately 85% of total by product generated. It is rich in cellulose and non-cellulosic polysaccharides which have to be recycled to produce ethanol (Aliyu and Bala, 2011). However, in some countries this biomass has been mainly used as cattle feed (Jay *et al*., 2008). Hydrolysis (0.6N HNO_3) and partial digestion (enzyme) of 20% spent grains for 18 h yields about 27g/L of glucose. Fermentation of this hydrolysates with *Pichia stipites* and *Kluveromyces marxianus* resulted in 8.3 and 5.9g/L of ethanol, respectively (White *et al.*, 2008).

2.4. Potential or possible substrates used for bioethanol production

Ethanol can be produced from a variety of substrates. Substrates used for ethanol productions exist in different forms across the world. However, the predominant and currently utilized substrates for ethanol production across the worlds are; corns in US, China and Slovakia; sugarcane in Brazil, China, Argentina, Colombia, Thailand and Australia; wheat in Germany, France, Spain, India, Canada, Sweden, Denmark and Australia; sugar beet in France and Czech Republic; rye in Poland; and Cassava in Thailand, which can also be generally categorized as sugary substrates, starchy substrates and lignocellulosic substrates (Timilsina and Shrestha, 2010). Some of the resources used for bioethanol and biodiesel productions are indicated in Figure 1 (Demirbas, 2008).

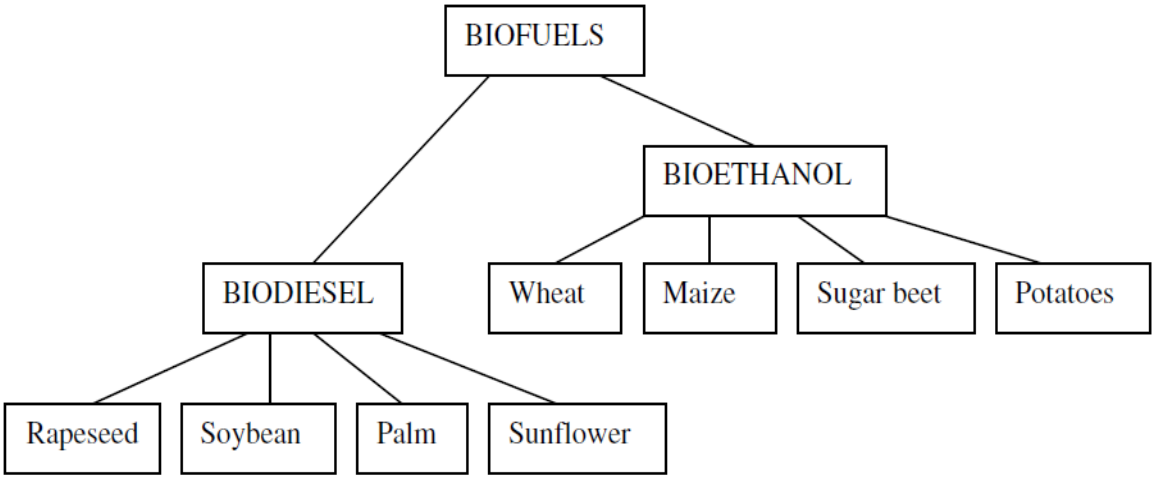


Figure 1: Resources of bioethanol and biodiesel. Source: (Demirbas, 2008)

2.4.1. Sugary substrates

Sugary substrates are important feedstocks to produce bioethanol. The well-known sugary substrates used for ethanol productions are of two types; namely, sugarcanes and sugar beets (Araújo *et al*., 2017). In most of the cases, direct production of bioethanol from such substrate is uneconomical (Gnansounou *et al.*, 2005). Yet, their byproducts are the main substrates used for production of ethanol in the world (Macrelli *et al.*, 2012).

2.4.1.1. Sugarcanes

Sugarcane is an agricultural crop that is mainly used as a feedstock to produce sucrose or sugar throughout the world. Bioethanol production from sugarcane is uneconomical due to the fact that the net money generated from the sale of the ethanol after the process and the net money obtained from the sale of sugar are incomparable i.e. the money obtained from the sale of a sugar is profitable (Humbird *et al.*, 2011). Instead waste generated from the sugarcane processing industry is good feedstocks for the production of ethanol (Fekadu, 2007).

2.4.1.2. Sugar beets

The beets used for ethanol productions are originally known as sugar beet (*Beta vulgaris*) which was originated from Europe. It is a plant whose tuber contains a high concentration of sucrose. Besides this there are nonfood-beets, which would not be an efficient feedstock for the production of sugar used for human consumption, but it is still one of the best raw materials utilized for the production of ethanol (Woiciechowski *et al*., 2016). A study done by Bowen *et al.* (2010) indicates that about 14,700 kg of ethanol is produced from the consumption of 168,000kg of raw sugar beet which indicates that approximately half of the sucrose in sugar beets was converted to ethanol. This investigation proves that sugar beets have the potential to produce ethanol.

2.4.2. Starchy substrates

Starchy substrates exist in many varieties and dominate world agricultural crop land. Basically, they are found in the form of cereals like; corn, barley, wheat, sorghum, rye, and root crops like; potatoes and cassava.

2.4.2.1. Cereals

2.4.2.1.1. Corn

Corn (maize) is the fundamental staple food that can be grown in a range of climates from tropical to temperate and may be sensitive to frost. It is a main feedstock used in US for ethanol

production (Araújo *et al*., 2017). Corn plants dominate the US total crop land. This indicates that in US biofuel production is mainly correlated with cultivation of corn which is a dominant feedstock. In addition to it, wheat and soy beans are also used for biofuel production in US with a low share of land as compared to land allocated for the cultivation of corn as shown in Figure 2 (Plevin, 2009).

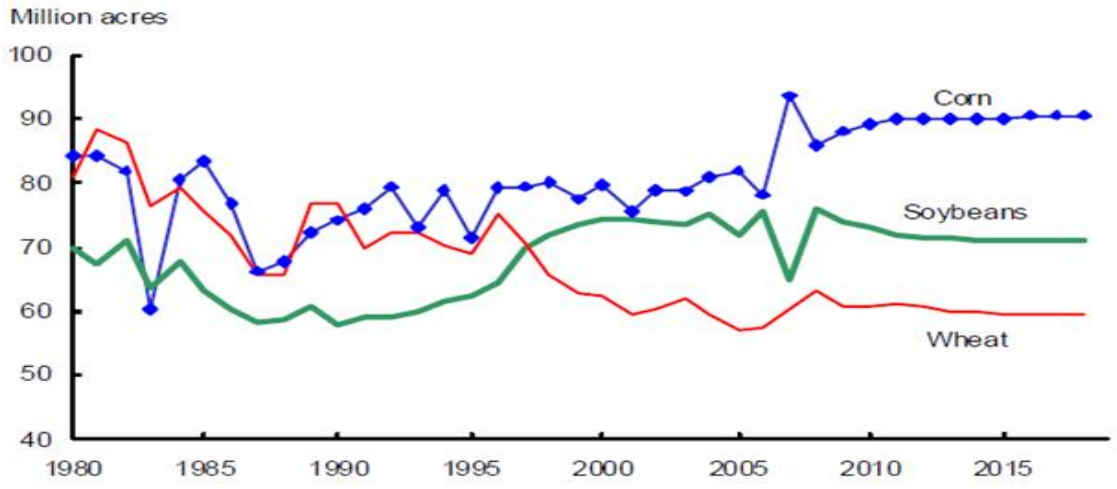


Figure 2: Planted area given for: corn, wheat and soybeans in United States. Source: (Plevin, 2009)

2.4.2.1.2. Barley

Barley is a potential candidate for industrial ethanol production since its ethanol yield is comparable to that of wheat but below that of American corn, which is currently the preferred industrially cheaper feedstock compared to wheat. Very high gravity (VHG; 30% solids) fermentations using both conventional and Stargen 001 enzymes for starch hydrolysis were carried out as simultaneous saccharification and fermentation (Białas *et a1*., 2010). A VHG traditional fermentation approach utilizing jet-cooking fermentation revealed that two of barley types; dehulled bold and Xena barley produced ethanol concentrations higher than that produced by wheat (12.3, 12.2 and 11.9%, respectively) but lower than that produced by corn (13.8%). In addition to this VHG-modified Stargen-based fermentation of dehulled bold barley demonstrated comparable performance (14.3% ethanol) relative to that of corn (14.5%) and wheat (13.3%) (Gibreel *et al*., 2009).

A new fermentation process known as EDGE (enhanced dry grind enzymatic) for ethanol production from thoroughbred, a winter barley variety with high starch content, was developed. In this process, in addition to the normal starch-converting enzymes, two accessory enzymes were used to solve the β-glucan problem (Fujita *et al.*, 2002). First, β-glucanases were used to hydrolyze the β-glucans to oligomeric fractions, thus significantly reducing the viscosity to allow good mixing for the distribution of the yeast and nutrients. Next, β-glucosidase was used to complete the β-glucan hydrolysis and generate glucose, which was subsequently fermented in order to produce additional ethanol (Vohra *et al*., 2014). Through this EDGE process, 30% of total dry solids could be used to produce 15% (v/v) ethanol (Nghiem *et al*., 2010).

2.4.2.1.3. Wheat

Ethanol is produced from wheat grain via the conventional hydrolysis and fermentation process. Using grain wheat to produce ethanol is uneconomical due to its competition with food. However, wheat straw is a waste material left after a process of harvest which was a substrate for ethanol production (Lesage and Van de Graaf, 2016). It was possible to produce ethanol from wheat straw which was pretreated with 3% H_2O_2 and 2% NaOH at 130^{o}C for 60 min. The hydrolysates was further treated with commercial cellulose digesting enzyme and after the process of saccharification substrate were seeded with *Saccharomyces cerevisiae* for three days of fermentation under anaerobic condition and produced 44g/L of ethanol (Irfan *et al.*, 2014; Kahr *et al.*, 2012). The basic process for bioethanol production from wheat grain and straw is shown in Figures 3 and 4, respectively**.**

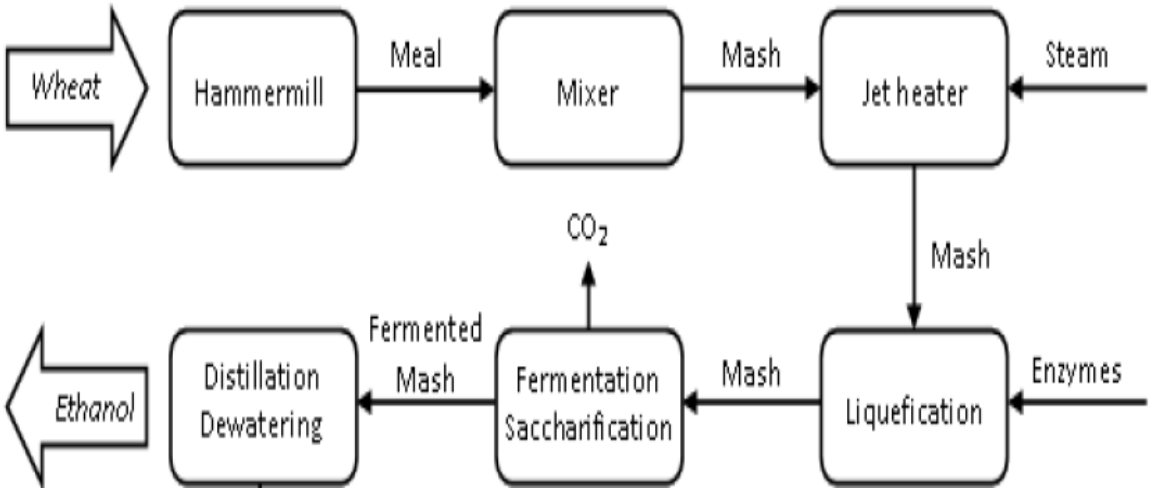


Figure 3: Flow chart showing bioethanol production from wheat raw materials. Source: (Kahr *et al*., 2012)

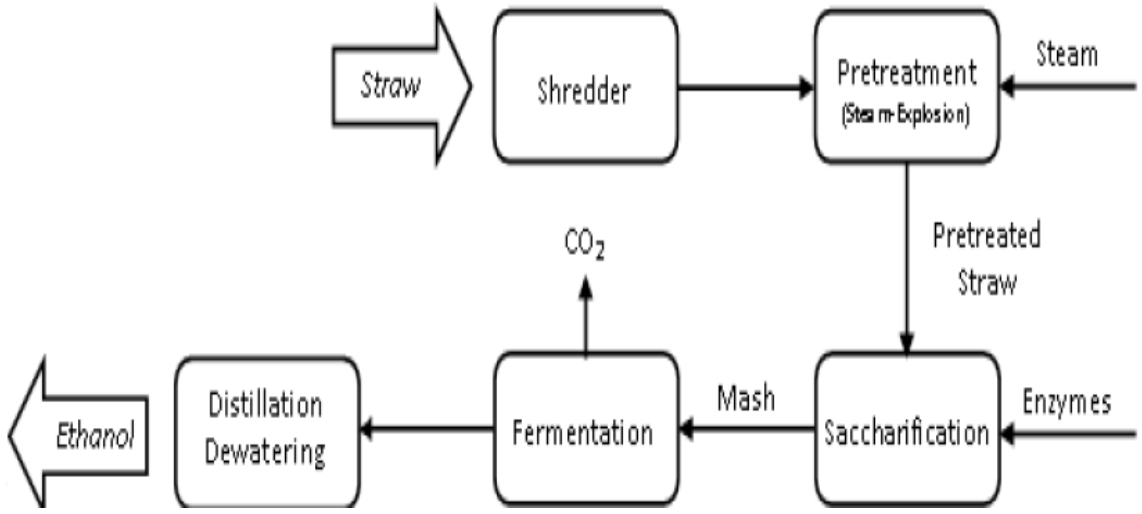


Figure 4: Flow chart showing bioethanol production from straw. Source: (Kahr *et al.*, 2012)

2.4.2.1.4. Sorghum

Sorghum is the fifth most important cereal crops worldwide and it is a major source of agricultural residues in tropical regions that would serve for bioethanol production (Rooney *et al*., 2007). Bioconversion of whole sorghum crop residues comprising stalks, leaves, peduncles and panicles to ethanol has great potential for improving ethanol yield per sorghum crop cultivated and for sustainable biofuel production (Nasidi *et al*., 2016). Furthermore, effective pretreatment of sorghum lignocellulosic biomass is central to the efficiency of subsequent fermentation to ethanol and efficient bioconversion of whole sorghum residues to ethanol and this provides a sustainable route for utilization of crop residues thereby providing a non-food feedstock for industrial-scale bioethanol production (Kim and Day, 2011).

Sweet sorghum (*Sorghum bicolor*) is a promising non-food energy crop. A study done by Liu *et al*. (2015) showed that production of ethanol from sweet sorghum is economically feasible as compared to ethanol production from sunflower and cotton at saline-alkali sites in Shandong (Wudi County) provinces of China. Sweet sorghum is a sugar crop, similar to sugarcane and sugar beets, that may show promise as a source of sugar for ethanol fermentation **(**Whitfield *et al.*, 2012).

Sorghum is underutilized as a source of renewable feedstock for bio-energy, which however, is a major cereal crop in US. Wang *et al.* (2008) studied factors impacting ethanol production from sorghum and they develop Energy Life Cycle Analysis Model (ELCAM) to quantify and prioritize the saving potential from the factors they obtain. Protein digestibility, level of

extractable protein, protein and starch interaction, mash viscosity, amount of phenolic compounds, ratio of amylose to amylopectin and formation of amylose-lipid complexes in the mash are key factors affecting ethanol production from sorghum. However, ELCAM showed a positive net energy value of 22400kJ/gallon ethanol.

2.4.3. Roots

2.4.3.1. Potatoes

Potato is high value starchy crop used as a source of food. During processing, potatoes are wasted, the waste from restaurant, hotels, chips shops and potato processors can be utilized as growth media for the fermentation processes and production of bioethanol because of the fact that waste potato contain 0.095g/ ml of starch and potato peels contain 0.084g/ ml of starch which were able to produce 32% (v/v) and 27.5% (v/v) of bioethanol, respectively (Fasil, 2015). The fermentation path way of potato in to ethanol is indicated in Figure 5.

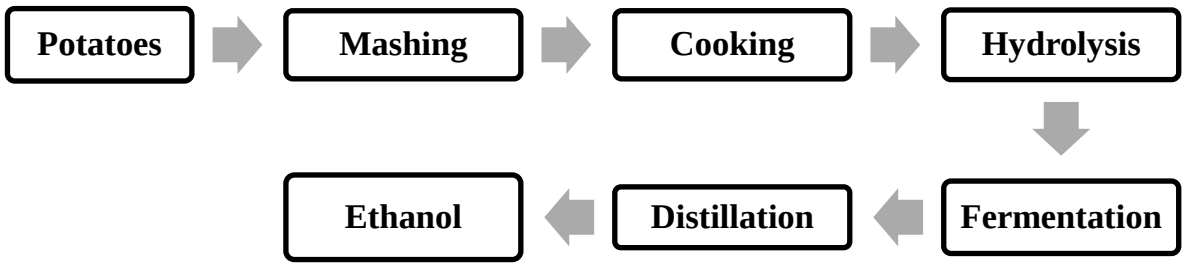


Figure 5: Ethanol production steps from potatoes using yeast. Source: (Memon *et al*., 2017)

2.4.3.2. Cassava

Cassava is a shrub with tuberous roots. A study done by Akponah *et al.* (2013) presented that cassava waste is a good substrate used in the production of bioethanol through fermentation seeded with strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis. Zymomonas mobilis* produced maximum ethanol as compared to *Saccharomyces cerevisiae*. *Zymomonas mobilis* produced about 15.0% and 16.5% (w/v) of ethanol using substrates treated with acid and enzyme respectively after 48h of fermentation. The same study indicated that *Saccharomyces cerevisiae* produced 2.9% and 2.84% (w/v) of ethanol using substrates treated with acid and enzyme respectively after 48h of fermentation.

2.4.4. Lignocellulosic substrates

Now a days, lignocellulosic substrates are promising feedstocks for the production of ethanol (Sun and Cheng, 2002). This is because of the fact that lignocellulosic substrates are non-edible and cannot compete with food (Thiruvengadathan, 2017; Timilsina and Shrestha, 2010). Ethanol production from lignocellulosic hydrolysates is an economically feasible process that requires microorganisms that produce ethanol with a high yield from all sugars present (hexoses as well as pentoses) and have high ethanol productivity in lignocellulosic hydrolysates, i.e., can with stand potential inhibitors (Hodzic and Karlsson, 2017). Depending on the type of lignocellulosic hydrolysates, the composition of inhibitors will differ and their influence on the microorganisms and the fermentation performance will consequently vary (Olsson and Hahn-Hägerdal, 1996). Currently the main lignocellulosic feedstocks used for bioethanol productions include forest residue, agricultural waste resource and paper waste (Anwar *et al.*, 2014). The fermentation path way of lignocellulosic feedstocks in to ethanol is as indicated in Figure 6.

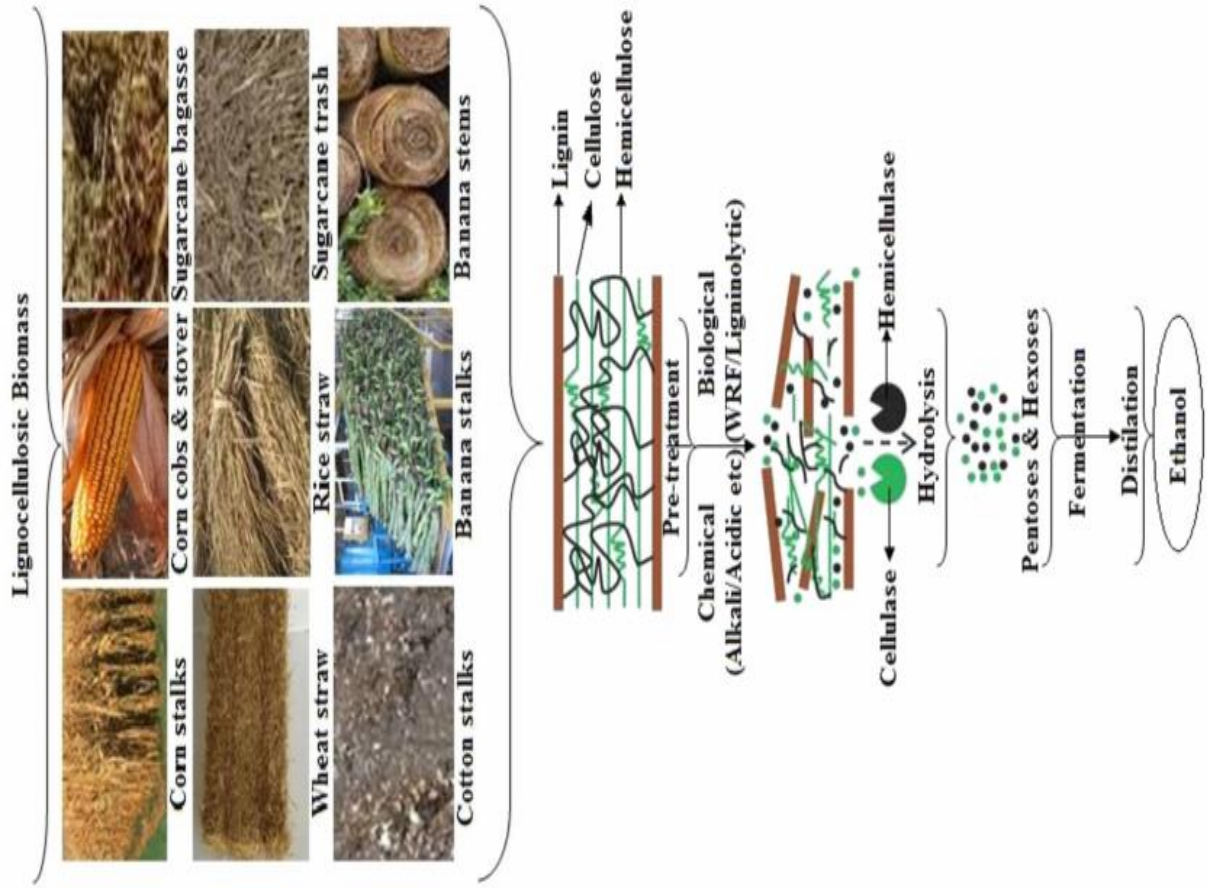


Figure 6: Ethanol fermentation path way from lignocellulosic biomass. Source: (Anwar *et al*., 2014)

2.4.4.1. Forest residues

Forest residue is one of the most cost effective, abundant, harvested year round and worldwide feedstock which makes it advantageous for bioethanol production (Hasunuma and Kondo, 2012). Forest residues, especially, those from soft wood species, are highly recalcitrant to biochemical conversion. This strong recalcitrance is removed by sulfite pretreatment. Sugar degradation to inhibitors is as reduced using a novel approach of "pH profiling" by delaying acid application in the pretreatment phase. Sever acid pretreatment step result in sugar degradation to inhibitors causing difficulty or problematic during fermentation at high solid loadings (Cheng *et al*., 2015). The same author gets improved carbohydrate yield in pretreated solids and reduced fermentation inhibitors with pH profiling resulted in a terminal ethanol titer of 48.9 ± 1.4g/L and yield of 297 ± 9L/tone, which are substantially higher, i.e., by 27% in titer and by 38% in yield, than those of a control run without pH profiling as indicated in Figure 7.

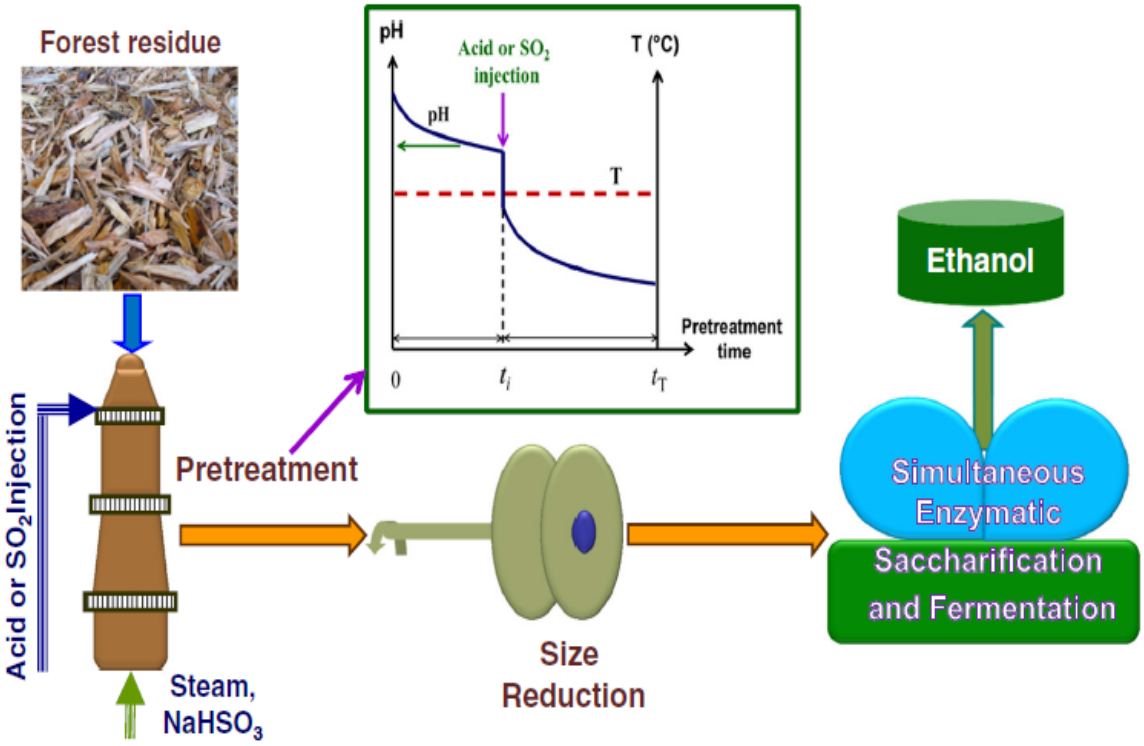


Figure 7: A schematic experimental block flow diagram with the illustration of the "pH profiling" concept in sporl for high titer and ethanol production without detoxification from a douglas-fir forest residue. Source: (Cheng *et al.*, 2015)

2.4.4.2. Agricultural waste resources

Agricultural waste resources are a good substrate for ethanol production. Agro-waste from maize plant *Zea maize*; corncob, corn stalk, corn husk was subjected to pretreatment process using acid hydrolysis to remove lignin which act as a physical barrier to cellulolytic enzymes (Braide *et al*., 2016). The same authors suggest that fermentation of hydrolysates with *Saccharomyces cerevisiae* produced a maximum ethanol concentration of 6.17, 4.17 and 3.45% (w/v), respectively after 72h of fermentation, indicating that agricultural waste material generate wealth compound from waste. The stages involved in the production of ethanol from agricultural waste product are indicated in Figure 8.

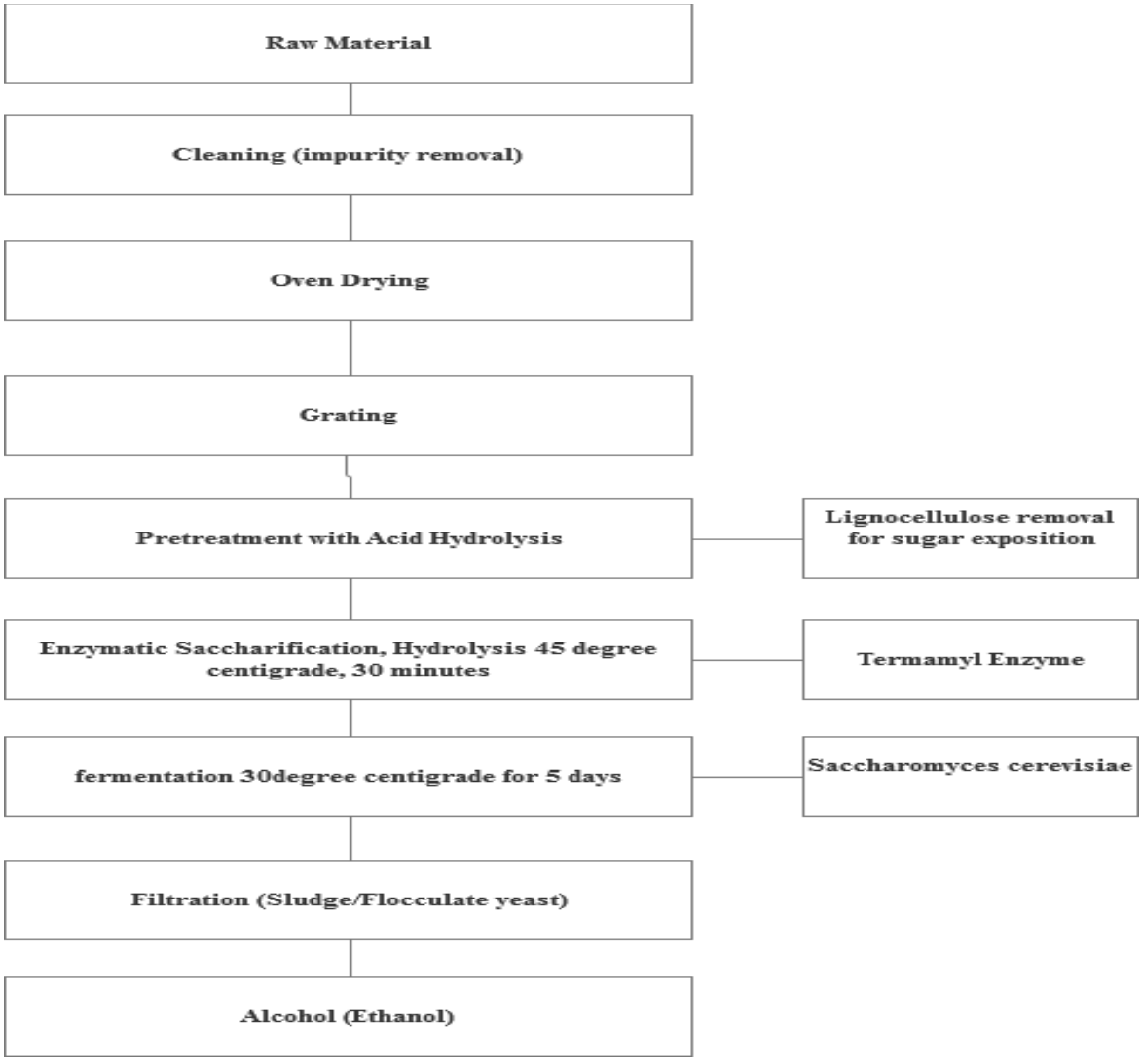


Figure 8: Flow chart indicating ethanol production from corncob, cornhusk, and cornstalk. Source: (Braide *et al.*, 2016)

2.4.4.3. Paper waste

Paper is one of the largest constituent of municipal and industrial solid waste which becomes a severe problem for disposal in developed and developing countries due to shrinking land fill capacity (Byadgi and Kalburgi, 2016). Today, managing solid waste is an important and challenging task (Badgie *et al*., 2012). Waste paper is a lignocellulosic feedstock which has the potential to be used as an excellent alternative feedstock for ethanol production. Acid and enzyme was separately used to hydrolyze the substrate (office paper and newspaper) and the hydrolysates were used to produce ethanol of 11.15 and 6.65g/L with the productivity of 0.32 and 0.28 (g of ethanol/L/h) using *Saccharomyces cerevisiae,* respectively (Sivakumar, 2017). Thus, the improved yields achieved through the pretreatment with 0.5% H_2O_2 and subsequent ethanol production suggested that the wastepaper could serve as a potential feedstock for bioethanol production.

2.4.5. Industrial wastes

2.4.5.1. Molasses

Molasses are non-crystallizable residue remaining after crystallizing sucrose, which has its own additional advantages, i.e., it is relatively inexpensive raw material, readily available and already in use for industrial ethanol production. Molasses contains about 45–50% total sugars, of which 30–33% are cane sugar or sucrose and the rest are reducing sugars (Fekadu, 2007).

Molasses is a widely used substrate for bioethanol production at global level. A combination of urea as a nutrient source and sugar in 0.25% and 20% (w/v) concentrations, respectively produced a high yield (11% (w/v)) of ethanol from sugarcane molasses (Gasmalla *et al*., 2012). Similarly from sugarcane molasses two commercial baker's yeast (New Aule alcohol yeast and New Aule baker's instant dry yeast) produced 74.8g/L and 102.854g/L with a productivity of 2.078g/L/h and 3.359g/L/h, respectively (Mayzuhroh *et al*., 2016).

2.4.5.2. Industrial food processing waste

Industrial food waste is currently processed as animal feeds and organic fertilizers (Wang *et al*., 2011). Large amounts of solid food wastes are buried in landfills, while liquid food wastes are released into public sewer systems (Beede and Bloom, 1995). The selection of an appropriate process to recover materials and energy from food wastes largely depends on the characteristics of the waste, the desired forms of bioenergy and bio-products, the economic feasibility (Wang, 2013). Although food waste (FW) can serve as a valuable substrate containing large amounts of

organic materials such as soluble sugar, starch and cellulose, it is recognized as an environmental pollutant and the hydrolysis of solids in food waste still serves as a rate limiting step in its biological processes (Moon *et al.*, 2009).

Food waste is made up of materials intended for human consumption that are subsequently discharged, lost, degraded or contaminated that has been possibly used for ethanol production (Canter *et al*., 1996). The problem of food waste is currently on an increase, involving all sectors of waste management from collection to disposal; identifying sustainable solutions extends from all contributors to the food supply chains, agricultural and industrial sectors, as well as retailers and final consumers (Williams, 2005). A series of solutions may be implemented in the appropriate management of food waste and prioritized in a similar way to waste management hierarchy. The most sought-after solutions are represented by avoidance and donation of edible fractions to social services. Food waste is also employed through industrial processes for the production of biofuels or biopolymers (Girotto *et al.*, 2015).

Most of the time industrial food waste can be disposed off without any pretreatment stage in to a nearby environment. As a result, there has been a paradigm shift recently, from disposing of waste to using it. There are several biological processing strategies that produce bioenergy or biochemical while treating industrial and agricultural wastewater, including methanogenic anaerobic digestion, biological hydrogen production, microbial fuel cells and fermentation for production of valuable products (Angenent *et al*., 2004).

2.5. Pretreatment of substrates for bioethanol production

Biomass derived from corn has become one of the primary feedstocks for bioethanol production for the past several years in the US (Nguyen *et al*., 2017). However, the argument of whether to use it as a food or biofuel has led to a search for alternative non-food sources (Christou *et al*., 2012). Consequently, industrial research efforts have become more focused on low-cost large-scale processes for lignocellulosic feedstocks originating mainly from agricultural and forest residues along with herbaceous materials and municipal wastes (Li *et al*., 2007). Although cellulosic-derived biofuel is a promising technology even if there are some challenges interfering with bioconversion processes to reach to the optimal production capacity with minimum capital for investment. Effective pretreatment is fundamental for optimal successful hydrolysis and downstream operations. Several pretreatment methods, namely, physical,

chemical or microbiological have been used to remove the recalcitrant cell wall material of lignocellulosic biomass depending on the raw material being extracted (Limayem and Ricke, 2012). Effects of such pretreatment methods are shown in Figure 9.

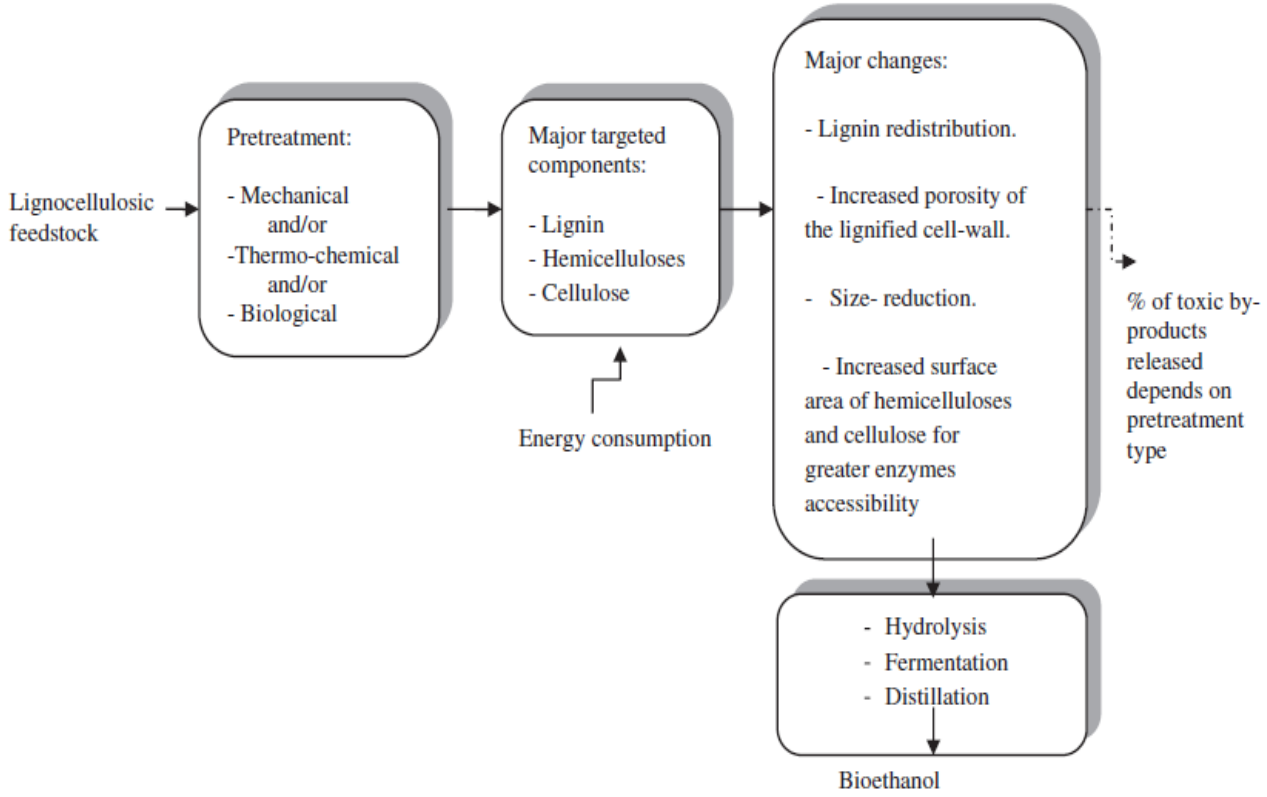


Figure 9: Pretreatment methods, process and their effects. Source: (Limayem and Ricke, 2012)

2.5.1. Physical pretreatment

Various pretreatment techniques change the physical and chemical nature of lignocellulosic feedstocks for ethanol production. Physical pretreatment does not use any chemical agents and any of biological organisms to alter the physical and chemical nature of lignocellulose (Agbor *et al*., 2011). It typically includes un-catalyzed steam explosion, liquid hot water pretreatment, mechanical break and high heat radiation, of which the former three pretreatment methods are common than the later (Zheng *et al*., 2009). The equipment for uncatalyzed steam-explosion is commercially available which rapidly heat up the biomass particles under high-pressure saturated steam to promote hydrolysis and it is one of only a very limited number of cost effective pretreatment (Kumar *et al*., 2009). The cost of ethanol production from lignocellulosic

feedstocks are relatively high based on the current pretreatment technologies due to low yield of ethanol and high cost of hydrolysis process (Alvira *et al*., 2010).

2.5.2. Chemical pretreatment

Chemical pretreatment is the most studied pretreatment technique among the other pretreatment techniques. The primary goal of chemical pretreatment is improving the biodegradability of cellulose and hemicelluloses by removing lignin and decreases the degree of polymerization and crystallinity (Pérez *et al*., 2002). The common chemicals used in pretreatment include steam-explosion, acid, base, organosolv, pH controlled liquid hot water and ionic liquids (Vallejos *et al*., 2017).

Thermo chemical extrusion, particle size reduction and alkaline pretreatments are potential pretreatments for lignocellulosic ethanol production. Yet, crystallinity index, lignin and hemicelluloses contents, total phenolic content, furfural and degree of polymerization are the main factors affecting enzymatic saccharification and fermentation. In thermo chemical treatment when lower temperature (100-130^{o}C) is used it is better to increase a pretreatment time to above 90 min (Myat and Ryu, 2016).

Dilute acid hydrolysis is a fast and easy to perform. Additionally, many scientific studies were reported in the hydrolysis of lignocellulosic feedstocks using dilute acid to produce ethanol. However, it is hampered by non-selectivity, by-product formation and has some disadvantages such as corrosion of material, neutralization of hydrolysates before fermentation, formation of degraded products, fermentation inhibitors and disposal of neutralization salts (Mosier *et al*., 2005).

2.5.3. Microbial pretreatment

Bio-conversions of lignocellulosic biomasses to ethanol are significantly influenced by the structural and chemical complexity of biomass. Cellulose and hemicelluloses were hydrolyzed in to fermentable sugars through well planned conversion technologies and fermentation process to get a high yield of ethanol (Cheng *et al*., 2015).

Instead of depending on pretreatment of substrate with different chemicals (acid, base, etc.) and separate enzyme to hydrolyze and release monosaccharides from starch and lignocellulosic feedstocks, it is possible to use different microbes in the same fermentation process to produce

ethanol. One of this methods is the use of consolidated bio-processing technique, which is a promising technology for cost competitive biofuel production by choosing and combining cellulase, lignocellulase and amylase producing microbes in single step of fermentation process in which one of the microbes degrade lignin, the other cellulose and the remaining one degrades starch (Ammara *et al*., 2017).

A polymer (cellulose, hemicellulose and lignin) is degraded by a variety of microorganisms which produce a battery of enzymes that work synergistically. To establish cost effective fermentation of lignocellulosic biomass, the consolidation of saccharifcation and fermentation process is a desirable strategy, but requires the development of microorganisms capable of cellulose and hemicellulose hydrolysis and target chemical production (Hasunuma *et al*., 2013). Such an endeavor solution requires a large number of prerequisites to be realized, including engineered microbial strains with cellulosic activity, high product yield, productivities and titers, ability to use many carbon source and resistance to toxic chemicals released during pretreatment of lignocellulosic biomass. Among many microorganisms white rot fungi (WRF) are known to break down lignin with the aid of extracellular peroxidase and laccase enzymes (Bugg *et al*., 2011). *Trichoderma reesei* is an important fungal microbes involved in bio-consolidated process (Xu *et al*., 2009). Similarly, fermentation is increased through *Aspergillus. niger and S. cerevisiae* co-culture technique (Abouzied and Reddy, 1986; Manikandan and Viruthagiri, 2010).

2.6. Production of bioethanol

Bioethanol production can be done through either microbial fermentation or industrial technologies.

2.6.1. Industrial bioethanol production

Most ethanol is manufactured at present by catalytic hydration of ethene, and in the process ethene is treated with steam at 573K and 60atm pressures in the presence of phosphoric acid, catalyst (Gokel, 2004). Industrial production of bioethanol is done petro-chemically through the acid-catalyzed hydration of ethylene (Kang *et al*., 2014). Similarly over 83% ethanol can also be produced through syngas using Cu/SiO_2 catalyst, but stability of catalyst is another problem (Gong *et al*., 2012). Ethanol production from dimethyl oxalate through syngas process is shown

in Figure 10. Additionally, less than 4% of ethanol is synthetically produced from oil, while the rest is produced by fermentation from bio resources (Gasmalla *et al*., 2012).

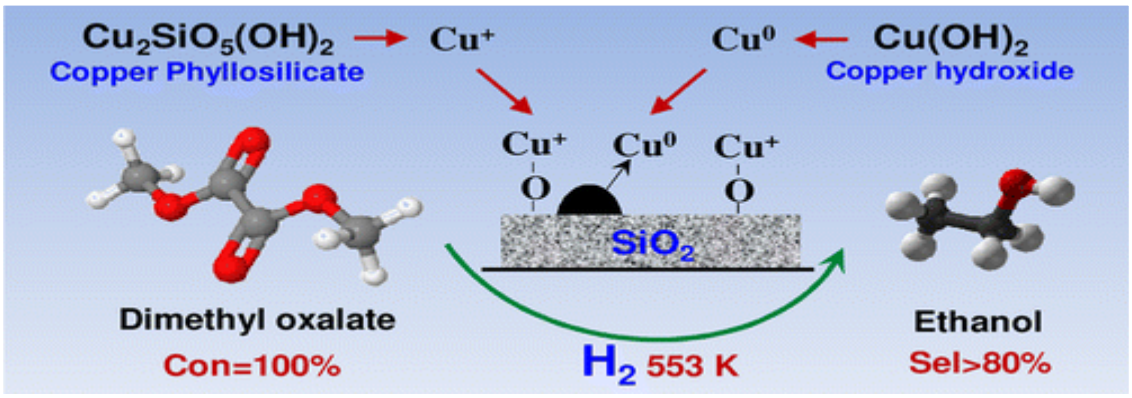


Figure 10: Ethanol production from dimethyl oxalate through syngas using Cu/SiO_2 catalyst. Source: (Gong *et al*., 2012)

2.6.2. Microbial bioethanol production

Microorganisms are good machineries to produce important biological molecules in the universe. There are several microbes existing in the environment having different catalytic efficiency and different roles. Different microbes also follow different pathway to convert one product to the other, while others may not. Therefore, isolation and selection of appropriate microbes to get the target product is an essential task. Ethanol can be produced both by bacteria and yeast (Kang *et al*., 2014). This is the reason why both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases and hemicellulases enzymes. Most emphasis have been placed on the use of fungi because of their capability to produce copious amount of cellulases and hemicellulases which are secreted to the medium for easy extraction and purification (Maki *et al*., 2009).

2.6.2.1. Yeast

Yeast is a single celled, microscopic eukaryotic fungus, which is grouped under the phylum Ascomycota. It undergoes asexual reproduction through budding and sexual reproduction through conjugation. Certain yeast strains are better adapted to the environmental conditions which is associated with producing alcoholic beverages and have evolved protective cellular membrane characteristics which induce tolerance to high ethanol concentrations (Acero and Brandao, 2017). The two commonly employed yeast for bioethanol productions are *Kluveromyces marxianus* and *Saccharomyces cerevisiae*.

2.6.2.1.1. *Kluveromyces marxianus*

Kluveromyces marxianus is commonly used yeast strains used for the production of ethanol from cheese whey (Moreira *et al*., 2015). Cheese whey is one of a by-product waste materials from dairy industry, which mainly contain 4.8% fermentable sugar (lactose) and other side components, which may is also a good substrate for production of ethanol (Ariyanti *et al.*, 2014; Das *et al*., 2016). *Kluveromyces marxianus* produce a high yield (7.9626g/L) of ethanol from whey in feed-batch fermentation process at 30^{o}C and can be improved through both mutation and genetic engineering to a superior strain; for example, *Kluveromyces marxianus* is treated in a temperature between 35^{o}C-40^{o}C and were able to get thermo tolerant species which was able to produce high ethanol using yeast extract as an additional source of organic nitrogen for the growth and reproduction of microorganisms from deprotonated whey as shown in Figure 11 (Yanase *et al*., 2010). Thermo tolerant alcohol producing yeast can grow up to 52^{o}C with a maximum growth rates in liquid culture at 40^{o}C, producing high concentration of ethanol, 5.7-7% (w/v) at 45^{0}C and 5-5.5% (w/v) at 50^{o}C when growing on 14% glucose (Banat *et al*., 1992).

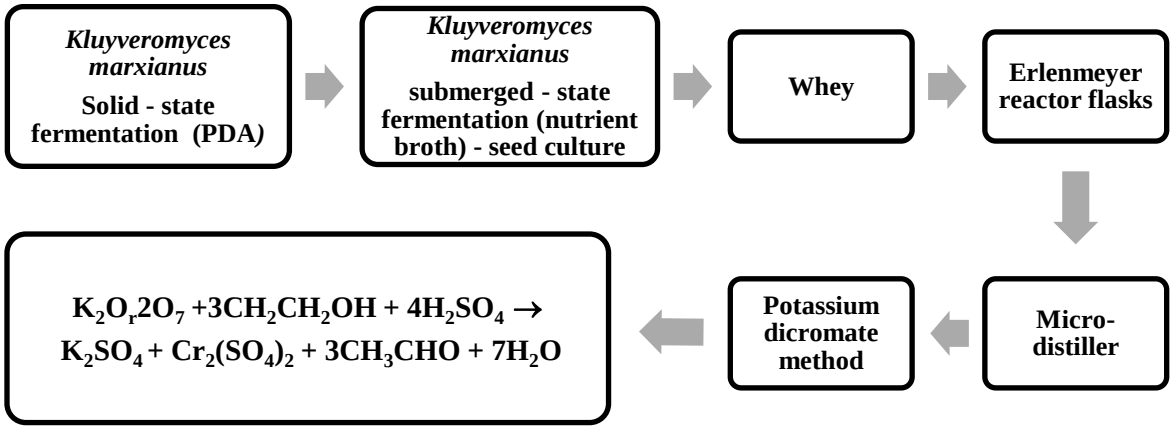


Figure 11: A general experimental design used for production of ethanol using *Kluveromyces marxianus*. Source: (Banat *et al*., 1992)

2.6.2.1.2. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is the commonly used strains in industrial microbiology and biotechnology for production of different compounds (Nofemele *et al*., 2012). Among different compounds produced by *Saccharomyces cerevisiae* one is bioethanol. Production of bioethanol is found to be the ability of yeast to break down six carbon molecules such as glucose to in two carbon components, such as ethanol, without proceeding to the final oxidation product which is

carbon dioxide (Matsakas *et al*., 2014). *S. cerevisiae* is one of the most effective ethanol producing microorganisms having several advantages including high ethanol productivity, high ethanol tolerance and tolerance to inhibitory compounds in lignocellulosic substrates (Goshima *et al.*, 2013). The optimal temperature for *S. cerevisiae* to produce ethanol range from 25-35^{o}C, but if fermentation occurs at higher temperature (40-50^{o}C), large scale commercial production become economical since it reduces contamination and is suitable in tropical countries (Karimi *et al*., 2006). *S. cerevisiae* is a good yeast converting glucose in to ethanol as indicated in Figure 12.

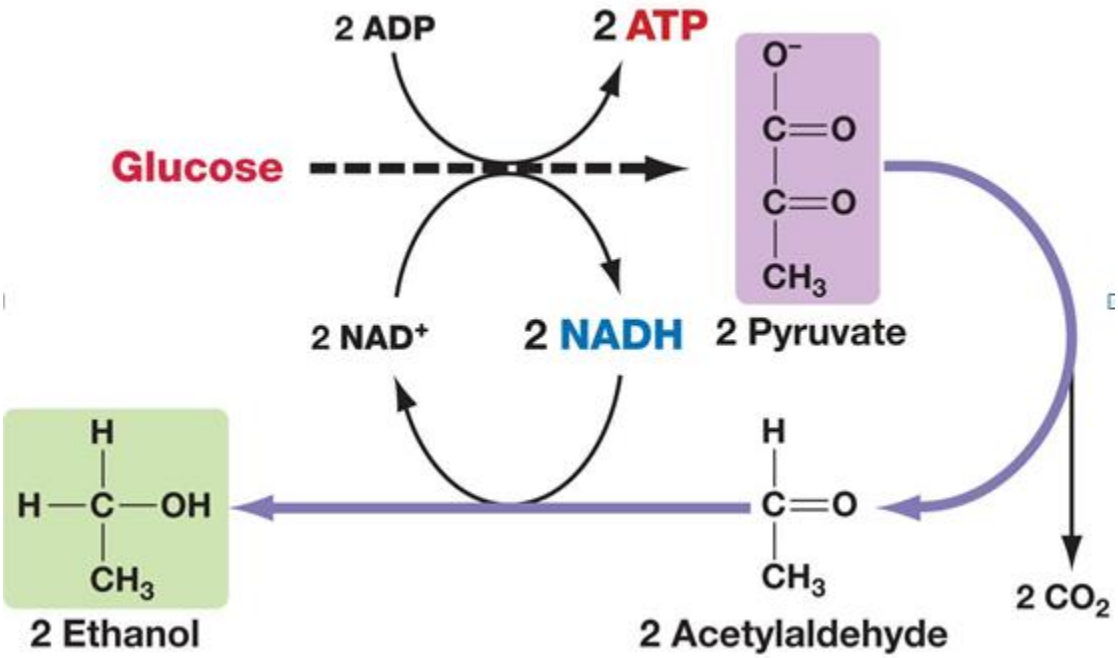


Figure 12: Alcohol fermentation of monosaccharide's using *Saccharomyces cerevisiae*. Source: (Buglass, 2011)

2.6.2.2. Bacteria

A bacterium is the other groups of microorganisms involved in fermentation of bioethanol. Currently, *Zymomonas mobilis* is a highly studied bacterium utilized for bioethanol production.

2.6.2.2.1. *Zymomonas mobilis*

Zymomonas mobilis is a desirable industrial biocatalyst with a characteristic of high ethanol tolerance and broad pH range for production (Rogers *et al*., 2007). It is common strain of bacteria used to produce ethanol from pretreated waste containing reducing sugar. *Z. mobilis* produces high yield of ethanol, from fermentation of glucose and fructose (Dien *et al*., 2003).

Similarly, Z. *mobilis* produced 16.46g/L of ethanol by using acid-steam pretreatment of deseeded sunflower head waste at a pH value of 6.5 and temperature of 30^{o}C (Sivasakthivelan *et al*., 2014).

Z. mobilis also produce ethanol from sugarcane molasses as a feedstock. About 9.3% (v/v) of bioethanol was obtained with a production efficiency of 90.5% from 16g/100 ml of sugar, at pH value of 5.0 and fermentation temperature of 34^{o}C (Khoja *et al*., 2015). The basic fuel and chemical fermentation pathway carried out by *Z. mobilis* from different biomass feedstocks is indicated in Figure 13 as of He *et al*. (2014).

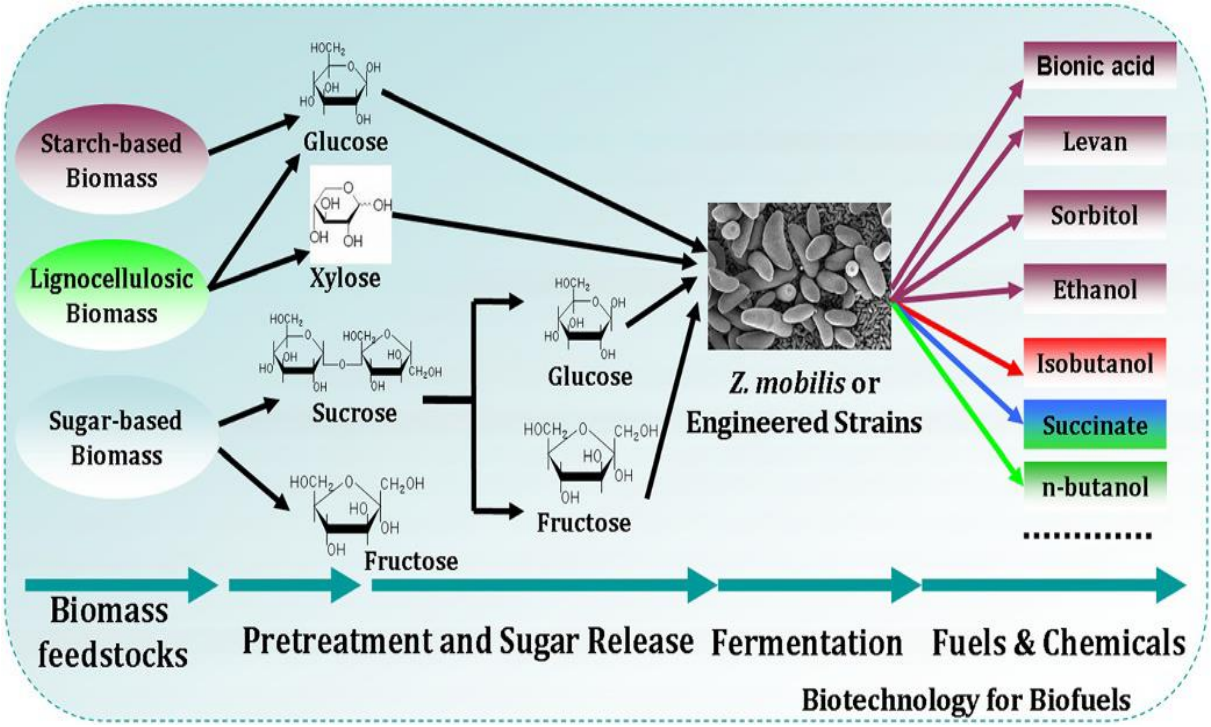


Figure 13: Fermentation of different biomass to different fuels and chemicals by *Zymomonas mobilis*. Source: (He *et al*., 2014)

Fermentations utilizing strains of *Z. mobilis*, in place of traditional yeasts, have been proposed due to their ethanol yields being close to a theoretical value. The best conditions for ethanol production were 200g/L of total reducing sugars, temperature of 30^{o}C, static culture and 48h of fermentation time to get 55.8g/L of ethanol (Cazetta *et al*., 2007).

Studies on the growth of *Z. mobilis* revealed that high concentrations of glucose (10-25%) can be efficiently and rapidly converted to ethanol in batch culture. By comparison with *S. carlsbergenesis*, *Z. mobilis* had specific glucose uptake rates and specific ethanol productivities

several times greater than the yeast. *Z. mobilis* also had ethanol yields of up to 97% of a theoretical value (Lee *et al*., 1979).

2.7. Factors affecting bioethanol production

Ethanol production can be influenced by intrinsic factors like: culturing medium, dissolved oxygen, immobilization, temperature, sugar concentration, tolerance to ethanol, organism used and other micronutrients (Fakruddin *et al.*, 2012). A maximum yield of ethanol were reached when reducing sugar concentration between 5-6%, a temperature of 30^{o}C and pH value between 4-5 was used when *S. cerevisiae* is used for production (Arroyo-López *et al*., 2009).

S. cerevisiae shows maximum specific growth rate and maximum specific ethanol production rate between 30^{o}C and 45^{o}C with different initial glucose concentration. The maximum sugar conversion at 30^{o}C after 72h of incubation is 48, 59.9, 28.3, 13.7 and 3.7% for 20, 40, 80, 160 and 300kg/m^{3} of glucose concentrations, respectively (Lin *et al*., 2012). The same author reported that highest specific ethanol production rate for all batch experiments is achieved at pH 5.0 and gave an ethanol conversion efficiency of 61.93%. The pH value in between 4-5 was the optimum range for the ethanol production process and formation of acetic acid was increased when the pH was below 4.0, while butyric acid was produced when the pH was higher than 5.0 (Lin *et al*., 2012; Narendranath and Power, 2005). The presence of oxygen affects ethanol production. In the presence of oxygen ethanol could be utilized by the yeast as carbon source after other nutrients become depleted (Lin and Tanaka, 2006).

S. cerevisiae is traditionally used for alcoholic beverage and bioethanol production; however, its performance during fermentation is compromised by the impact of ethanol accumulation on cell vitality (Nevoigt, 2008). Understanding the molecular events that occur in yeast during ethanol stress response is important, as it has been established that the stress response can be improved, resulting in more rapid adaptation to ethanol assault and increased stress tolerance (Cray *et al.*, 2015). Evidence for this lies in the acquisition of stress tolerance, where cells attain the ability to more effectively with stand severe stress conditions. Pre exposure of yeast to a sub lethal amount of stressing agent can stimulate an adaptive response resulting in transient resistance to higher levels of the same stress compared to cells without pre exposure (Stanley *et al*., 2010).

Similarly, product formation (ethanol) has an inhibitory effect on yeast cells. Ethanol levels between 0.8-1.8g/L was non inhibitory; ethanol levels between 1.9-2g/L is an inhibitory and ethanol concentration above 2g/L shows no yeast growth (Arroyo *et al*., 2010).

Lack of industrially suitable microorganisms for converting biomass in to fuel ethanol has traditionally been cited as a major technical road block to developing a bioethanol industries (Dien *et al*., 2003). In addition to this, some toxic compounds produced during hydrolysis of raw material and fermentation were removed to improve ethanol yield; however, removing toxicity will add cost and complexity to the process and generate additional waste products (Liu *et al*., 2005). So, it is necessary to isolate yeast strains that can tolerate toxic compounds and produce high yield of ethanol.

2.8. Purification of bioethanol

Among bioenergy, bioethanol is very important in many countries because it can be used directly or mixed with gasoline in combustion engines. The production of bioethanol in a fermentative process usually gives a dilute solution from which the bioethanol must be obtained in high concentration in order to be used as a biofuel (Pacheco-Basulto *et al*., 2012). A major challenge in the production of ethanol is the high energy cost associated with the separation of ethanol from the large excess of water (Simo *et al*., 2008).

2.8.1. Distillation

Fermentation by-products are mostly separated or removed by a process of distillation. Distillation is the most dominant, widely used and recognized industrial purification technique of ethanol. Distillation process utilizes the differences of volatilities of components in a mixture. The basic principle is that by heating a mixture, low boiling point components are concentrated in the vapor phase early than the other mixes. By condensing this vapor, more concentrated less volatile compound is obtained in liquid phase (Baeyens *et al*., 2015; Vander, 2007). Distillation is one of the most efficient separation techniques. However, it contains several problems. One is separation of volatile compounds. In ethanol production, a distillation tower is designed to separate water and ethanol effectively. Water is obtained from the bottom of the tower and ethanol is obtained from the top of the tower. It is expected that impurities with similar boiling points to ethanol lodges in ethanol even after distillation. The other problem is that, it is costly because distillation is a repetition process of vaporization and condensation (Awoke, 2017).

2.8.2. Ozonation

Ozone is a tri-atomic molecule containing three oxygen atoms. Ozone has strong oxidation potential and can decompose various compounds. Decomposition of compound could change both physical and chemical properties of compounds like increasing volatility, biodegradability and decrease in toxicity. Oxidation of ethanol can also be expected by ozone, which can remove impurities without a significant damage on ethanol (Onuki *et al.*, 2008). There are still some problems with the process of ozonation since it could generate new compounds or by-products which should be removed after ozonation by post- ozonation treatments (Onuki *et al.*, 2015).

2.8.3. Pressure swing adsorption

The pressure swing adsorption is attractive due to its energy and cost efficiency, increase productivity and recovery (Guan and Hu, 2003; Jeong *et al*., 2012; Pruksathorn and Vitidsant, 2009). A zeolite molecular sieve is being increasingly employed to remove the water to produce pure ethanol by a process known as pressure swing adsorption. The mixture of ethanol and water is passed through a column containing pellets of a molecular sieve of 3Å (i.e. the pores have diameters of 3 angstroms, $3x10^{-10}$m or 0.3nm).

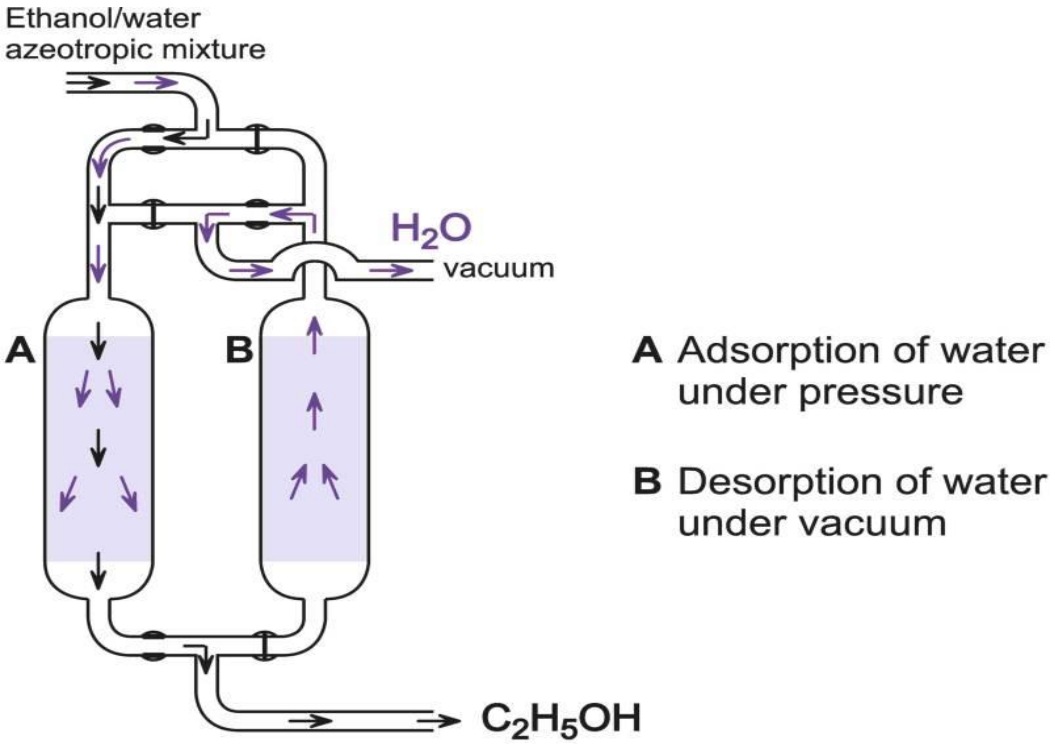


Figure 14: Purification of ethanol by pressure swing adsorption using a zeolite. Source: (Simo, 2013)

Although both ethanol and water are polar, only water molecules (diameter 0.28nm) can pass through the pores as the diameter of ethanol molecules is too large (0.44nm). Thus, the water molecules enter through the pores and are trapped in the cages of zeolite. The ethanol passes through the column and is collected (Katzen *et al*., 1999). In one method, which is usually used, the ethanol-water mixture is passed through the column is the gas phase at about 420K, under pressure (4atm) (Figure 14). The water vapor passes through the pores and is adsorbed, as a liquid, in the cages. The term 'swig' is used because two columns are in parallel. In Figure 14, the liquid solution of ethanol and water is passed through a column of zeolite and the pure ethanol is collected. After several hours, the bed is drained and heated to over 500K using a stream of heated nitrogen. Then the water is expelled (Simo, 2013).

2.9. World bioethanol production

First generation biofuel helped to demonstrate the potential for large-scale production, distribution and use of plant based fuels (Timilsina and Shrestha, 2010). However, since such fuels were and continue to be made from edible crops, first generation processes have also raised a number of concerns regarding land use choices; food vs. fuel issues and environmental impacts of vast-scale production of edible crops (RFA, 2017).

More than 85% of the world ethanol production was produced from United States and Brazil which contributes 21.8 billion gallons or 82.5 billion liters in 2011 G.C. United States was the major producer of ethanol contributing 50.3 billion liters in 2011 G.C from corn whereas Brazil produces 21.2 billion liters (Kang *et al*., 2014; Yacob, 2013).

In 2014, ePure member companies produced 6.6 billion liters of ethanol, compared to 5.8 billion liters in 2013, a production increase of approximately 13%. From this production 86% (5.6 billion liters) was fuel ethanol while 1.4% (1 billion liters) was for traditional markets, with an equal share between beverage and industrial applications (ePURE, 2015).

In 2015, the US produced an estimated 14.7 billion gallons (56 billion liters) of ethanol and 1.3 billion gallons (4.8 billion liters) of biodiesel, thus ethanol production alone was equivalent to 527 million barrels of crude oil, or 31% of US crude oil import. Ethanol production was estimated to impact 40% of US corn production and to account for 26% of US harvested cropland (Nguyen *et al*., 2017).

Global ethanol production is projected to increase modestly during the outlook period from about 115.6 billion liters in to 2015 to nearly 128.4 billion liters by 2025 (Figure 15). More than half of this increase is expected to originate from Brazil mostly to fill domestic demand and the second largest contributor to the expansion in ethanol production is Thailand (OECD, 2011). Furthermore, in the United States, ethanol production will increase in 2017 and 2018 to meet the stronger demand induced by low crude oil prices and implied higher gasoline use and then decrease slightly because of lower transportation fuels demand, but the country is expected to remain the major ethanol producer and exporter followed by Brazil (Figure 15).

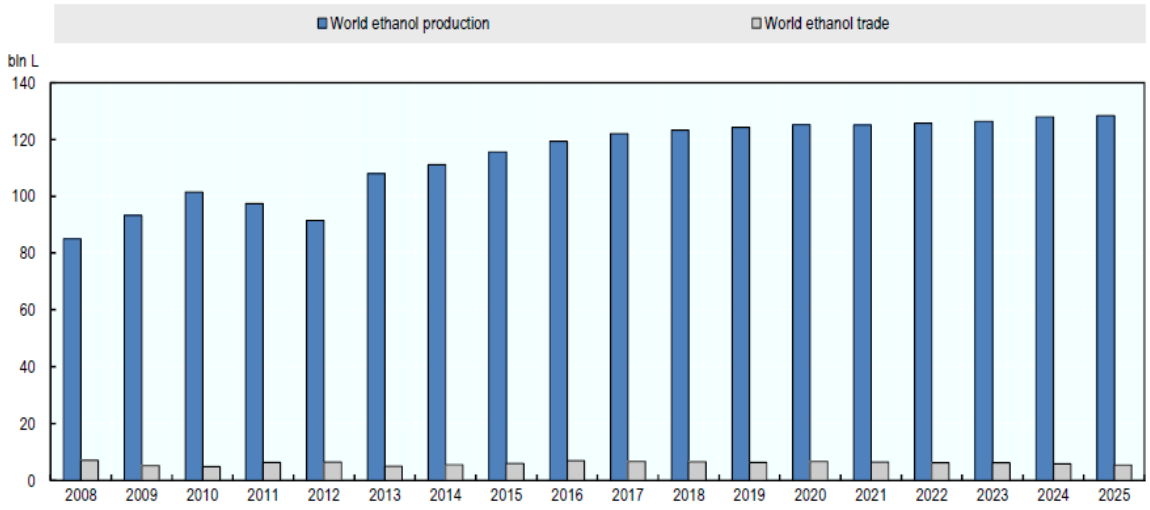


Figure 15: World ethanol production and trade. Source: (OECD, 2011)

2.10. Current status of Ethiopian bioethanol production

The energy system in Ethiopia is characterized by the predominance of biomass fuels which account for nearly 94% of the total national energy consumption (Shanko, 2009). The demand for modern energy sources such as petroleum fuels is increasing with increase in population and economic growth, even though the share of petroleum fuel is about 7% of the total consumption, the increasing demand for it and the associated price hike have hit the national economy very hard (Lakew and Shiferaw, 2008). In 2015/2016 Ethiopia imported 3,501,758.8 metric tons of refined petroleum and about 3.8 million metric tons of refined petroleum was imported for 2017/2018, i.e. gasoil, gasoline, jet A-1/kerosene, light fuel oil and heavy fuel oil (Birhanu and Ayalew, 2017).

Because of this reason, Ethiopia was among the first Sub-Saharan countries to devise a policy strategy in response to the increased demand for biofuels. However, concerned stake holder's

criticized the Ethiopian biofuel strategy policy due to the problem related with type of biofuel feedstock, the availability of land and the institutional frame work (Portner *et al.*, 2014).

As part of the bioethanol road map, the government has established a blending of 10% share of fuel ethanol in the SI-engine vehicles at the capital, Addis Ababa, where, around 70% of the imported gasoline is consumed. Additionally, a target of 15% share was set starting from 2015 so as to tackle foreign currency loss, energy insecurity and climate change (Yacob, 2013) (Table 2). However, currently this road map or target was not meet due to different challenges partly indicated below.

Table 2: Current mandate and future planned biofuel policies in Africa

Country	Current mandate	Planned target
Angola	E10	…
Ethiopia	E10	E20
Kenya	E10 (in Kisumu)	…
Malawi	E10	…
Mauritius	…	E5 (implementation date not firm)
Mozambique	E10	…
Nigeria	E10 voluntary std	E10
South Africa	E2, B5	...
Sudan	E5	…
Zambia	...	Considering E10, B5
Zimbabwe	E5 (up to E15 as energy crop production	E20

Source: (Biofuel Digest, 2016 cited from Nguyen *et al.*, 2017)

Fincha sugar factory produce 110,000 tons of sugar and 8000m^3 of ethanol or 34,782L/day with annual operation day of 230 days. It will able to produce 270,000 tons of sugar and 20,000m^3 ethanol after the expansion projects carried out on both its sugarcane plantation and sugar mill is getting finalized (Gebreegziabher and van Kooten, 2013). Metehara sugar factory produces an average of 136,692 tons of sugar and 12500m^3 of ethanol beginning from 2011 and produced 60,000L/day with annual operation day of 230 days. Through its expansion program it will be able to produce 25,500m^3 of ethanol. Wonji produce 75,000 tons of sugar, but currently no ethanol and through its expansion program it will able to produce 174,000 tons of sugar and

10,299m^3 ethanol per annum in the future. Tendaho, Kessem, Kuraz, Beles, Wolkyit and Arjo Dediessa sugar development project will produce more sugar and ethanol when they become fully operational (Yacob, 2013).

The fact is that, bioethanol production in Ethiopia goes with development of projects in sugar factory. There are about 33 biofuel projects that are under pre-implementation or implementation phase at both public and private sector engaging at investment in the country which have to produce sugar as a main product for the country and molasses as a substrate for ethanol production (Table 3).

Table 3: Summary of projects operating in Ethiopia as of December, 2012

	Ethanol	Biodiesel	Total
Number of projects authorized	8	25	33
Number of projects implemented	3	1	4
Total area authorized (ha)	0[2]	575,902	575,902
Current area under cultivation (ha)	47,280	15,000	62,280
Planned area for cultivation (ha)	430,280	575,902	1,006,182
Current output of biofuel (m^3)	20,000,000	0	20,000,000
Planned output of biofuel in (m^3)	130,316,000	NK	130,316,000

NK = not known, Source: (Locke and Henley, 2013)

2.11. Challenges of bioethanol production in Ethiopia

The government of Ethiopia has set up a state-owned Mineral, Petroleum and Biofuel Corporation with the goal of establishing biofuel as well as fossil fuel and mining operations in the country (Locke and Henley, 2013). Currently, the vast majority of biofuels used in the country come from molasses-based ethanol following different problems or challenges attempts at jatropha and castor for biodiesel (Wendimu, 2016). Biofuels and renewable energy remain a key part of the landlocked country's policies to achieve energy independence. Explorations over the past 40 years have indicated potential mineral and fossil fuel resources could be viably extracted as well. Furthermore, due to different factors such as political unrest in the fossil fuel producing countries, the price of the fuels is rising. All the aforementioned factors and other challenges lead to search for alternative fuels which are renewable, environmentally friendly and cost effective (Portner, 2013; Portner *et al*., 2014).

In Ethiopia, the country has blended a total of 59.6 million liters of ethanol during the past six years (2011-2016), saving $46.9 million of gasoline imports thanks to the E10 policy (Birhanu and Ayalew, 2017). Ethanol is produced at the Metehara and Fincha sugar factories but expansion is underway at most of the country's sugar mills (Tekle, 2008). Though Nile Oil was the first company to begin ethanol blending with E5 starting from 2008, but now Oil Libya and National Oil Company is also blending E10 with benzene starting from 2011. But, the country didn't reach its E15 goals as planned for the year 2015 due to the lack of ethanol supplies following delays in upgrading sugar mills (Lane, 2014). As a result, the E10 policy will remain in place until other policy is set in the future. In fact, the Ethiopian Sugar Corp. has 4 million liters of stock on hand, enough for almost four months' supply. But, it's still not clear if the country's car fleet can handle E15 and E20 policy, because of the shortcomings associated with type of biofuel feedstocks, availability of the land, the institutional frame work, political unrest in the country (Guta, 2012).

In addition to this, Ministry of Water, Irrigation and Energy, states that the delay in the new sugar factory projects were a major factor contributing to the shortage of bioethanol production. In line with this factor, increasing demand of bioethanol in beverage industry, cosmetic factories and low production capacities of the current factories contributed to the shortage of ethanol in the country that equally shares for the fall of E15 for the planned year (Kamp and Forn, 2016).

On July 21, 2017, Ethiopian Mineral, Petroleum and Biofuel Corporation announced that the construction of 2 ethanol production plants that consume 1.4 billion birr was started in 2017 (Gebreyohannes *et al*., 2017). The factories will help generate ethanol from waste products obtained in the process of producing sugar to be blended with petroleum. According to him, the plants would help to cut foreign currency spending. Besides, the fuel consumption of Ethiopia is growing from 10-15 percent annually. The construction of the new plants is expected to be finalized in 2019 (Tadesse *et al*., 2018).

The other most important challenge is the requirements of efficient microorganisms which are able to ferment a variety of sugars like pentoses and hexoses as well as their ability to tolerate different stress conditions (Agbogbo and Coward-Kelly, 2008). However, different genetic or metabolic engineering should be employed to improve the bioethanol producing

microorganisms in order to overcome different stress conditions or to get superior desired bacterial or yeast strains having a good capability for ethanol production (Zaldívar *et al.*, 2001). So, several rounds of modification and evaluation have to be done in near the future to come up with high yields of ethanol producing strains of microorganisms.

2.12. Screening wild yeasts for bioethanol production

A great of yeast biodiversity is found in distillery environments which could be an important source of yeast strains. This is because during yeast cell recycling, selective pressure (an adaptive evolution) is imposed on cells, leading to strains with higher tolerance to the stressful conditions of the industrial fermentation. As a result, evidence is presented showing the positive impact of selected yeast strains in increasing ethanol yield and reducing production costs, due to their higher fermentation performance (high ethanol yield, reduced glycerol and foam formation, maintenance of high viability during recycling and very high implantation capability into industrial fermenters).

Even though selecting indigenous strains is considered an attractive strategy to guarantee high product yields and population homogeneity during industrial fermentations, this approach is laborious and not always successful. As appointed by Basso *et al.* (2008) after a 12 years yeast selection program, among 14 selected strains exhibiting high performance in laboratory trails, only few presented high implantation capabilities when re-introduced to the industrial process. Probably, laboratory-screening procedures do not simulate all the stressing agents faced by the yeast in industrial-scale process. Other important factor could be the variations that occur in industrial fermentations from one season to another. It is worthwhile to point that each distillery has peculiar process features, imposing different stresses with different magnitudes to the fermenting yeast. This may explain that a given strain performs well only in a few distilleries (sometimes in only one), and just for one or a couple of seasons.

In a search for more efficient ways of producing fuel ethanol, 2 strains of yeast *Saccharomyces cerevisiae* (RA-74-2) and *Kluyveromyces marxianus* (RA-912) showing superior fermenting ability at high temperature were isolated from soils and wastewaters by an enrichment culture method. RA-74-2 was able to grow at up to 43°C and sustain similar fermenting ability from 30 to 40°C; it also tolerated 30% (w/v) glucose and 10% (v/v) ethanol, apparently higher than the tolerances of 9 industrial yeast strains used at alcohol factories at a time. When it was used to

ferment 20% w/v glucose medium at 40°C, ethanol concentration reached 86.6 g/L (85% of theoretical) in 96 h. The thermotolerant yeast RA-912 was able to grow at 48°C and showed identical growth between 35 and 45°C. On 20% glucose at 45°C the ethanol concentration reached 42.3 g/L (41.5% of theoretical) in 72 h. The results show that RA-74-2 can be adopted for ethanol production where there is significant expense on cooling, and suggest that RA-912 may be applied in either SSF (simultaneous saccharification and fermentation) or flash-fermentation process. RA-912 may be used as a gene donor for the development of thermotolerant ethanol-producing yeasts (Sohn and Seu, 1994).

Ethanol production from wheat straw (WS) hemicellulose acid hydrolysate using an adapted and parent strain of *Pichia stipites* NRRL Y-7124 showed that adaptation of the yeast to the hydrolysate resulted in further improvement in ethanol yield and productivity compared to parent strain of *Pichia stipites* NRRL Y-7124. The maximum yield was 0.41±0.01 g of ethanol per gram of wheat straw, equivalent to 80.4±0.55% theoretical conversion efficiency (Nigam, 2001).

Adaptation of the fermentation yeasts to the lignocellulosic hydrolysate prior to fermentation is suggested as an alternative approach to detoxification. Increases in fermentation rate and ethanol yield by adapted microorganisms to acid pre-treated lignocellulosic hydrolysates have been reported in some studies. Another approach to alleviate the inhibition problem is to use genetic engineering to introduce increased tolerance by *Saccharomyces cerevisiae*, for example, by overexpressing genes encoding enzymes for resistance against specific inhibitors and altering co-factor balance. Cloning of the laccase gene followed by heterologous expression in yeasts was shown to provide higher enzyme yields and permit production of laccases with desired properties for detoxification of lignocellulose hydrolysates. A combination of more inhibitor-tolerant yeast strains with efficient feed strategies such as fed-batch will likely improve lignocellulose-to-ethanol process robustness (Parawira and Tekere, 2011).

One wild strain (*Saccharomyces cerevisiae*) was selected based on its growth tolerance to simultaneous elevated temperature and AFEX™ (Ammonia Fiber Expansion) degradation products. The strain is engineered with two copies of the *Scheffersomyces stipitis* xylose reductase, xylitol dehydrogenase and xylulokinase genes. The strain is compared to the benchmark 424A (LNH-ST) strain in ethanol production and xylose fermentation in standard

lab medium and AFEX pretreated corn stover (ACS) hydrolysates. The engineered strain showed higher cell viabilities and produced a similar amount of ethanol (51.3 g/L) compared to the benchmark 424A (LNH-ST) strain (Jin *et al*., 2013).

Wild type *Saccharomyces cerevisiae* ITV-01 yeast was employed to obtain respiratory deficient (RD) which was induced by exposure to chemical agents (ethidium bromide). *S. cerevisiae* ITV-01 RD-B14 (lacking cytochrome c), obtained using bromide ethidium, had the highest alcoholic fermentation efficiency (95%) and 97% viability under the tested conditions (150 gL^{-1} glucose, pH 3.5). The respiratory deficient mutation is a tool for improving the ethanol production (Ortiz-Muñiz *et al.,* 2012).

In conclusion, it can be said that any selected strain must be evaluated in industrial conditions in as many as possible distilleries for many seasons. The most important and rare attribute of superior yeast starters is the implantation capability, since good fermentation profile (at laboratory screening experiments) is more easily found among indigenous and even laboratory strains.

2.13. Strain improvement in bioethanol producing yeast

A number of different strategies have been applied to engineer yeasts capable of efficiently producing ethanol from xylose, including the introduction of initial xylose metabolism and xylose transport, changing the intracellular redox balance, and overexpression of xylulokinase and pentose phosphate pathways (Matsushika *et al*., 2009). Genome shuffling is a powerful strategy for rapid engineering of microbial strains for desirable industrial phenotypes. industrial yeast strain SM-3 is improved by genome shuffling to increase the thermotolerance and ethanol tolerance of a while simultaneously enhancing the ethanol productivity. After three rounds of genome shuffling, the best performing strain, F34, which could grow on plate cultures up to 55°C, was obtained. It was found capable of completely utilizing 20% (w/v) glucose at 45–48°C, producing 9.95% (w/v) ethanol, and tolerating 25% (v/v) ethanol stress (Shi *et al*., 2009). Similarly, several different large-scale genome shuffling strategies to obtain novel hybrids with increased ethanol tolerance and fermentation capacity is made. Several of the novel hybrids show best-parent heterosis and outperform the commonly used bioethanol strain Ethanol Red, making them interesting candidate strains for industrial production (Snoek *et al.*, 2015).

The strategy carried out by Hou (2010) show that through sexual and asexual reproduction of a yeast to itself genome shuffling can be possible without polyethylene glycol-mediated protoplast fusion. As stated by him after three rounds of genome shuffling, the best performing strain S3-10 was obtained on the special plate containing a high ethanol concentration. It exhibits substantial improvement in multiple stress tolerance to ethanol, glucose and heat. The cycle of fermentation of S3-10 was not only shortened, but also, ethanol yield was increased by up to 10.96% compared with the control in very-high-gravity (VHG) fermentations.

Yeasts can also ferment xylose through redesigning the yeast metabolic pathway for fermenting xylose to ethanol, including cloning three xylose-metabolizing genes, modifying the genetic systems controlling gene expression, changing the dynamics of the carbon flow, etc. This has made it possible because genetically engineered *Saccharomyces* yeasts as such that they are able to overcome some of the natural barrier present in all microorganisms, such as the synthesis of the xylose metabolizing enzymes not to be affected by the presence of glucose and by the absence of xylose in the medium (Ho *et al*., 1999).

Global transcription machinery engineering (gTME) is an approach for reprogramming gene transcription to elicit cellular phenotypes important for technological applications. The gTME is made to *Saccharomyces cerevisiae* for improved glucose/ethanol tolerance, a key trait for many biofuel programs. Mutagenesis of the transcription factor Spt15p and selection led to dominant mutations that conferred increased tolerance and more efficient glucose conversion to ethanol. The desired phenotype results from the combined effect of three separate mutations in the *SPT15* gene [serine substituted for phenylalanine ($Phe^{177}Ser$) and, similarly, $Tyr^{195}His$, and $Lys^{218}Arg$] (Alper *et al*., 2006).

Ideally the most productive way of improving the whole-cell biocatalysts is by evolution of the entire cell genome. Genetic improvement techniques applied in new and optimized wine strains construction, paying particular attention to blind and whole genome strategies, such as the sexual recombination and genome shuffling (Giudici *et al*., 2005). Genetic engineering and evolutionary adaptation to increase glycolytic flux coupled with transcriptomic and proteomic studies have identified targets for further modification, as have genomic and metabolic engineering studies in native xylose fermenting yeasts (Van Vleet and Jeffries, 2009).

Thermotolerant yeast, *Kluyveromyces marxianus*, which has high growth and fermentation at elevated temperatures, was used as a producer of ethanol from cellulose. The strain is genetically engineered to display *Trichoderma reesei* endoglucanase and *Aspergillus aculeatus* β-glucosidase on the cell surface, which successfully converts a cellulosic β-glucan to ethanol directly at 48°C with a yield of 4.24 g/l from 10 g/l within 12 h. The yield (in grams of ethanol produced per gram of β-glucan consumed) was 0.47 g/g, which corresponds to 92.2% of the theoretical yield. This indicates that high-temperature cellulose fermentation to ethanol can be efficiently accomplished using a recombinant *K. marxianus* strain displaying thermostable cellulolytic enzymes on the cell surface (Yanase *et al*., 2010).

3. Materials and Methods

3.1. Raw materials

3.1.1. Grain waste flour, *tella* spent, pineapple peels and papaya peels

About 2 kg grain waste flour (F) or (a mix of different grains like corn, *teff*, sorghum, barley flour, etc.) was collected using a polyethylene plastic bag (Falcon, Saudi Arabia) from three different milling house room in Addis Ababa, Ethiopia. About 5 kg of *tella* spent (T) was kindly provided by *tella* vendors from Lega Dadi. *Tella* was brewed from soaked barley (*bikil*), roasted barley (*enkuro*), corn, sorghum and *daggusa* milled together (*yetella kitta*) which were pooled together in a jar and baker yeast were added and were allowed to ferment for three days under anaerobic condition. *Tella* spent was collected after sloping brewed *tella* (personal communication). So, it was expected to contain polysaccharides (starch, cellulose, hemicellulose and lignin) and yeasts. Stalks were separated from *tella* spent and dried in open air for a week. About 10 kg of pineapple peels (A) and papaya peels (P) were separately collected from juice processing shops from Addis Ababa, Ethiopia.

3.2. Pretreatment methods

3.2.1. Physical pretreatments

Physical pretreatments of F, T, A and P was done by grinding or milling to reduce the particle size and increase surface area, to facilitate the access of sulfuric acid to the biomass surface. A mix of grain waste flour (F), T, A and P were separately dried in open air for a week to reduce the moisture content, ground it with mortar and pestle (Porcelain, China) and sieved with 0.417mm opening sieve (Tyler screen standard scale, US), reground, further screened and about 1.5kg was stored for hydrolysis in sealed polyethylene plastic bag (Falcon, Saudi Arabia) from each of separate raw material at room temperature following the method of Sun and Cheng (2005) and Xu *et al*. (2011).

3.2.2. Acid hydrolysis

Acid hydrolysis of each separate raw materials treatment was separately done as follows. Raw material was hydrolyzed in a 250 ml flask (Bomex flak, England) loaded with 40 g sieved F, T, A and P (dry weight base) as a method described in Ačanski *et al*. (2014) and then 200 ml of 0% (control group), 1%, 1.5% and 2% dilute sulfuric acid was added as a catalyst in solid to liquid ratio of 1:5 (w/v%). Then it was incubated at 110^{o}C in a universal hot air oven (Arbon

Scientific instruments, India) for 90 min in accordance with a method described by Manzoor *et al*. (2012) and treatment design indicated in Table 4.

Table 4: Experimental design for H_2SO_4 pretreatment of substrate at 110^oC for 90 min and run at different fermentation time

Experimental code and Acid concentration	Fermentation time (h)
F/T/A/P [0%]	72
F/T/A/P [1%]	72
F/T/A/P [1.5%]	72
F/T/A/P [2%]	72
F/T/A/P [0%]	120
F/T/A/P [1%]	120
F/T/A/P [1.5%]	120
F/T/A/P [2%]	120
F/T/A/P [0%]	168
F/T/A/P [1%]	168
F/T/A/P [1.5%]	168
F/T/A/P [2%]	168

Key: F/T/A/P = separate grain waste flour (F), *tella* spent (T), pineapple peels (A) and papaya peels (P) treatment, F/T/A/P: H_2SO_4 or dH_2O was 1:5 (w/v %)

After the process of hydrolysis, the hydrolysates of each separate raw material were cooled to room temperature. The pH of the mash was adjusted to 5.0 by adding 2N NaOH and/or 2N HCl. The content of each separate treatment was filtered through cheese cloth with a pore size of (cotton cheese cloth, China). From each of separate triplicate treatments about 5 ml of mash was transferred to separate test tube. Finally, each of separate treatments was sterilized in autoclave (Selecta J.P., Spain) for 30 min at 121^oC and 15lb pressure.

3.3. Analytical methods

Grain waste flour (F) starch content analysis was done following Starch Assay Kit protocol as stated in Abcam (2012). Reducing sugar in the hydrolysates before and after fermentation of F, T, A and P was determined by the dinitrosalicylic acid (DNS) method using glucose as a standard following a protocol done by Miller (1959). Change in pH value was measured using pH meter (ATC Bante instruments, China).

3.3.1. Starch content analysis of grain waste flour

Starch standard curve preparation

In order to determine starch content in grain waste flour, first starch standard curve was prepared. Starch standard was prepared by dissolving 10 mg of starch in 1000 µl of distilled water. Starch solution (0, 40, 80, 120, 160 and 200 µl) was separately added to in separate test tubes and the volume in each separate test tube was adjusted to 1000 µl with hydrolysis buffer to form 0, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ ml of starch standard. Development mix (50 µl) was also prepared by mixing 46 µl hydrolysis buffer (50 mM sodium phosphate, pH 4.7), 2 µl developer enzyme mix (Amyloglucosidase and Glucose Oxidase) and 2 µl OxiRed Probe together in 25 ml flask. Development mix (1000 µl) was separately added to test tube containing starch and blank. The mix or solution was kept at room temperature for 30 min. by protecting it from sun light in cupboard. The assay was conducted in duplicates. After a given period of time, the absorbance of the solution was measured using spectrophotometer at 570 nm against a reagent blank. The starch standard curve was plotted against starch concentration in mg/ ml vs. OD reading at 570 nm.

Grain waste flour starch content analysis.

For starch analysis protocol both soluble and resistant starches were extracted through two steps as soluble and insoluble starch extraction.

A. Soluble starch extraction

About 1 g of grain waste flour was ground with mortar and pestle (Porcelain, China). From it any free glucose and small polysaccharide was washed off with 2 ml of 90% ethanol by warming it at 60^{o}C for 5 min in water bath (Gemmy industrial Corp, Taiwan). The contents were centrifuged at 10,000 rpm for 2 min. using centrifuge machine (Changsha Yingtai instruments, China). The pellet was kept and supernatant was decanted. The wash was repeated twice.

Soluble starch was extracted from the pellet with 2 ml of dH_2O and was heated in boiling water bath (90^{o}C) for 5 min. The solution was cooled under running tap water. Finally, the solution was spun at 10,000 rpm for 2 min. to remove insoluble materials. The supernatant was soluble starch, it was decanted to clear empty test tube and the pellets were kept in the test tube.

B. Insoluble starch extraction

The insoluble pellet from part (A) was subjected to 10 N KOH solution and was boiled in water bath (90^oC) for 5 min. The solution was cooled and neutralized by adding 2 ml of 10 N H_3PO_4 very slowly. The contents were spun at 10,000 rpm for 2 min. to remove insoluble materials. Again, the supernatant was insoluble starch and decanted to clear test tube.

For starch testing

Solution (A) and solution (B) were pooled and mixed together in order to give a total starch. From the mixed content, 1000 µl was added to 9000 µl of hydrolysis buffer (50 mM sodium phosphate buffer) and mixed together. Finally, 1000 µl of the diluted solution was transferred to test tubes from mixture. Developer mix (1000 µl) (hydrolysis buffer, developer enzyme mix, OxiRed Probe) as separately added to test tube containing starch and blank. The mix was kept at room temperature for 30 min. while protecting it from sun light by putting it in a cupboard. The absorbance of the solution was measured at 570 nm using spectrophotometer (Spectrum Lab. 725s, India) against a reagent blank. The analysis was carried out in duplicates. The percentage of starch was calculated using equation 1.

$$\textbf{[Starch in mg/ ml]} = \left[\frac{\textbf{Corrected Absorbance – y intercept}}{\textbf{Slope}}\right] \textbf{X Dilution Factor……Equation 1}$$

3.3.2. Reducing sugar analysis in each raw material hydrolysates

Glucose standard curve preparation

Glucose standard curve was prepared by dissolving a known concentration of glucose in distilled water. Glucose standard was prepared by dissolving 10 g of D-glucose in 1 L of distilled water (10,000 mg of glucose in 100 ml of distilled water). Glucose solution of 0, 25, 50, 75, 150, 200 and 250 µl were added in to separate test tubes and the volume in each test tube was adjusted to 1 ml with assay buffer (50 mM sodium acetate buffer) to form 0, 2.5, 5.0, 7.5, 15.0, 20.0, 25.0 mg/ ml of glucose standard. To each of separate test tube containing glucose standard and the blank solution 2 ml of 3, 5-dinitro salicylic acid (DNS) solution was added.

DNS solution contains: 1% DNS, 0.2% crystalline phenol and 0.05% sodium sulphite which were added simultaneously and dissolved by stirring in 100 ml of 1% NaOH solution. Finally, 2% sodium potassium tartarate was dissolved in the mixture. The contents were mixed and

boiled in water bath at 90^{o}C (Gemmy industrial Corp, Taiwan) for 5 min. The solution was cooled under running tap water. The absorbance of the solution was measured using spectrophotometer (Spectrum Lab. 725s, India) at 540 nm against a blank reagent. The glucose standard curve was plotted against glucose concentration in mg/ ml vs. OD reading at 540 nm. The spectrophotometer was turned on and run for exactly 30 min. First the blank solution was measured and calibrated to zero before the measurement of each separate treatment series. The blank was poured off from cuvette; and the cuvette was rinsed with distilled water 3 times. The solution from each separate treatment was poured to in cuvette and its OD was measured. The assay was conducted in duplicate treatments.

Reducing sugar determination from F, T, A and P hydrolysates

Raw material hydrolysates obtained through H_2SO_4 pretreatment process, reducing sugar yield before and after fermentation, change in pH after fermentation time and ethanol yield from each separate F, T, A and P treatments were determined. After pretreatment stage, the pH of each separate hydrolysates was adjusted to 5.0 and each was filtered separately using cheese cloth (cotton cheese cloth, China) to remove solid residues. About 5 ml of hydrolysates from each separate treatment was centrifuged at 10,000 rpm for 15 min. using centrifuge machine (Changsha Yingtai instruments, China) and the clear supernatant from each separate hydrolysates was taken for reducing sugar estimation following a procedure done by Sivasakthivelan *et al*. (2014).

From each separate treatment 50 µl was diluted in 950 µl of assay buffer (50 mM sodium acetate buffer) to form 1000 µl solution. DNS solution (2 ml) was added to each separate test tube containing hydrolysate to form a total volume of 3 ml solution. The solution in each separate test tubes was boiled in water bath at 90^{o}C (Gemmy industrial Corp, Taiwan) for 5 min. The solution in each separate test tube was cooled under running tap water. The absorbance of a solution in each separate treatment was read at 540 nm by spectrophotometer (spectrum Lab. 725s, India) against a reagent blank. Finally, reducing sugar content in the hydrolysates of each separate raw materials (F, T, A and P) was analyzed by applying OD reading of raw material hydrolysates to the glucose standard curve. The assay was conducted from each separate triplicate treatment. The percentage of reducing sugar was calculated using equation 2:

$$\text{[Glucose in mg/ ml]} = \left[\frac{\text{Corrected Absorbance} - \text{y intercept}}{\text{Slope}}\right] \text{X Dilution Factor……. Equation 2}$$

3.3.3. pH reading

The pH of each separate fermention medium or mash was initially adjusted to pH 5.0 using 2 N NaOH and 2 N HCl before yeast inoculation for fermentation. A change in pH value after fermentation periods of 72, 120 and 168 h were measured using pH meter (ATC Bante instrument, China). The pH meter was first adjusted using buffer, pH 4.0 and 7.0 (Crison instruments S.A, Spain)

3.4. Isolations of ethanol producing yeasts from traditional beverages

Isolations of indigenous yeast from traditional beverages was conducted in Microbiology Laboratory, Kotebe Metropolitan University, Addis Ababa, Ethiopia. All chemicals used for chemical reactions were analytical grades.

3.4.1. Collection of samples

Indigenous yeast in this study was isolated from *areki difdif* and *tej* samples. Two samples of *tej* from Lega Dadi and Addis Ababa were collected in sterile bottles. Three samples of *areki difdif* from Lega Dadi and Addis Ababa were collected using sterile sample bottle. Samples were stored in the refrigerator at 4^0C for 24 h until it was further processed for recovery of indigenous yeasts. Samples of commercial baker yeast were purchased from super market which was produced by Turkey Company in Addis Ababa, Ethiopia. *Saccharomyces cerevisiae* (no accession number) which were originally isolated (characterized and highest ethanol producer; personal communication) from fruit was obtained from Ethiopian Biodiversity Institute.

3.4.2. Isolation of indigenous yeast from *areki difdif* and *tej* samples

Peptone water was prepared by dissolving 0.1 g of peptone in 100 ml of distilled water. Peptone water (9 ml) was transferred to separate screw caped test tubes, autoclaved at 121^0C for 15 min. and kept at 4^oC in refrigerator.

From each separate sample, locally known as *areki difdif* fermented for 6 days (sample, from Addis Ababa) and 7 days (sample, from Lega Dadi), 25 g was weighted aseptically. Each weighed sample was separately transferred to separate flask containing 225 ml of sterilized

peptone water diluents and was mixed thoroughly. From each mix, 1 ml of content was transferred to series of test tube that contained diluents (9 ml) using micropipette aseptically and each sample was ten-fold diluted.

From each separate sample (*tej*), 25 ml was taken using sterile measuring pipette and transferred to separate flask containing 225 ml of sterilized peptone water diluents and was mixed thoroughly. From each mix, 1 ml of content was transferred to series of test tube that contained diluents (9 ml) using micropipette aseptically as described above.

Two of commercial dry baker yeast starter cultures; one from Turkey instant baker yeast (TIBY) and the other from Aladdin's instant baker yeast (AIBY) (0.1 g/9.9 ml) were aseptically transferred to peptone water. The culture was shaken for 20-30 seconds to separate aggregate cells and get separate single colonies on malt extract agar. It was done to compare them with yeast isolate obtained from *areki difdif* and *tej* samples during ethanol production.

Malt extract agar (MEA) was prepared and autoclaved at 121^{o}C for 15 min. After cooling at 55^{o}C in water bath, 0.1 g/L chloramphenicol was added and then aseptically poured into sterile plates in laminar flow hood. The medium was allowed to solidify, the plates were labeled accordingly and put at 4^{o}C in refrigerator for 24 h following a procedure indicated in Passoth *et al*. (2009). This was done for all samples (for three of *areki difdif*, two of *tej* and two of commercial baker yeast samples).

Prior to using the medium (MEA), it was incubated for 3 h in incubator at 30^{o}C. Then after, 0.1 ml of sample from serial dilution of 10^{-4}, 10^{-6} (*areki difdif* samples and *tej* samples) was transferred to duplicate plates using sterile microppipeter and spread uniformly over solid medium aseptically using sterile glass spreader. Similarly, 0.1 ml of sample from two of diluted commercial dry baker yeast (TIBY and AIBY) was transferred to duplicate plates using sterile micropipette and spread uniformly over solid medium aseptically using sterile glass spreader. This have been done to adapt them to different ethanol concentration. Finally, the spread of MEA plates was inverted and incubated at 30^{o}C for 48 h in incubator (J.P. Selecta Incubator, Spain).

3.4.3. Purification of yeast isolates

Morphologically different yeast colonies were grown on malt extract agar plates after 48h of incubation time. Based on their surface morphology, 15 yeasts from each of *Areki difdif* and *tej* samples was chosen for purification stage. Similarly, one colony from each of TIBY and AIBY was picked for adaptation process. Each of them was repeatedly streaked in order to purify the isolates, which were then maintained on MEA slants for immediate use and in 15% glycerol for storage at -20^{o}C. All the yeast isolates undergo screening and adaptation process using different ethanol concentrations.

3.4.4. Adapting, screening and isolating yeast for ethanol productions

Repetitive cultivations with a stepwise increase in ethanol concentration were performed as follows using the method of Dinh *et al*. (2008). Adaptation and screening process were conducted in two steps. The cultivation of isolated indigenous yeast was carried out in Yeast Peptone Dextrose medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) containing different ethanol concentrations and then the culture was transferred to fresh medium containing the same ethanol concentration (repetitive cultivation). Then after, the culture was transferred to medium containing a higher ethanol concentration (series of concentration).

The culture was challenged initially with ethanol concentration set at 2.5% (v/v) and was transferred step by step to 5, 6, 8, 10 and finally to 12%. The adaptation and screening step of each yeast isolate separately was run for 48 h at 30^{o}C followed by spread plate method by transferring a 0.1 ml of pretreated yeast culture on fresh MEA medium to check growth of yeast. Each yeast isolate showing growth was passed to next higher ethanol concentrations. First, a single colony from each pure yeast isolate was inoculated to 2.5% ethanol containing liquid medium and was allowed to grow for 48 h at 30^{o}C. Then spread plate was made on fresh MEA medium by transferring 0.1 ml of pretreated culture aseptically and incubated for 48h at 30^{o}C.

A single colony of yeast showing growth on MEA was again inoculated to fresh medium containing the same ethanol concentration (2.5%) and incubated for 48h at 30^{o}C. Finally, 0.1 ml of pretreated culture was transferred, aseptically spread on fresh MEA and incubated for 48 h at 30^{o}C before going to next higher ethanol concentration. This procedure was repeated twice for 5% ethanol containing medium. In similar ways, one colony from each yeast isolate grown on

MEA medium was aseptically transferred, to 6%, then to 8%, then to 10% and finally to 12% ethanol concentration following pretreatment and incubation at 30^{o}C for 48 h. From each step of pretreatment, 0.1 ml of pretreated yeast culture was aseptically spread on fresh MEA medium to check growth of yeast. From each grown yeast again one colony was inoculated to fresh YPD medium containing higher ethanol concentration.

Before challenging each yeast to the next higher level of ethanol concentrations (above 6%), the morphology of each yeast isolate were checked in order to confirm whether the isolate were yeast or not according to the procedure described by Kurtzman *et al*. (2011). Selected yeast isolates were also observed for characteristic like cell shape, cell size and for budding characters following the procedure indicated in Tikka *et al*. (2013) and Umeh (2016) with slight modification as shown in Figure 16. The isolates grown in above 6% ethanol concentration were selected for subsequent studies. The adaptation and selection process were not employed for strain obtained from Ethiopian Biodiversity Institute at all since the strain was used as a control and/or for comparison purpose.

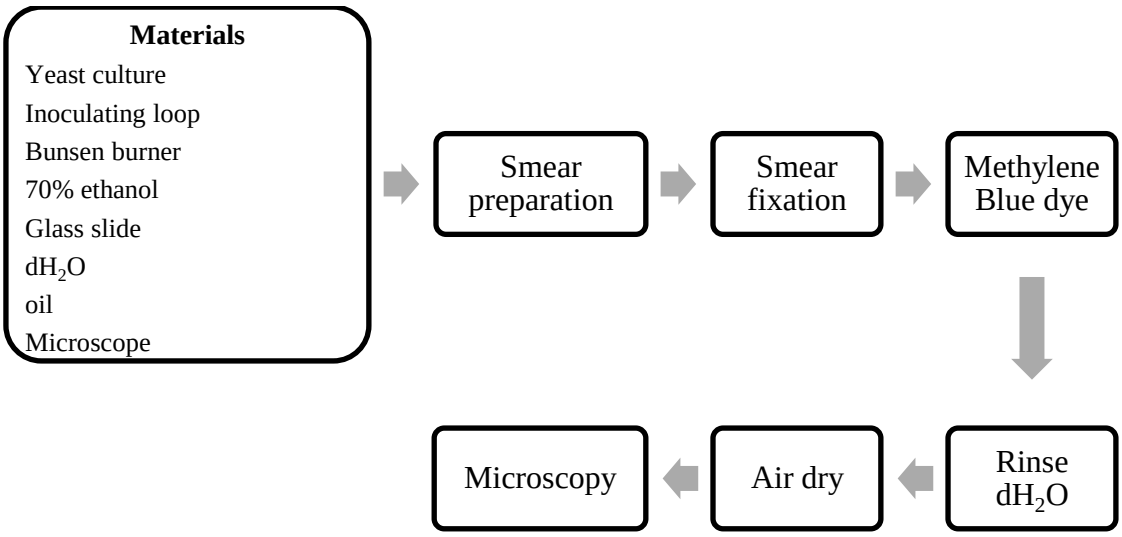


Figure 16: Microscopic staining protocols to determine shape and size of yeast isolate. Source: (Umeh, 2016)

3.4.5. Preparation and inoculation of starter culture

Streak plate was made from each of selected yeast isolates, two of commercial instant baker yeast and *Saccharomyces cerevisiae* on MEA solid medium and incubated at 30^{o}C for 48h. Yeast peptone dextrose (YPD) broth medium which was composed of 10 g/L of yeast extract, 20 g/L of peptone and 20 g/L dextrose was prepared. A single colony from each of selected pure

isolate (n=12), commercial baker yeast (n=2) and *Saccharomyces cerevisiae* was separately inoculated to 25 ml of YPD broth. They were labeled to reflect their source or origin as Ad for *areki difdif*, T for *tej*, AIBY for Aladdins instant baker yeast, TIBY for Turkey instant baker yeast and Biodiversity for *Saccharomyces cerevisiae*. The numbers before the abbreviation (e.g. 1Ad) indicates the sampling number and the number after the abbreviation (Ad-8) shows the isolate number, accordingly. The isolates were named 1Ad- 8, 1Ad- 10, 1Ad- 12, 2Ad- 8, 2Ad- 10, 3Ad- 10, 3Ad- 12, 1T- 8, 1T- 10, 1T- 12, 2T- 8, 2T- 12, AIBY- 10 and TIBY- 12. The medium was incubated for 48 h at 30^{o}C.

3.4.6. Determination of ethanol producing ability

All the yeast isolates grown on above 6% ethanol concentration were selected to produce ethanol. Ethanol yield from fermentation of D-glucose using each yeast isolate was measured by specific gravity method. D-Glucose solution (20%) was used as a medium for bioethanol production and a total of 200 ml of the medium was transferred to fermentation flask with a capacity of 500 ml (Pyrex flask, England). The medium was autoclaved at 121^{o}C for 15 min. and was cooled to room temperature. From each of selected yeast (n=12), commercial baker yeast (n=2) and *Saccharomyces cerevisiae* starter culture (20 ml) (2.07x10^{8}-2.8x10^{8}cfu/ml) was separately inoculated to a glucose solution in a separate flak aseptically for the test of ethanol production as a procedure indicated in Tropea *et al*. (2014). The medium was incubated at 30^{o}C for 120 h of fermentation time following the protocol given by Chatterjee *et al*. (2011). The assay was conducted in duplicate. Efficiency of yeast which was calculated using equation 3.

$$\textbf{Efficiency in } \% = \frac{\textbf{Actual ethanol yield}}{\textbf{Theoretical ethanol yield}} \textbf{X 100}\% \quad \textbf{Equation 3}$$

Theoretical ethanol yield was calculated from sugar degradation equation 4 as indicated in Hodzic and Karlsson (2017).

$$\underset{\textbf{1.11 g}}{\mathbf{C_6H_{12}0_6}} \longrightarrow \underset{\textbf{0.567 g}}{\mathbf{2C_2H_5OH + 2CO_2}} \quad \textbf{Equation 4}$$

3.4.7. Fractional distillation setup and process

To carry out the fractional distillation process, fractional column was fixed on a stand with clamp, at the top of fractional column with a thermometer was inserted. Through the other joint of fractional column, a condenser was connected with slight horizontal inclination, then a receiving volumetric flask was connected to the other end of the condenser. Thereafter, fractional distillation was carried out for fermented broth obtained from each of yeast isolate in order to separate ethanol. To separate ethanol from fermented broth, about 200 ml of fermented broth, 30 ml of distilled water and a few pieces of marble chips (calcium carbonate, China) was transferred into a 500 ml of distillation flask (Pyrex, 500 ml, England). The distillation flask was connected to the fractional column with J-SlL, 24/29 joint and the content was heated using a temperature between 78-90^{o}C. The distillation process was run for 35 min. and the distillate was collected in 75 ml volumetric flask till the volume almost reached the mark. The distillate was brought to room temperature and mixed thoroughly as indicated in FSSAI (2015).

Similarly, ethanol in all separate fermentation broth or F, T, A and P was recovered at the end of 72, 120 and 168 h fermentation periods. Before running a process of distillation first, the pH of each separate treatment was measured using pH meter (ATC Bante instrument, China). About 5 ml of solution from each of separate hydrolysates was taken to in a clean empty test tube for estimation of reducing sugar left in mash after fermentation.

3.4.8. Calculation and measurement of ethanol concentration

Clean, dry and empty pycnometer (50 ml, J569 lx, China) with stopper was weighed on electronic balance (Electronic Balance Scale, Oahu's Corporation Florham Park. N.J.7932, Switzerland) at 20^{o}C and recorded as (W). Then it was filled with distillate up to the brim and the stopper was gently inserted. The liquid spilled out was wipe out with water absorbing filter paper (SAN 3mm absorbent filter paper, China), weighed at 20^{o}C and was recorded as (W1). Next, distillate was poured off and pycnometer was washed with distilled water. Pycnometer was filled with distilled water in the same manner, weighed at 20^{o}C and recorded as (W2). The specific gravity of the distillate was calculated from equation 5 as stated by Itelima *et al*. (2013).

Specific gravity = $\frac{W1-W}{W2-W}$ **……Equation 5**

Where,

W - is the weight of empty pycnometer

W1 - is the weight of pycnometer and distillate

W2 - is the weight of pycnometer and distilled water

Finally, the corresponding alcohol percent by weight was taken from the table showing Specific gravity Vs Alcohol percent (FSSAI, 2015; IUPAC, 1965). The distillates specific gravity in all separate treatments (F, T, A and P) was also calculated and measured in the same manner.

3.5. Yeast inoculums preparation for fermentation of raw material hydrolysates

Based on ethanol yield testing (7.72%), the yeast isolate 1T-10 was selected for ethanol production from each raw material hydrolysates. The selected yeast culture was maintained on agar slant at 4^{o}C, consisting of malt extract (3 g/L), yeast extract (3 g/L), peptone (5 g/L) and agar (20 g/L) in 1L of distilled water. Before using the isolate for the fermentation experiment, the isolate was grown in 1000 ml fermentation flasks at 30^{o}C for 48h in a liquid media which consisted of glucose, 1 g/L; yeast extract, 3 g/L; peptone, 3.5 g/L; KH_2PO_4, 2 g/L; $MgSO_4.7$ H_2O, 1 g/L; NH_4Cl, 2 g/L at pH, 5.0 following the method described in Mojović *et al*. (2006). The number of cells in the starter culture was calculated after serial dilution and plating method.

3.6. Fermentation of grain waste flour, *tella* spent, pineapple peels and papaya peels hydrolysates for ethanol production

The hydrolysates of F, T, A and P that obtained through acid hydrolysis were subjected to fermentation by yeast isolate (1T-10) for ethanol production under anaerobic conditions in sealed flask. Batch fermentation type was carried out in the study. Yeast inoculation was carried out following the procedure of Liguori *et al*. (2015) with few modifications. Actively growing yeast culture (starter, $2.63x10^{8}$cfu/ml), 20 ml was aseptically transferred to each treatments of sterilized mash in 1:10 ratio. The fermentation time was run for 72, 120 and 168 h at 30^{o}C as a procedure indicated in Cutzu and Bardi (2017). The entire assay was performed in triplicate.

Frame work of the study design

Figure 17 below indicates the entire study design followed to run the experiments to produce ethanol from each of separate raw material (F, T, A and P) hydrolysates.

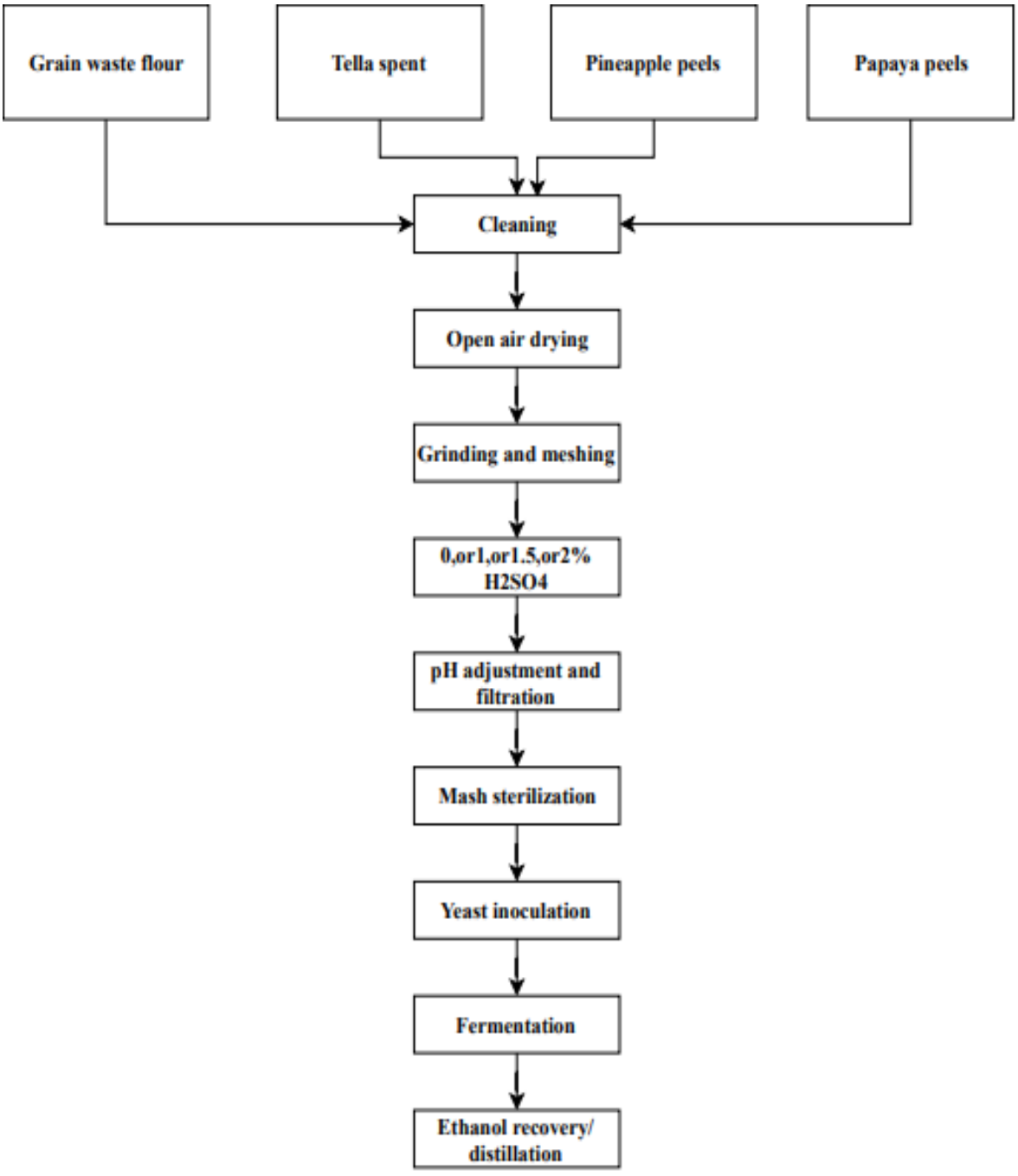


Figure 17: A general frame work of the study

3.7. Data analysis

A two-way ANOVA analysis was carried out to determine if there was significant difference among yeast isolate in ethanol production. Similarly, a two- independent factor (3 x 4) analysis of variance was conducted to evaluate the effect of Fermentation time (FT) with three levels (72, 120 and 168 h) and Acid concentrations (AC) with four levels (0, 1, 1.5 and 2%) in the production of ethanol from F, T, A and P using selected yeast isolate using LSD (p=0.05) for initial analysis. Analysis of variance was performed using Sigma plot software version 12.5. The Normality Test (Shapiro-Wilks test) and homogeneity of variance test was checked whether the assumptions underlying of the two-way ANOVA was met or not for each of separate ethanol yield produced from F, T, A and P. The AC and FT simple main effect ANOVA was also conducted to check whether there is a significant difference among each of acid concentration at each levels of FT and vice versa. In this ANOVA analysis, the Tukey test was applied, which is considered to be one of the most conservative *posthoc* comparison test (Howell, 2009) for pair wise mean comparison between the treatments. The dependent variable was ethanol percentage, with higher percentage indicating that higher yield of ethanol production. The result was given as mean ± SD, percentage and data were presented in the form of Tables and Figures.

4. Results and Discussion

4.1. Isolation and selection of indigenous yeast

Yeasts used in this study were isolated from locally made traditional beverages (*tej*) and another from locally made *areki difdif* as shown in Table 5. From each sample of *areke difdif*, 15 morphologically different yeasts were (based on the surface, pigmentation, size and margin of colonies) picked for screening. In similar ways, 15 different colonies were picked for selection using different ethanol concentration. A total of 75 yeast (n=45 from *Areki* samples; n=30 from *tej* samples) isolates was picked from countable plates containing about 30-300 colonies. Out of 75 yeast isolates; 58, 17 and 15 yeast isolates were found to be tolerant to 2%, 5% and 6% ethanol, respectively.

4.2. Morphological characterization of selected yeast

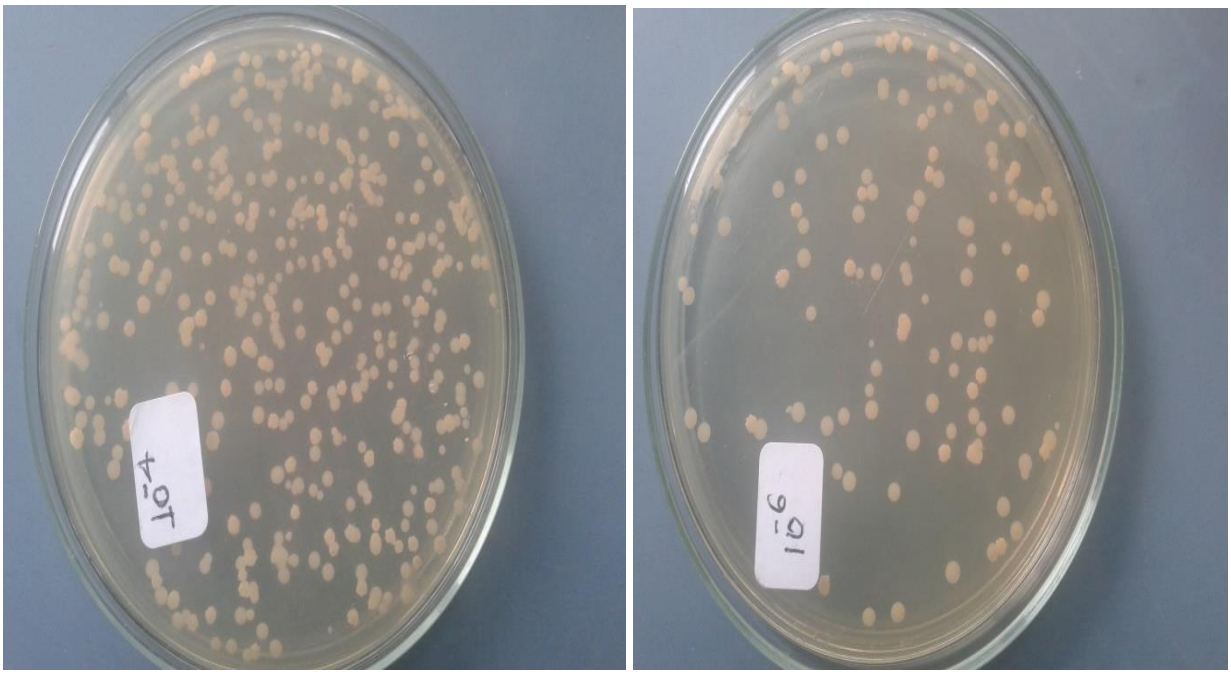
Colony morphological characters were used for preliminary identification of the selected yeast isolate (Table 5). All of the fifteen isolates selected and/or under selection with smooth or rough, white or creamy and oval or round colonies on MEA were found to be yeasts (Table 5 and Figure 18-20). Morphological characteristics of all the selected yeast isolates are shown in Table 5.

Table 5: Morphological characteristics of the 15 selected yeast isolates from *areki difdif* and *tej* samples grown in equal to or above 6% ethanol concentrations

		Colony features			
Source of yeast	Isolate code	Surface	Margin	Pigmentation	Shape and Size
Areki difdif	1Ad- 8	Rough	Irregular	Creamy White	Oval, Small
sample one	1Ad- 10	Smooth	Circular	White	Oval, Medium
	1Ad- 12	Smooth	Circular	White	Round, Medium
Areki difdif	2Ad- 8	Rough	Irregular	Creamy White	Oval, Small
sample two	2Ad- 10	Smooth	Circular	White	Round, Small
	2Ad-12	Rough	Irregular	White	Round, Large
Areki difdif	3Ad-8	Rough	Irregular	White	Oval, Medium
sample three	3Ad- 10	Smooth	Circular	White	Round, Medium
	3Ad- 12	Rough	Irregular	Creamy White	Oval, Small
Tej sample one	1T- 8	Smooth	Circular	White	Oval, Medium
	1T- 10	Smooth	Circular	White	Oval, Large
	1T- 12	Smooth	Circular	White	Ellipsoidal, Medium
Tej sample two	2T- 8	Rough	Irregular	Creamy White	Oval, Small
	2T-10	Rough	Irregular	Creamy White	Circular, Small
	2T- 12	Smooth	Circular	White	Ellipsoidal, Medium

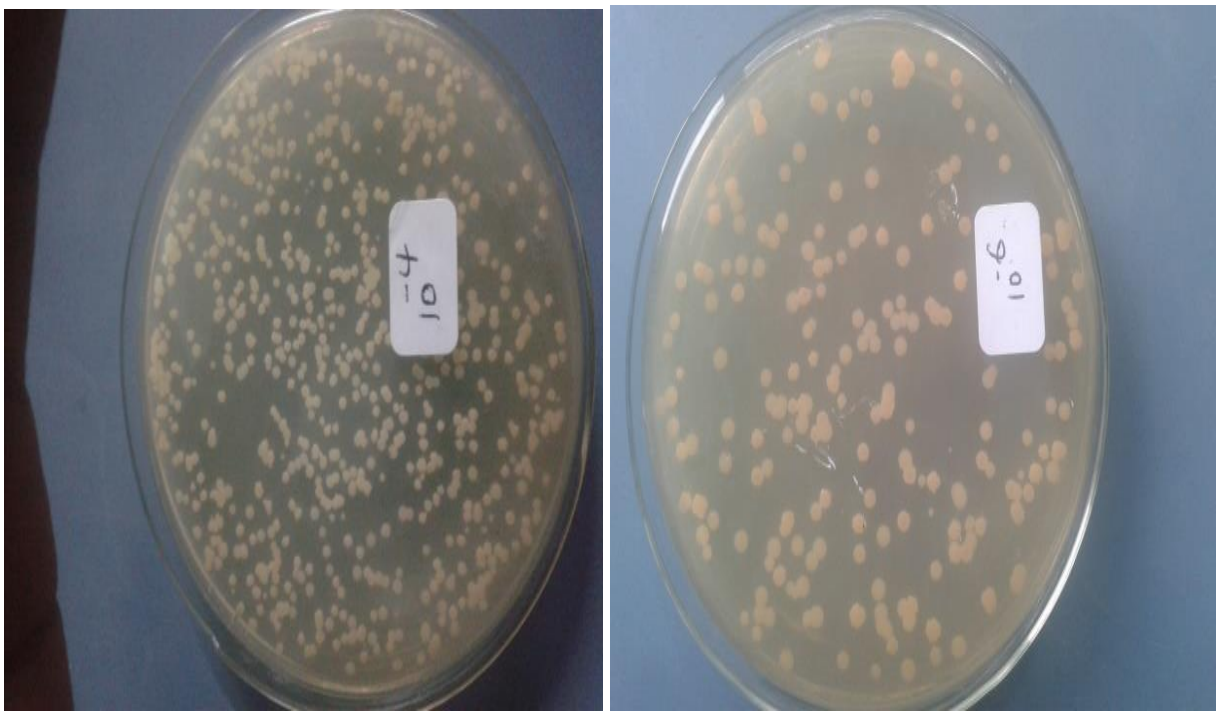
The study revealed that yeast isolates from *tej* and *areki difdif* sample displayed common morphological characters with theoretical yeast used for making beverages. The surface of colony was found to be smooth and rough, with circular and irregular margin (Table 5). They appeared to be white and cream white in color on malt extract agar (Figure 18 and 19). Staining of colony from each pure isolate using methylene blue showed budding character, oval and round shape with very small to large sized cells (Table 5). For instance, staining of single colony from 1A-10 yeast which was the second highest ethanol producer and its colonial features were characterized as smooth surface, circular margin and whitish pigment; and microscopically as medium sized oval yeast as shown in Figure 20A. In similar way, the colonial feature of the yeast chosen in this study, 1T-10 for production of ethanol from raw materials fermentation (F, T, A and P) had smooth surface, circular margin and whitish color and microscopically characterized as large sized oval yeast as shown in Figure 20B. S*accharomyces cerevisiae* is frequently used yeast to produce bioethanol with Ellipsoidal shape and budding morphological characters (Saito *et al*., 2004).

However, yeasts used in this study passed through different ethanol concentration treatment and show smooth surface, circular margin and whitish color and microscopically characterized as large sized oval yeast. The shape may be changed due to ethanol effect on the surface of cell membranes. The result has been supported by Casalone *et al.* (2005) that colony phenotype was exhibited following different environmental stimuli and are thought to be some strains grew with peculiar colony morphologies under different environmental stimuli and some showed colony morphology variations. The isolates from *tej* and *areki difdif* show different morphological characters. Additionally, ellipsoidal shape, with different yeast size distributions and populations kinetics (single or budding) are displayed by different isolates used for ethanol fermentations (Pons *et al.,* 1993).



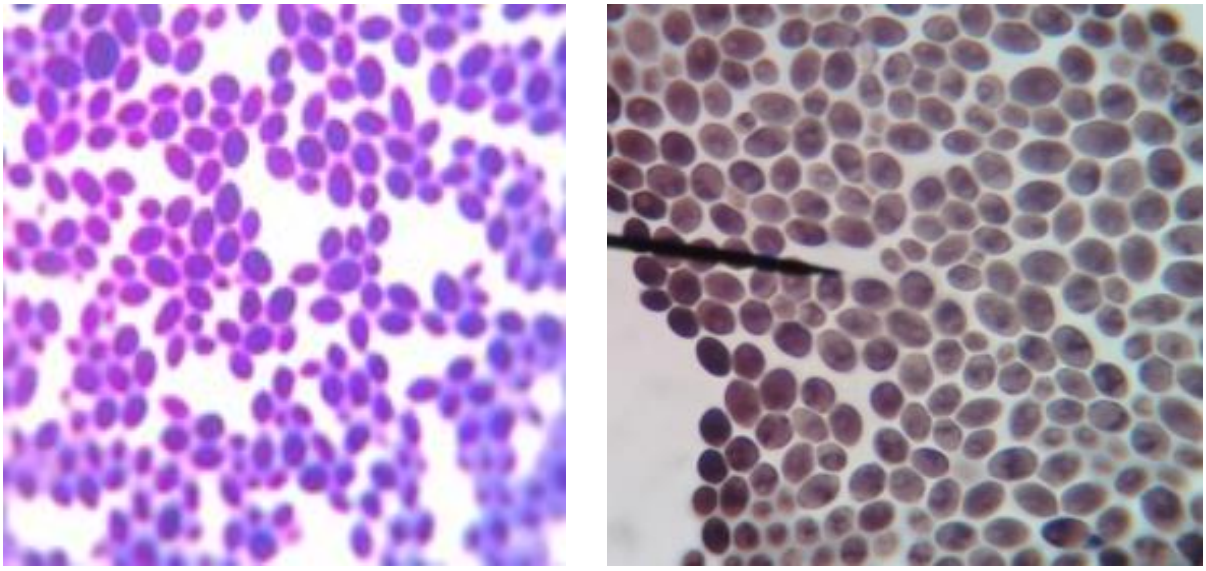
A B

Figure 18: Colonies of yeast isolates from *tej* on malt extract agar



A B

Figure 19: Colonies of yeast isolate from *areki difdif* on malt extract agar



A B

Figure 20: Microscopic observation of yeast cells in *areki difdif* (A) and *tej* (B)

4.3. Further screening and adaptation tests

The results in Table 6 show that out of 75 yeast isolates only 15 of them were grown at 6% ethanol concentration. Out of this, 12 (16%), 8 (10.67%) and 4 (5.33%) were able to grow at 8%, 10% and 12% (v/v) ethanol treatment, respectively.

Table 6: Yeast screening and adaptation tests

Source of yeast	Isolate code	Screening and adaptation process					
		Ethanol concentration in % (v/v)					
		2.5%	5%	6%	8%	10%	12%
Areki difdif sample one	1Ad- 8	+	+	+	+	-	-
	1Ad- 10	+	+	+	+	+	-
	1Ad- 12	+	+	+	+	+	+
Areki difdif sample two	2Ad- 8	+	+	+	+	-	-
	2Ad- 10	+	+	+	+	+	-
	2Ad- 12	+	+	+	-	-	-
Areki difdif sample three	3Ad- 8	+	+	+	-	-	-
	3Ad- 10	+	+	+	+	+	-
	3Ad- 12	+	+	+	+	+	+
Tej sample one	1T- 8	+	+	+	+	-	-
	1T- 10	+	+	+	+	+	-
	1T- 12	+	+	+	+	+	+
Tej sample two	2T- 8	+	+	+	+	-	-
	2T- 10	+	+	+	-	-	-
	2T- 12	+	+	+	+	+	+
Aladdins instant baker yeast	AIBY- 10	+	+	+	+	+	-
Turkey instant baker yeast	TIBY- 12	+	+	+	+	+	+
Saccharomyces cerevisiae	Biodiversity	control					

Eight of the yeast isolates, namely, 1Ad- 10, 1Ad- 12, 2Ad- 10, 3Ad- 10, 3Ad- 12, 1T- 10, 1T- 12 and 2T- 12 grew in up to 10% (v/v) ethanol concentration. Furthermore, yeast isolates 1Ad- 12, 3Ad- 12, 1T- 12, 2T- 12 and TIBY-12 (baker yeast) grew in 12% (v/v) ethanol concentration. Similar results were also presented by Piper (1995) that yeast ethanol stress tolerance could be increased through increasing their treatment above a threshold level of 4-6% (v/v) for strong induction. In addition, a study done by Zerihun (2016**)** showed that two isolates of indigenous yeast (Y3 and Y4) isolates from various fruits tolerated 10% ethanol concentration. On the basis of this test, a total of 15 yeast isolates (n=12 isolates grown in equal to or above 8% ethanol treatment), TIBY- 12, AIBY- 10 and *Saccharomyces cerevisiae* was chosen for their ability of ethanol production.

4.4. Selection of yeast for ethanol production

Ethanol producing ability of selected indigenous yeast isolates, TIBY- 12, AIBY- 10 and *Saccharomyces cerevisiae* from 20% D-Glucose as a carbon source at pH 5.0 was given in Table 7. Actual ethanol production yield in weigh percentage for yeast isolates 1Ad- 10, 1Ad- 12, 1T- 8, 1T- 10, 1T- 12 and TIBY- 12 using only D-glucose as a carbon source of ethanol at 120 h of fermentation time, at pH, 5.0 and 30^{o}C was found above 5% (Table 7).

Table 7: Ethanol yield in weight percentage from fermentation of 20% D-glucose by yeasts at pH, 5.0 and 30^{o}C

Source of yeast	Isolate code	AE%	TE%	E in %
Areki difdif sample one	1Ad- 8	3.55 ± .04	10.216	34.75
	1Ad- 10	6.52 ± .04	10.216	63.82
	1Ad- 12	5.21 ± .00	10.216	50.99
Areki difdif sample two	2Ad- 8	2.75 ± .04	10.216	26.92
	2Ad- 10	3.49 ± .04	10.216	34.21
Areki difdif sample three	3Ad- 10	4.53 ± .00	10.216	44.34
	3Ad- 12	3.67 ± .04	10.216	35.92
Tej sample one	1T- 8	5.30 ± .04	10.216	51.89
	1T- 10	7.72 ± .04*	10.216	75.58*
	1T- 12	5.08 ± .00	10.216	49.72
Tej sample two	2T- 8	3.32 ± .04	10.216	32.49
	2T- 12	4.87 ± .04	10.216	47.67
Aladdins instant baker yeast	AIBY-10	4.44 ± .04	10.216	43.46
Turkey instant baker yeast	TIBY-12	5.68 ± .04	10.216	55.59
S. cerevisiae	Biodiversity	3.38 ± .04	10.216	33.08

Key: Data is in mean ± SD of duplicate measurements, AE% = actual ethanol in weight %, TE% = theoretical ethanol in weight %, E = efficiency of yeast in %, *= significantly different

On the other hand, the other yeast isolates were found producing ethanol between 3-5%. Isolate 1Ad- 10 and 1T- 10 were observed to have actual ethanol production yield by weight as 6.52% and 7.72%, respectively. Similarly, isolates 1T- 10, 1Ad- 10 and TIBY- 12 displayed 75.58%, 63.82% and 55.59% of glucose to ethanol conversion efficiency, in that order. A result reported by Armanul *et al.* (2017) presented that indigenous yeast isolated from rotten fruit peel had the highest ethanol production capacity of 7.39% and 5.02% for pineapple and orange, respectively, at pH, 5.0 and 30^{o}C temperature in media with an initial reducing sugar concentration of 6.5% for pineapple and 5.5% for orange (with shaking).

Theoretically, it was known that most of the baker yeasts grow poorly at 4-5% ethanol (Attfield, 1997). However, fermentation carried out in this study showed that baker yeast (TIBY- 12) produced higher ethanol yield 5.68% (w/w). A possible explanation for this difference is that through adaptation, commercial baker yeast cells can gradually respond to the ethanol stress and increase their tolerance. Among all yeast isolates, 1T-10 (isolate from *tej*) was the most resistant yeast to ethanol (10%) and produced high yield of ethanol, it one was used for subsequent ethanol fermentation. A work done by Groves (2009) has also reported that yeast adaptation resulted in 2.6 fold increase in ethanol production compared to the unadapted parent strain. Adaptation is an effective and inexpensive means of improving ethanol yields (Nigam, 2001).

Yeast species are known to differ in their ability to produce and tolerate ethanol and although this phenomenon has been studied over the last few decades, the reason why some strains are more tolerant to ethanol than others remains unclear (Bai *et al*., 2008). But a recent study showed that *S. cerevisiae* possesses diverse strategies to counteract the stress produced by high ethanol concentrations such as changing the membrane composition to antagonize membrane fluidization (by increasing levels of ergo sterol); expression of factors that stabilize and/or repair denatured proteins; synthesis of proteins involved in the expression of stress-related genes; and increase in plasma membrane ATPase activity which counteracts the ethanol-induced proton influx (Nicolaou *et al*., 2010; Rocha, 2013). *S. cerevisiae* is recognized as the most predominant yeast in alcoholic beverages production and especially brewing yeast, where, in the later stages of fermentation, high ethanol levels 14.2% (v/v) are reached (Kabak and Dobson, 2011).

4.6. Starch content analysis in grain waste flour

Results of starch standard obtained from experimental studies was given in Appendix 1 and based on the standard generated, the starch content of grain waste flour was found to be 60.62%. This result is in agreement with results of other studies in which about 60.0% and 62.0% of starch was found in rice and wheat flour, respectively (Neves *et al*., 2006) and between 59.18-62.73% of starch was found in wheat and buckwheat flours (Ačanski *et al*., 2014). The biomass composition varies depending up on crop variety, agronomic practices used during cultivation, stage of harvest, climatic and geological condition of area from where samples are collected and post-harvest storage conditions (Sree *et al*., 1999).

Starch is a long chain homopolymer of D-glucose, which is hydrolyzed to obtain glucose syrup suitable for ethanol production (Öner *et al*., 2005). Acid hydrolysis is an important chemical modification that can significantly change the structural and functional properties of starch without disrupting its granular morphology (Singh *et al*., 2004). Glucose is slowly destroyed or destructed when heated in neutral solutions and the rate is accelerated by sulfuric acid but, too much greater extent by hydrochloric acid (Zheng *et al*., 2009). Because of this reason, sulfuric acid is chosen as a suitable hydrolyzing agent. So the final yield of bioethanol depends upon the amount of glucose (reducing sugar) monomers released via the hydrolysis of starch (Umagiliyage *et al.*, 2015).

4.7. Reducing sugar analysis

Concentration of reducing sugar in each of separate raw material hydrolysates before and after fermentation time was given in Table 8 which was calculated after a glucose standard curve given in Appendix 2. Treatment of grain waste flour (F), P, A and T with 2% (v/v) H_2SO_4 generated 29.7–30.8, 28.2–28.9, 16.2–16.6 and 10.3–10.5 percent of reducing sugars at 110^oC and 1:30 h of incubation time, respectively. Pretreatment of P, F, A and T with 1.5% (v/v) H_2SO_4 produced 24.7–25.9, 23.3–24.7, 14.8–15.5 and 8.4–8.6% reducing sugars at 110^oC and 1:30 h of incubation time, respectively. In addition, treatment of F, P, A and T with 1% (v/v) H_2SO_4 generated 16.1–16.3, 12.9–14.1, 9.2–10.4 and 7.1–7.4 percent of reducing sugars at 110^oC and 1:30 h of incubation time, respectively.

Moreover, the control group or 0% (v/v) H_2SO_4 at 110^oC for 1:30h of incubation time resulted in the release of 6.7–8, 5.7–6.7, 5.3–6.5 and 5.2–5.3 percent of reducing sugars from P, F, A and

T, respectively. The study revealed that treatment of raw materials with dilute sulfuric acid released higher sugar concentration as compared to the control group. Perhaps, pretreatment of raw materials with 2% (v/v) H_2SO_4 was the best in terms of generating higher amount of reducing sugar as compared to other diluted sulfuric acid. Similarly, 27–28% (w/v) of reducing sugars were produced from finger millet flour (Pradeep *et al.*, 2010). A study done by Sun and Cheng (2005) have indicated that concentration of reducing sugar in the hydrolysates of Bermuda grass and rye straw was shown increased with increasing the pretreatment time and acid concentration. This is because of the fact that, higher acid concentrations cleaved the bond within both amylopectin and amylose chain to greater extent as compared to lower acid concentration (Wang *et al*., 2003). In addition, about 16.7 g of reducing sugar was produced from 100 g of starch free bran through dilute sulfuric acid hydrolysis (Chotěborská *et al*., 2004). This is also supported by Hoover *et al*. (2010) where reducing sugar yield is increased with increasing pretreatment time and concentration of acid due to better breakdown of glycosidic linkages, thereby altering the structure and properties of the native starch.

Table 8: Concentration of reducing sugar before and after fermentation time

$[H_2SO_4]$	Fermentation time (h)	Concentration of reducing sugar							
		Before fermentation				After fermentation			
		F	**T**	**A**	**P**	**F**	**T**	**A**	**P**
0%	72	6.7	5.3	6.5	8.0	1.9	0.4	1.5	1.7
	120	6.4	5.0	5.3	6.7	0.1	0.3	0.2	0.8
	168	5.7	5.2	5.3	6.9	-0.4	-0.7	-1.3	-0.8
1%	72	16.3	7.1	9.2	12.9	10.1	0.9	4.4	6.6
	120	16.2	7.4	10.4	14.1	7.3	0.7	4.3	6.3
	168	16.1	7.1	10.4	13.8	5.0	0.4	2.5	4.0
1.5%	72	24.4	8.5	15.0	24.7	11.7	1.2	6.8	14.7
	120	23.3	8.6	15.5	25.9	8.2	1.0	5.0	13.0
	168	24.7	8.4	14.8	24.7	5.3	0.5	3.0	9.0
2%	72	29.8	10.4	16.6*	28.2	25.7	1.7	8.3	16.5
	120	29.7	10.3	16.3	28.9*	17.5	1.2	6.9	14.5
	168	30.8*	10.5*	16.2	28.7	12.0	0.4	3.0	9.7

Key: data is a mean of triplicate measurments, [RS] = concentration of reducing sugar in %, F = grain waste flour, T = *tella* spent, A = pineapple peels, P = papaya peels, *= significant difference

4.8. Changes in pH after fermentation process

Changes in pH value after the fermentation process of each separate treatment was shown in Figure 22-25. Once drop in pH value to 4.5 and then holding a constant pH value was observed in grain waste flour treated with 0% H_2SO_4 for ethanol production during fermentation time of 72, 120 and 168 h, whereas a general trend of decreasing was observed for the other substrates treated in the same way. Generally, decreasing in pH value with increasing fermentation time was observed in the case of 1%, 1.5% and 2% H_2SO_4 pretreatment of F, T, A and P (Figure 22–25). Fermentations of grain waste flour hydrolysates obtained from 1%, 1.5% and 2% (v/v) H_2SO_4 pretreatment showed a decrease in pH from 5.0 to 4.8, 4.5 and 4.2; from 5.0 to 4.7, 4.5 and 3.9; and from 5.0 to 4.7, 4.6 and 4.5 during fermentation time at 72, 120 and 168h in that order (Figure 22–25). Fermentations of *tella* hydrolysates obtained from 0%, 1%, 1.5% and 2% (v/v) H_2SO_4 pretreatment showed a decrease in pH from 5.0 to 4.2, 3.9 and 3.8; from 5.0 to 4.2, 3.9 and 4.0; from 5.0 to 4.1, 3.9 and 3.7; and from 5.0 to 4.1, 4.0 and 3.8 during fermentation time at 72, 120 and 168h, respectively (Figure 22–25).

Fermentations of pineapple peels hydrolysates obtained from 0%, 1%, 1.5% and 2% (v/v) H_2SO_4 pretreatment showed a decrease in pH from 5.0 to 4.6, 4.8 and 4.7; from 5.0 to 4.8, 4.7 and 4.8; from 5.0 to 4.7, 4.8 and 4.6; and from 5.0 to 4.7, 4.7 and 4.7 during fermentation time at 72, 120 and 168h in that order (Figure 22–25). Fermentations of papaya peels hydrolysates obtained from 0%, 1%, 1.5% and 2% (v/v) H_2SO_4 pretreatment showed a decrease in pH from 5.0 to 4.6, 4.5 and 4.5; from 5.0 to 4.4, 4.5 and 4.6; from 5.0 to 4.6, 4.5 and 4.4; and from 5.0 to 4.4, 4.5 and 4.6 during fermentation time at 72, 120 and 168h, respectively (Figure 22–25). The results show that as a fermentation time increased the pH value of the fermentation medium was dropped. The result was in agreement with Efiuvwevwere and Akona (1995) that as a period of fermentation time increased, acidity in the fermentation medium was also increased, thereby increasing ethanol yield. This is because during fermentation process, along with ethanol and carbon dioxide, fermenting yeast cultures may also produce aldehydes, organic acids, esters, organic sulfides, carbonyl compounds that will affect the pH value of the fermentation medium (Hazelwood *et al*., 2008).

Furthermore, a work done by Teramoto *et al*. (2002) shows that *S. cerevisiae* ET99, *S. cerevisiae* W4, *S. cerevisiae* K7 decreased the pH of fermentation medium from 4.5-3.8, 4.5-3.9, and 4.5-

3.8 at 30^{o}C, respectively. Production of more acid causes a pH to be decreased. The results of the study also show that ethanol production was increased as a pH value of the medium is decreased. This was also supported by a work of Hancioğlu and Karapinar (1997) that during ethanol fermentation the pH value of the fermentation medium was going to be decreased with the increase in ethanol yield. Over all the pH of the fermentation medium in the study was found to be between 3.7-4.8. In similar ways a pH value between 4–4.8 is regarded as the operational limit for the anaerobic ethanol production process when *S. cerevisiae* is used in fermentation process (Hwang *et al*., 2004). A work done by Akin-Osanaiye *et al.* (2008) also indicated that optimum temperature of 30^{o}C and pH of 4.5 is used to produce about 2.82–6.6% (v/v) of ethanol using *S. cerevisiae* at 72 h of fermentation time. Lastly a pH value 4.5 was found to be the best when hydrolysates from pineapple was used for ethanol production (Nigam, 2000).

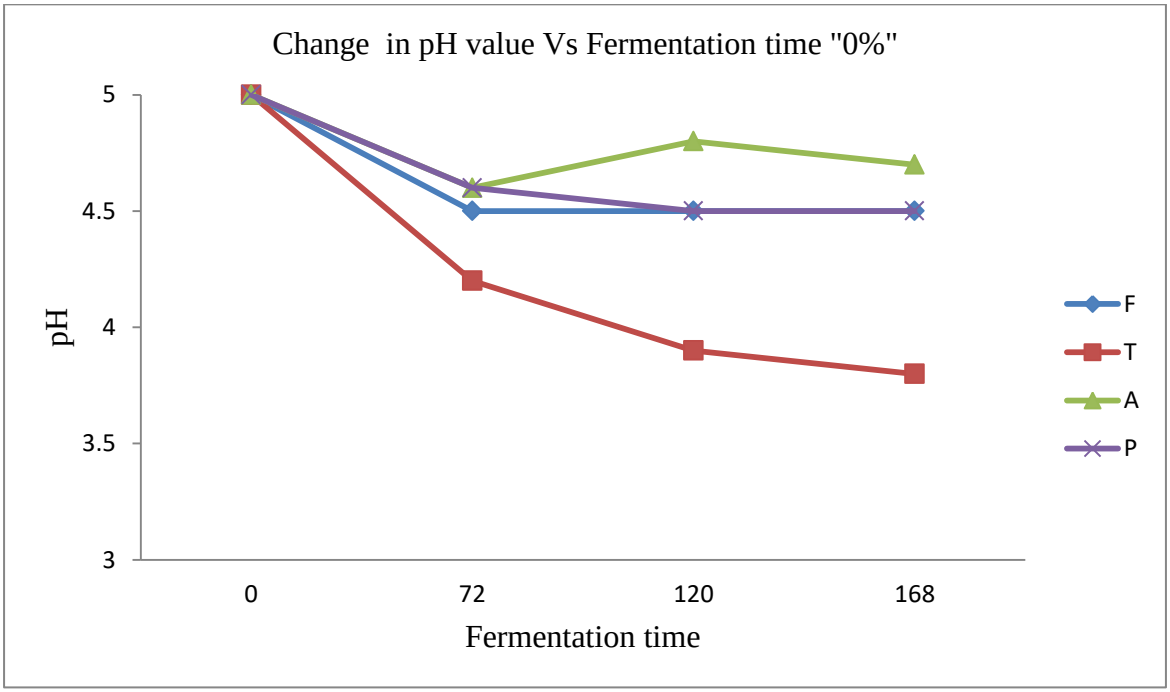


Figure 21: Effect of fermentation time on pH during fermentation of medium obtained from 0% acid pretreatment

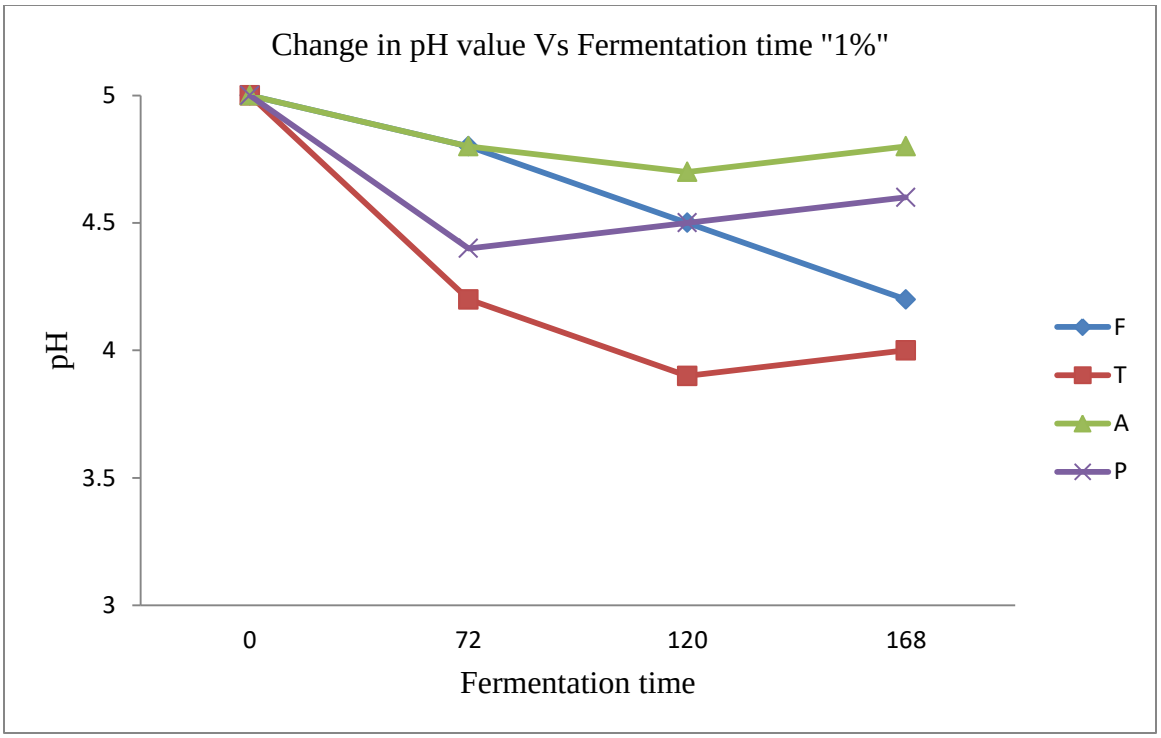


Figure 22: Effect of fermentation time on pH during fermentation of medium obtained from 1% acid pretreatment

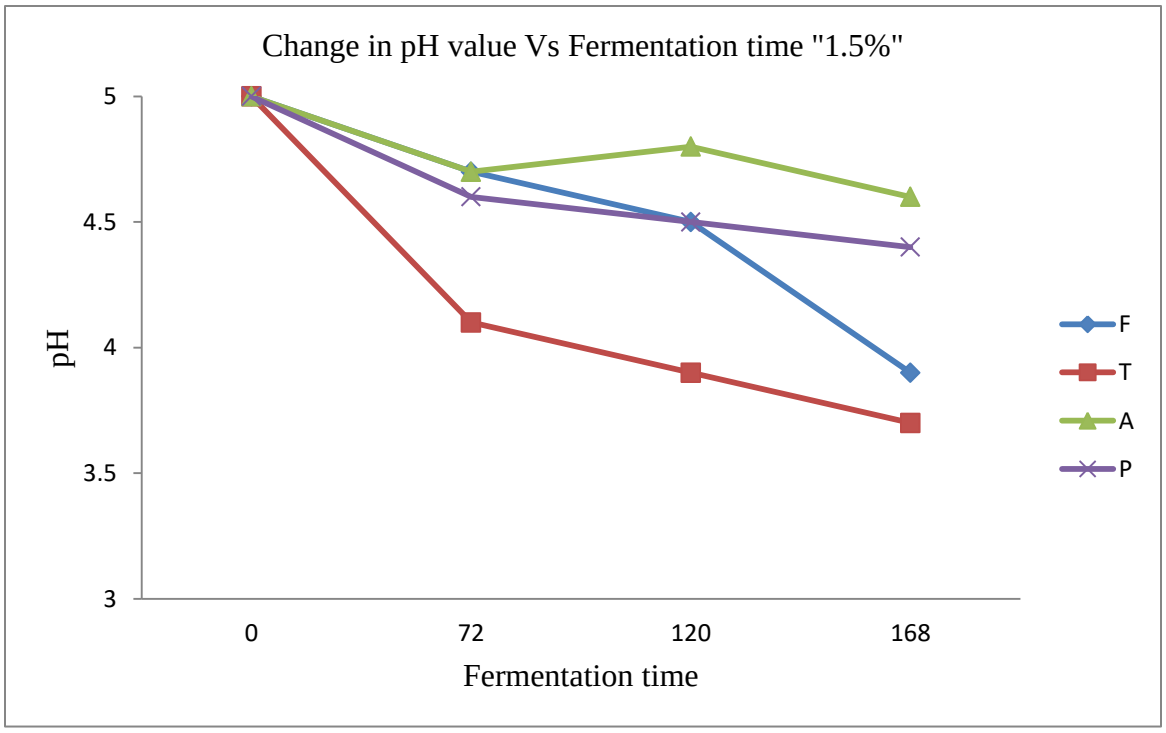


Figure 23: Effect of fermentation time on pH during fermentation of medium obtained from 1.5% acid pretreatment

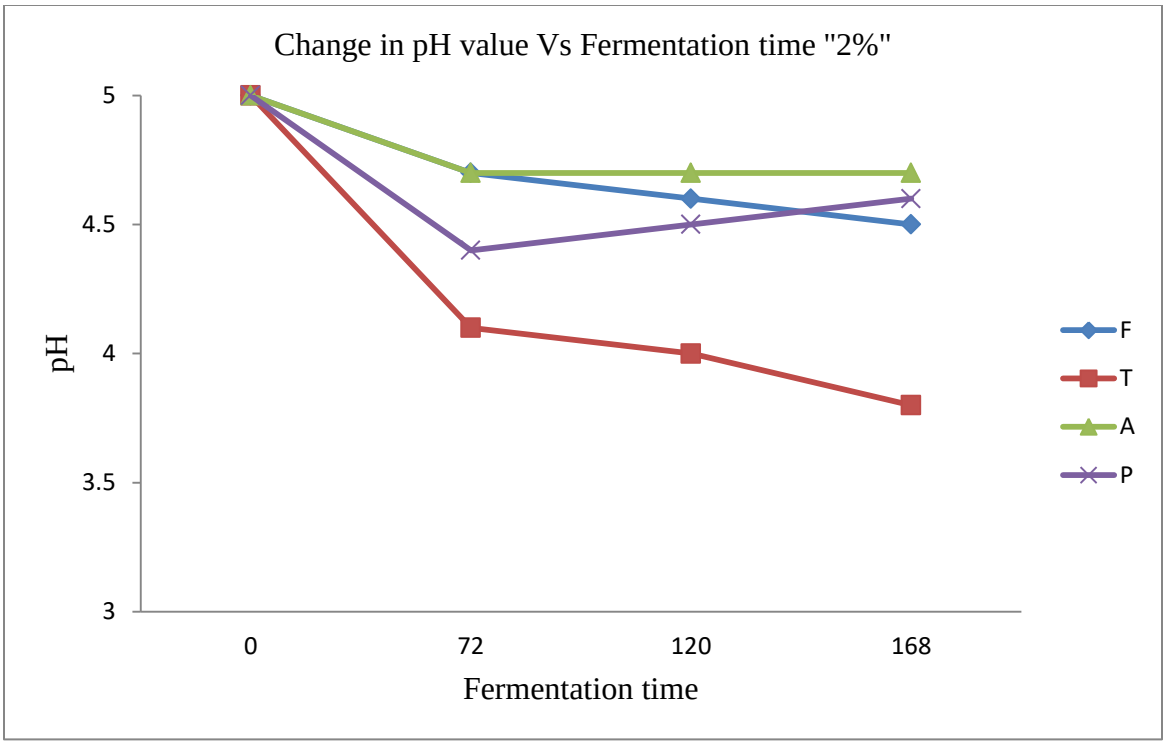


Figure 24: Effect of fermentation time on pH during fermentation of medium obtained from 2% acid pretreatment

Concentration of reducing sugar levels before and after fermentation time

The results showed that reducing sugar levels continued to decrease with increasing fermentation time (Figure 26-29). The average (9 treatments) of reducing sugar levels at the beginning of fermentation period were 5.17, 7.20, 8.50 and 10.4%; 5.7, 10, 15.1 and 16.4%; 7.2, 13.6, 25.1 and 28.6%; 6.27, 16.2, 24.13 and 30.1% when *tella* spent, pineapple peels, papaya peels and grain waste flour were pretreated with 0%, 1%, 1.5% and 2% H_2SO_4 at 110^oC for 90 min., respectively (Figure 26-29). In all cases, fermentation of the medium with the yeast isolate 1T-10 decreased concentration of reducing sugar (Figure 26–29). This result is supported by Lamb *et al*. (2018) that about 31.31±1.73 g of reducing sugars per 100 g of dry *Saccharina latissima* are produced upon enzymatic hydrolysis and concentration of reducing sugar is decreased to greater extent during the production of 0.42 g of ethanol per gram of reducing sugar using *S. cerevisiae*.

In addition, higher sugar yield (55.2 g of reducing sugar from 100 g) dry potato peel is produced at pretreatment temperature of 135^oC using 10% (w/w) H_2SO_4 and it was found that concentration of reducing sugar was decreased as a fermentation time increased (Lenihan *et al*.,

2010). Furthermore, raw dairy manure pretreated with 3% sulfuric acid at 110^{o}C for 1 h produced glucose yield of 11.32 g/100 g and is highly consumed by yeast to produce ethanol (Wen *et al*., 2004). In similar ways a study done by Yedro *et al.* (2015) indicated that a total quantity of 20.0 to 23.1% (w/w) sugars were recovered from grape seeds pretreatment at 90^{o}C for 60 min. using diluted acid with decreased concentration of reducing sugar as a fermentation time increased.

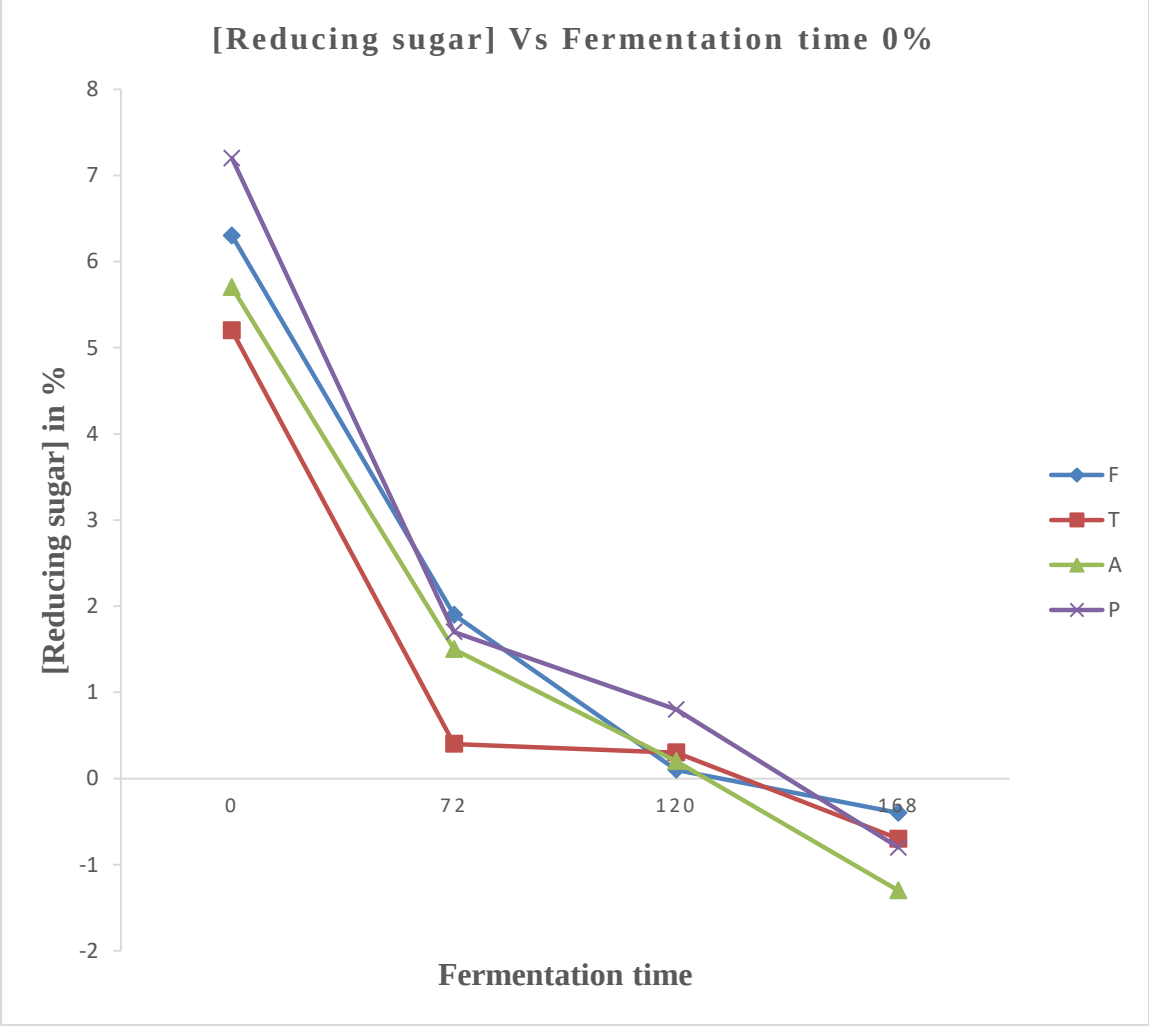


Figure 25: Effect of fermentation time on concentration of reducing sugar during fermentation of hydrolysates obtained from 0% acid pretreatment of raw materials

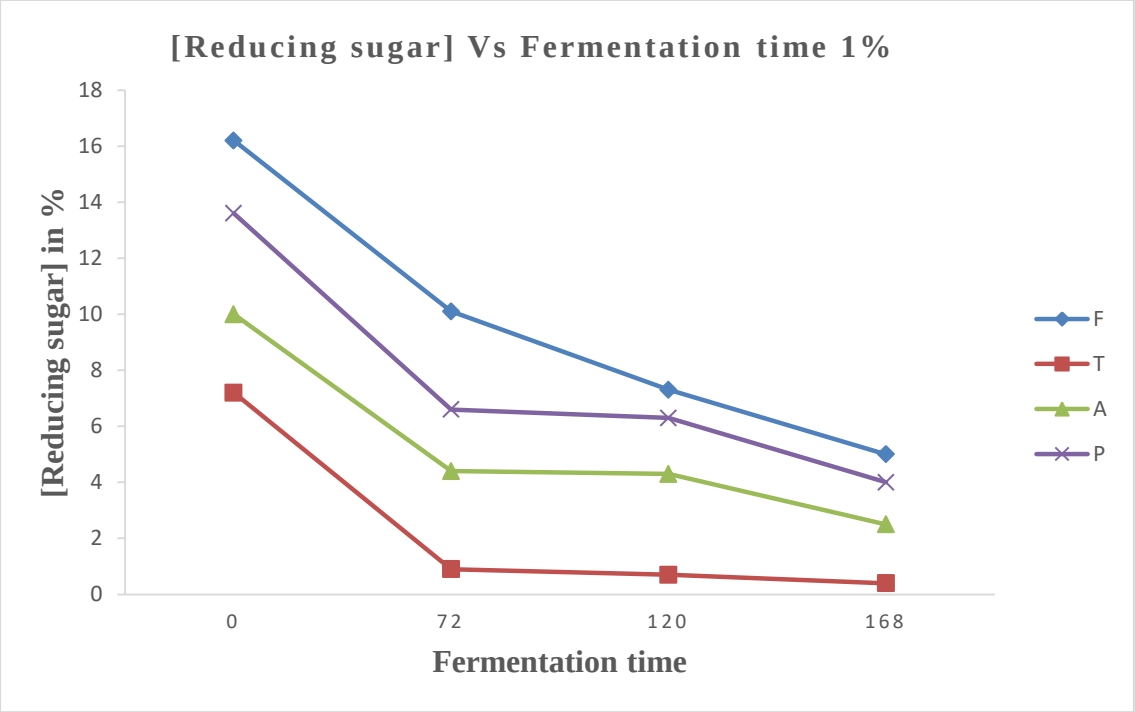


Figure 26: Effect of fermentation time on concentration of reducing sugar during fermentation of hydrolysates obtained from 1% acid pretreatment of raw materials

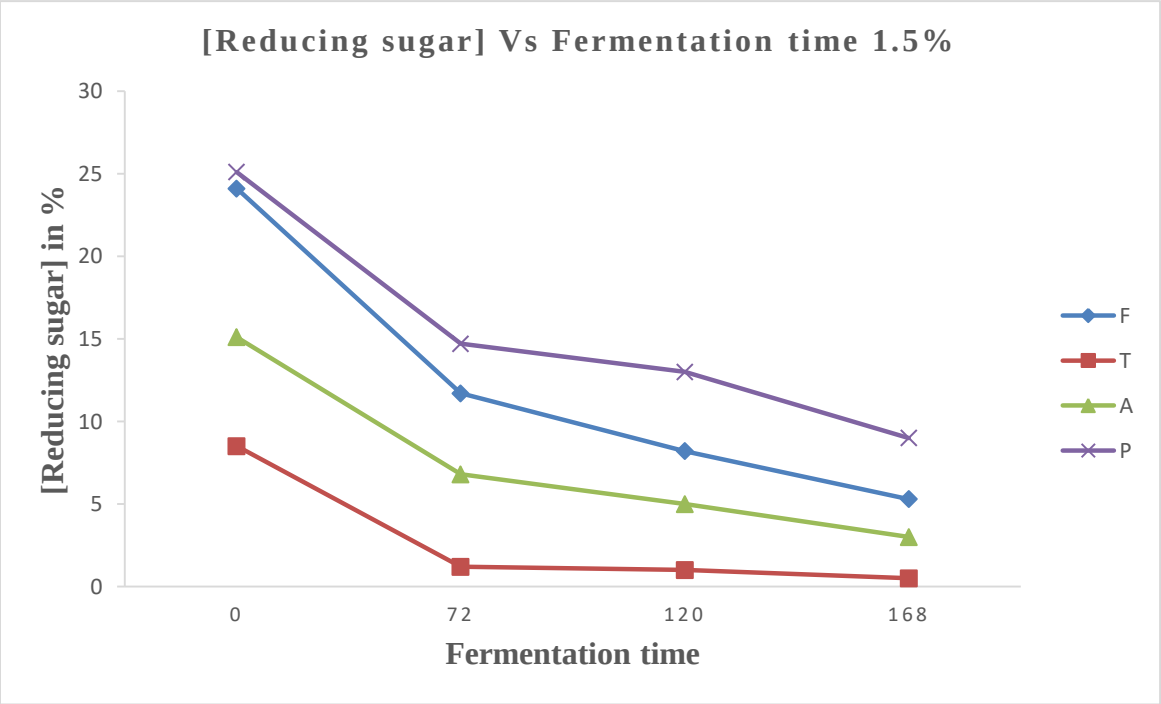


Figure 27: Effect of fermentation time on concentration of reducing sugar during fermentation of hydrolysates obtained from 1.5% acid pretreatment of raw materials

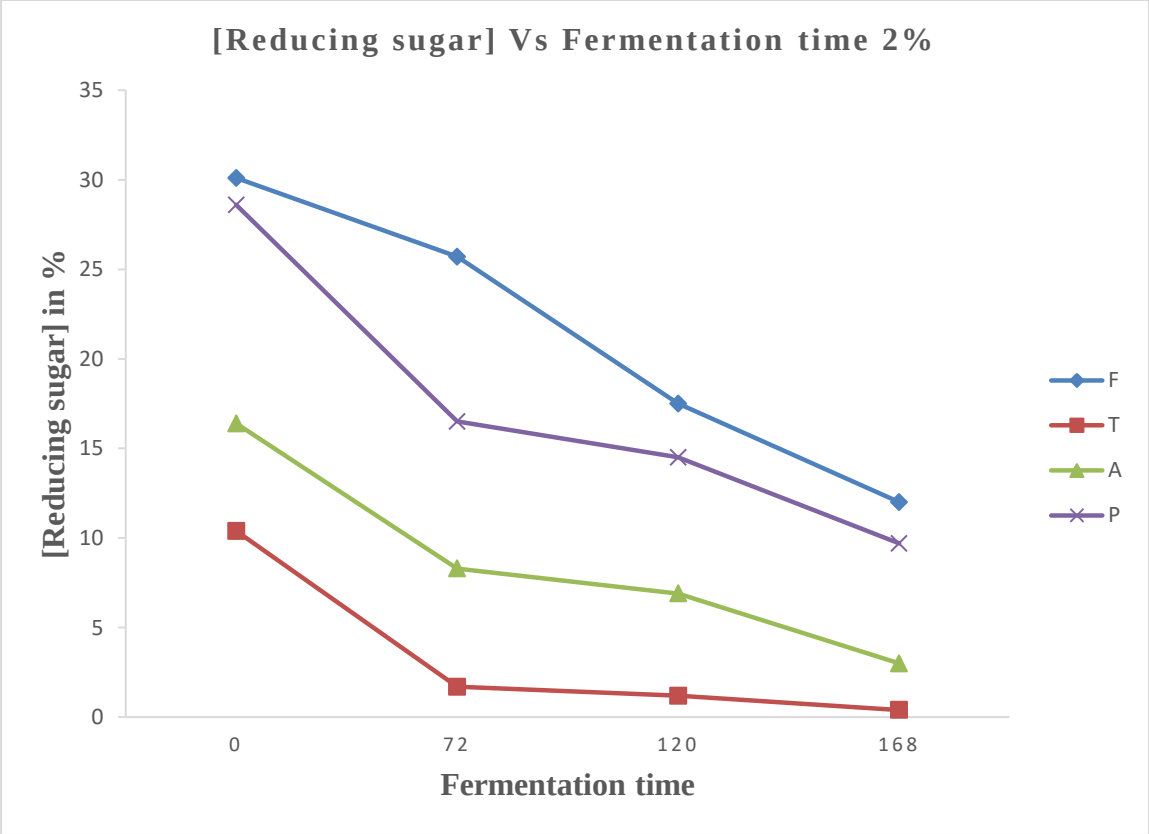


Figure 28: Effect of fermentation time on concentration of reducing sugar during fermentation of hydrolysates obtained from 2% acid pretreatment of raw materials

4.9. Ethanol production

The means of ethanol yield calculated as a function of the two factors for F, T, A and P and/or effects of each separate acid and fermentation time on ethanol yield are presented in Table 9, respectively. Ethanol production from grain waste flour pretreated with 1.5% and 2% acid was increased as fermentation time increased to 168 h, but grain waste flour pretreated with 1% acid increases ethanol production only up to 120 h of fermentation time. Ethanol production from *tella* spent pretreated with 1% and 2% produce high ethanol yield when fermented for 72 h and continues to decrease as fermentation time increased to 168 h. However, *tella* spent pretreated with 1.5% acid was increased as fermentation time increased to 120 h and continues to decrease as fermentation time increased to 168 h.

Ethanol production from pineapple peels pretreated with 1% and 2% acid was increased as fermentation time increased to 120 h of fermentation time and continues to decrease as

fermentation time increased to 168 h, however high yield of ethanol was produced from pineapple peels pretreated with 1.5% acid at 72 h of fermentation time and it keep on decrease as fermentation time increased. Similarly, ethanol production from papaya peels pretreated with 1% and 1.5% acid was increased as fermentation time increased to 120 h of fermentation time and going to decrease as fermentation time increased to 168 h, but papaya peels pretreated with 2% acid produced at 72 h of fermentation time and keep on decrease as fermentation time increases. Highest yield of ethanol was produced in combinations of; 2% H_2SO_4 pretreatment of grain waste flour fermented for 168 h (1.380%), 2% H_2SO_4 pretreatment of *tella* spent fermented for 72 h (4.813%), 2% H_2SO_4 pretreatment of pineapple peels fermented for 120 h (7.883%) and 2% H_2SO_4 pretreatment of papaya peels fermented for 72 h (6.400%).

Table 9: Means of ethanol yield in weight percentage as a function of fermentation time and acid concentration

[H_2SO_4]	FT	Mean of ethanol yield in weight percentage			
		F	**T**	**A**	**P**
0%	72	1.146	1.518	2.723	1.760
	120	1.111	1.070	2.003	1.453
	168	1.056	0.695	0.423	0.070
1%	72	1.217	2.333	3.487	2.203
	120	1.238	1.907	4.753	3.643
	168	1.198	1.650	3.623	3.447
1.5%	72	1.230	2.053	6.597	4.887
	120	1.255	3.850	5.903	5.970
	168	1.301	2.553	4.130	4.077
2%	72	1.322	4.813*	4.753	6.400*
	120	1.358	4.030	7.883*	6.380
	168	1.380*	3.083	6.097	5.607

Key: data is a mean of triplicate measurments, FT = fermentation time, F = grain waste flour, T = *tella* spent, A = pineapple peels, P = papaya peels, *= highly significant ethanol

Grain waste flour, *tella* spent and papaya peels pretreated with 2% H_2SO_4 produced mean values of ethanol yield 1.322%, 1.358% and 1.380%; 4.813%, 4.030% and 3.083%; 6.400%, 6.380% and 5.607% at 72, 120 and 168 h of fermentation time, respectively. The corresponding length of fermentation period and ethanol yields of grain waste flour, *tella* spent and papaya peels pretreated with 2% H_2SO_4 were found significantly higher than the ethanol of similar separate sample pretreated with 0, 1, 1.5% H_2SO_4 and fermented for corresponding comparable lengths of time. However, pineapple peels pretreated with 2% H_2SO_4 produced mean values of ethanol yield 4.753%, 6.097% and 7.883% at 72, 168 and 120 h of fermentation time, respectively. But, 1.5% H_2SO_4 pretreated pineapple peels gives higher ethanol yield than 2% H_2SO_4 in 72 h of fermentation time. The results were in agreement with that of Reddy and Reddy (2005) with increasing the duration of fermentation time from 3-5 days ethanol productivity from horse gram flour is increased through better utilization of reducing sugar.

The simultaneous saccharification and fermentation process of raw starch from damaged quality wheat and sorghum grains yield 4.4% and 5.5% (v/v) ethanol respectively in four days utilizing crude amylase prepared from *B. subtilis* VB2 and amylolytic yeast strain *S. cerevisiae* VSJ4 (Suresh *et al*., 1999). A research done by Cutzu and Bardi (2017) states that 4.26 ± .27% of ethanol were produced from peacles at 72 h of fermentation time. The same result was also presented by Triwahyuni *et al.* (2015) that 4.74% were produced in 72 h of fermentation time from empty fruit banch. The best ethanol was produced at 72 h of fermentation time and 2% H_2SO_4 pretreatment of papaya peels. Similarly, between 2.82–6.60% (v/v) of ethanol is produced from papaya fruit waste using baker yeast at 30^oC, pH of 4.5 and reach a peak 72 h of fermentation time (Akin-Osanaiye *et al.,* 2008).

Higher yield of ethanol was obtained when pineapple peels was pretreated with 2% H_2SO_4 and a fermentation time of 120 h. The results were in close agreement with Arumugam and Manikandan (2011) that 7.82% were produced from mango peels when the hydrolysates was fermented for 120 h of fermentation time. A close result were also presented by Reddy *et al*. (2011) that around 5.13% ethanol was produced by *Saccharomyces cerevisiae* from pineapple in 72 h of fermentation time. The maximum amount of ethanol (641mg/g) produced by free cells when straw is pretreated with microwave-assisted 5% H_2SO_4 hydrolysis and (543.51mg/g) from tomato waste using immobilized cells with microwave-assisted 7% HCl hydrolysis (Akafarna,

2015). Similarly, Olugbenga and Ibiyemi (2011) reported that 1.9% of ethanol is produced from acid treated breweries spent grain.

Generally, pretreatment of grain waste flour, *tella* spent, pineapple peels and papaya peels with 1%, 1.5% and 2% acid produced ethanol yield as fermentation time increased from 72 h to 120 h and continued to decrease as it is increased to 168 h except grain waste flour pretreated with 1.5% and 2% acid. Pineapple peels pretreated with 1.5% H_2SO_4 is effective in ethanol production if it was fermented for 72 h. A close result (7.97 ± .7%) and (8.71 ± .38%) of ethanol were presented by Cutzu and Bardi (2017) from kiwifruit and papaya respectively in 96 h of fermentation time. A similar result was also presented by Itelima *et al*. (2013) that about 8.34%, 7.45% and 3.98% of ethanol is produced in 9 days of fermentation time from pineapple, banana and plantain respectively. In similar ways Tropea *et al.* (2014) indicates that about 3.9% (v/v) ethanol is produced from pineapple fruit waste. Variations in fermentation periods had the greatest impact on the percentage of ethanol and the volume of ethanol produced showed best fermentation period for optimal ethanol production (Amadi and Ifeanacho, 2016).

5. Conclusions

It was shown that challenging the isolates successively with different ethanol concentration can be a useful method for screening yeast isolates with good bioethanol production potentials. From yeast isolate, 1T-10 which was isolated from *tej* produced a significantly higher yield of ethanol compared to other isolates. Grain waste flour, *tella* spent, pineapple peels and papaya peels pretreated with 2% H_2SO_4 were shown to be promising substrates for ethanol production when their separate hydrolysates were fermented with yeast isolate 1T-10 for 168, 72, 120 and 72 h of fermentation time in that order.

6. Recommendations

Further studies must be done on isolated yeast (1T-10) regarding;

- → Its genetic make-up.
- → Different carbohydrate fermentation ability.
- → Its optimum pH value and incubation temperature for production of bioethanol.

Yeast coculture or mix from different isolates have to be prepared and tested whether they produce higher yield of ethanol or not compared to the yeast isolate 1T-10. The study revealed that it is possible to produce bioethanol from grain waste flour, *tella* spent, pineapple peels and papaya peels; however, the economic feasibility should be studied especially for that of grain waste flour and *tella* spent. Furthermore, bioethanol production from the mix of each raw materials has to be carried out to get a maximum and optimal yield of ethanol.

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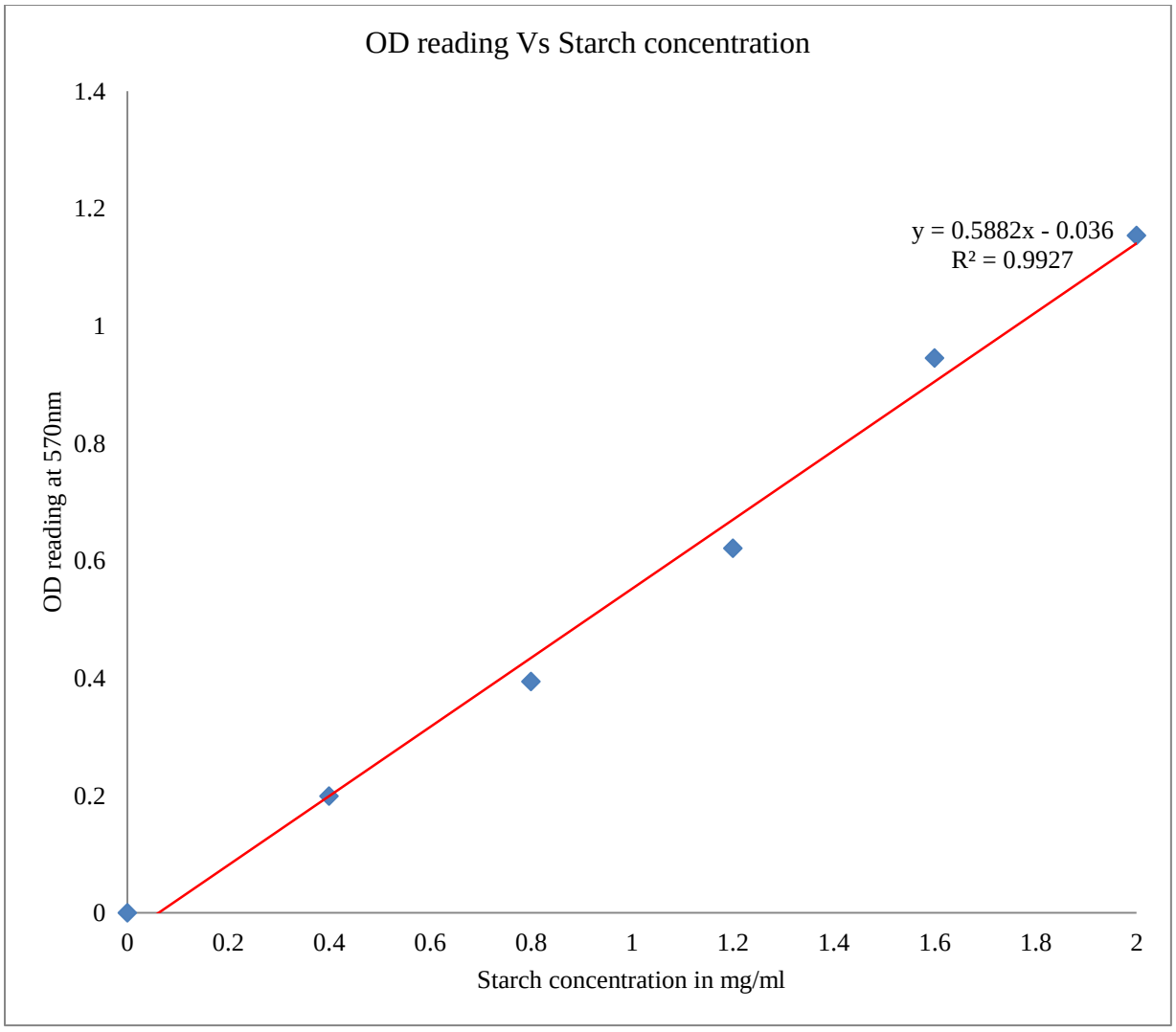
Appendix 1

Starch standard curve

Starch standard Optical density reading

[Starch] in mg/ ml	OD reading at 570nm
0	0
0.4	0.199
0.8	0.394
1.2	0.621
1.6	0.945
2	1.154

Data is a mean of duplicate treatments



Starch standard curve at 570nm spectrophotometric OD reading

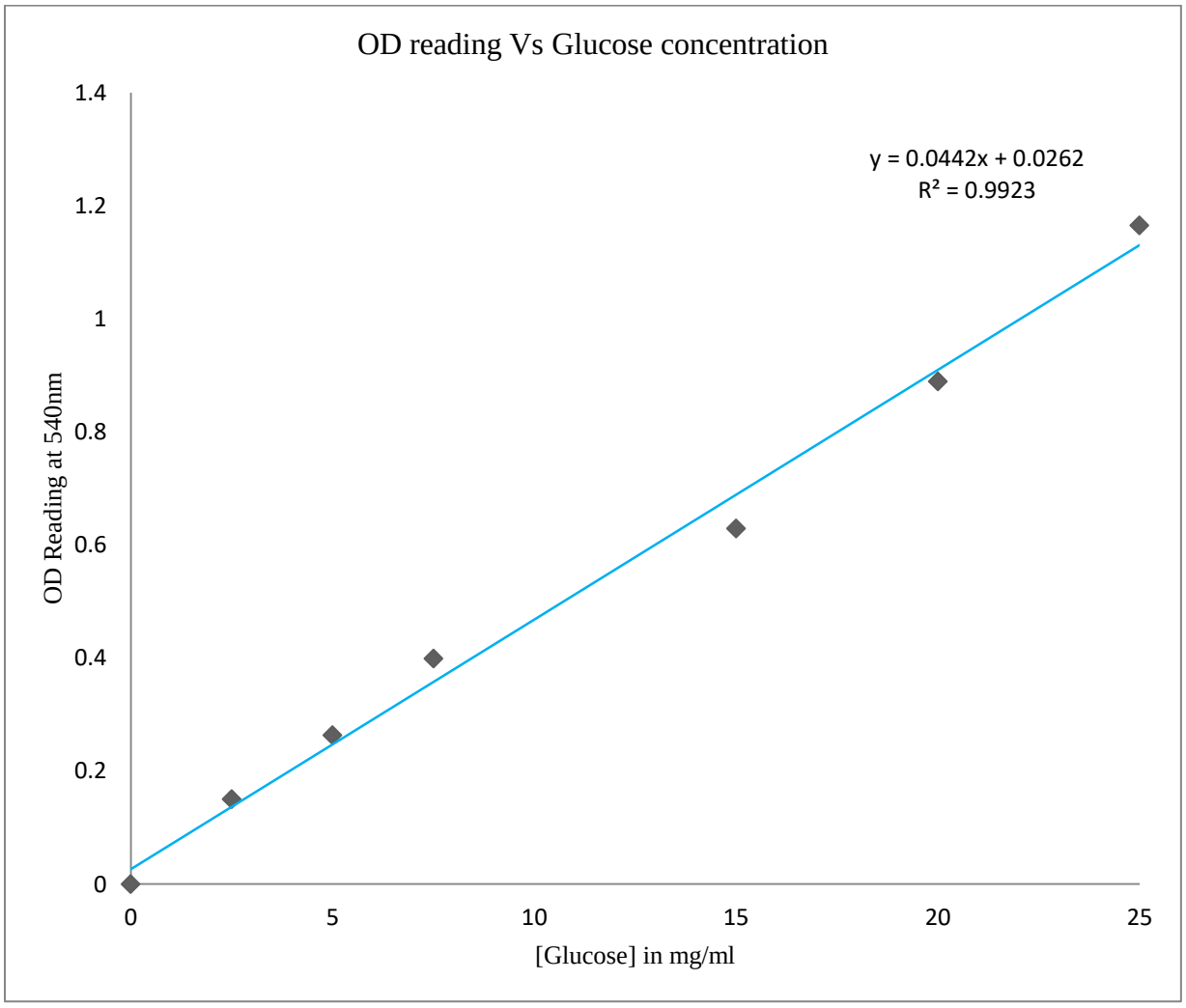
Appendix 2

Glucose standard curve

Glucose standard Optical density reading

[Glucose] in mg/ ml	OD reading at 540 nm
0	0
2.5	0.15
5	0.263
7.5	0.399
15	0.629
20	0.889
25	1.165

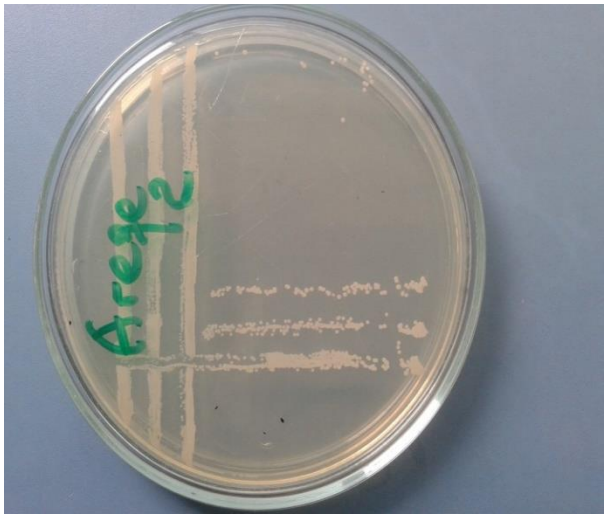
Data is a mean of duplicate measurements

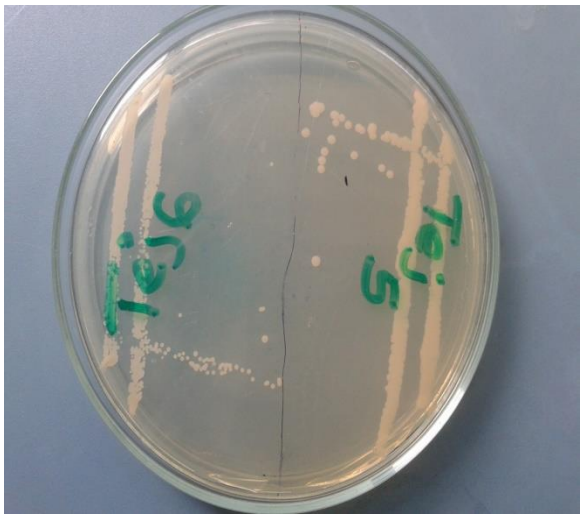


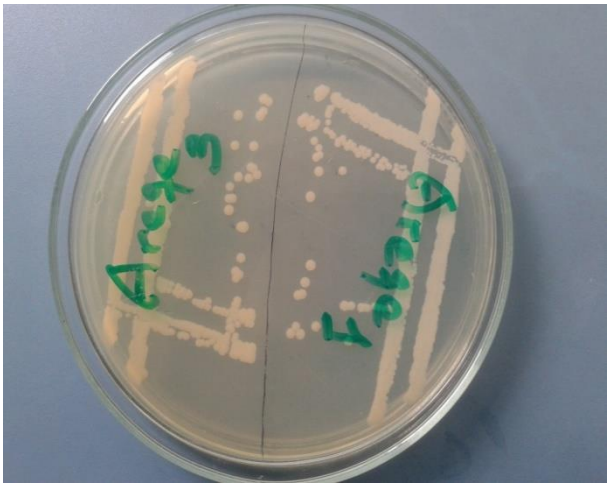
Glucose standard curve at 540 nm spectrophotometric OD reading

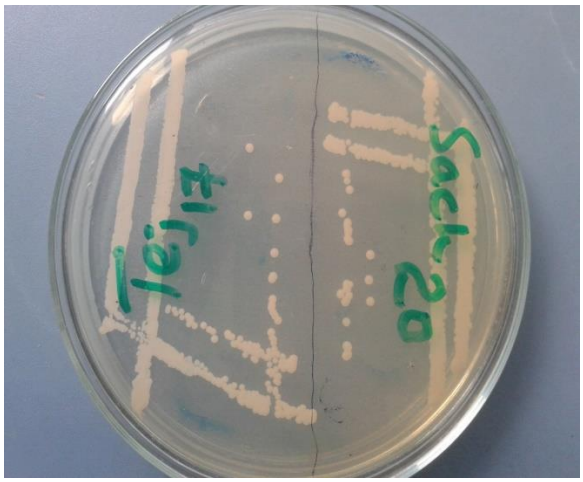
Appendix 3

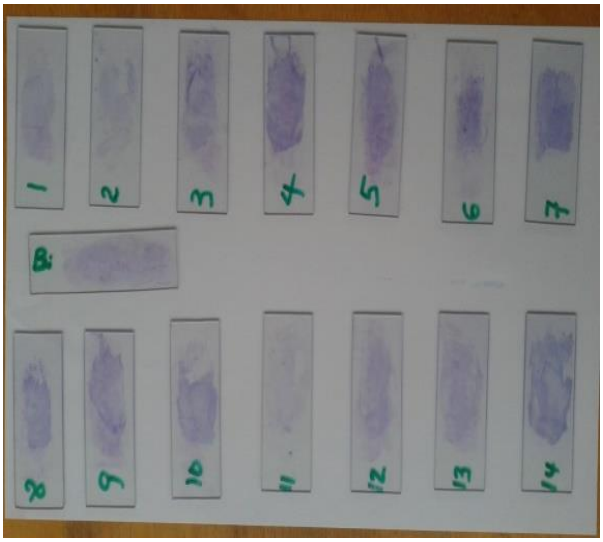
Figures of selected experiments


Arexe 12


Tej 5
Tejs 6


Arexe 3
Arexe 4


Sack 20
Tejs 14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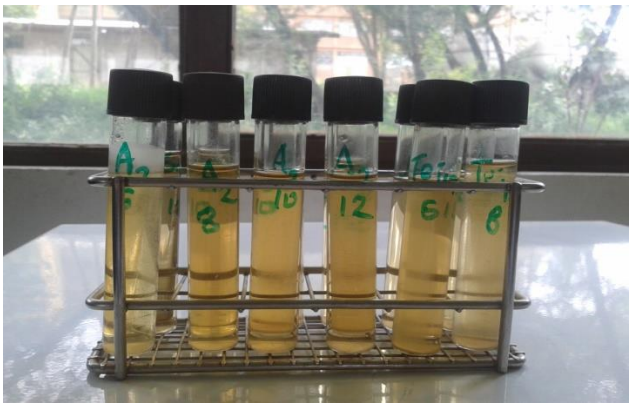
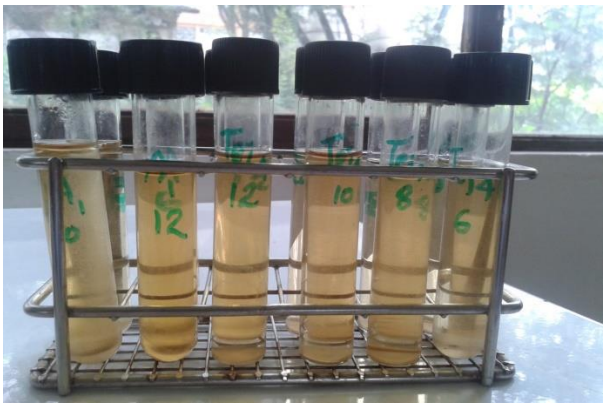
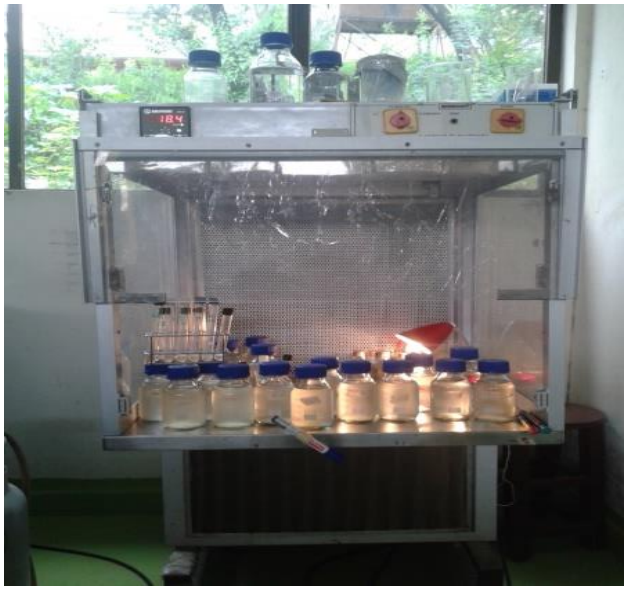
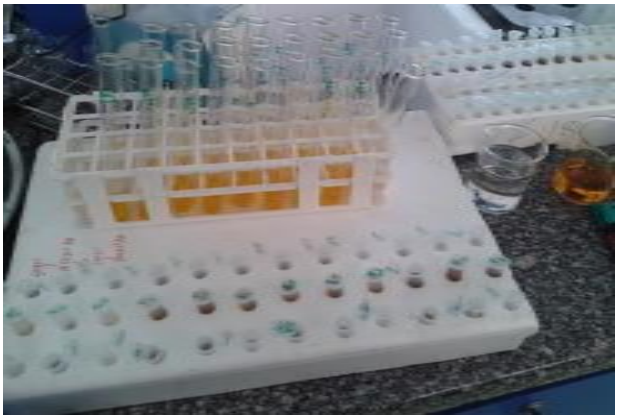


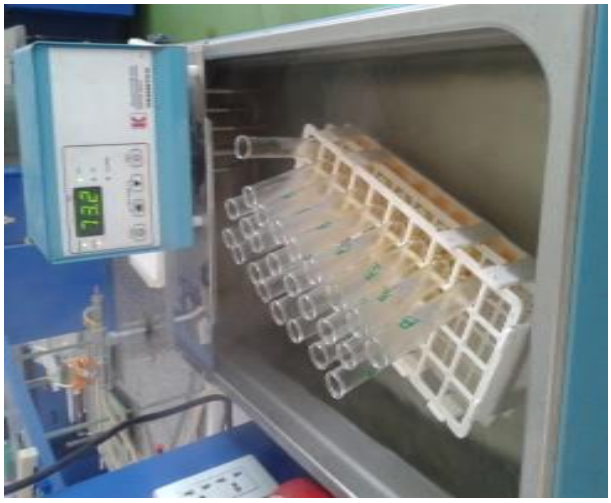


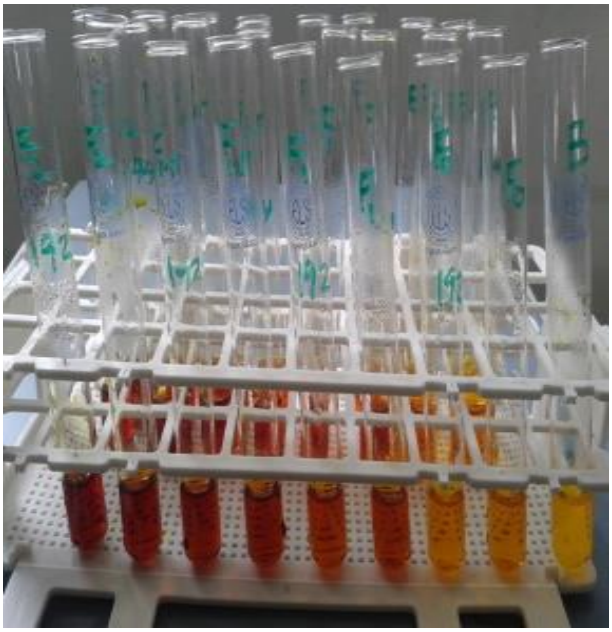



DNP-
ELECTRIC CONSTANT TEMPERATURE
INCUBATOR
POWER




250ml





Appendix 4

Specific gravities of alcohol-mixtures table at 20°/20°C

Specific gravity (20/20)	*Per cent* *by weight*	*by volume*	*G in* 100ml	*Specific gravity* (20/20)	*Per cent* *by weight*	*by volume*	*G in* 100ml
1·0000	0·00	0·00	0·00	0·9950	2·72	3·43	2·70
0·9999	0·05	0·07	0·05	0·9949	2·78	3·50	2·76
0·9998	0·11	0·13	0·11	0·9948	2·84	3·57	2·82
0·9997	0·16	0·20	0·16	0·9947	2·89	3·64	2·87
0·9996	0·21	0·27	0·21	0·9946	2·95	3·71	2·93
0·9995	0·26	0·33	0·26	0·9945	3·01	3·78	2·98
0·9994	0·32	0·40	0·32	0·9944	3·06	3·85	3·04
0·9993	0·37	0·47	0·37	0·9943	3·12	3·92	3·10
0·9992	0·42	0·54	0·42	0·9942	3·18	3·99	3·15
0·9991	0·48	0·60	0·48	0·9941	3·23	4·07	3·21
0·9990	0·53	0·67	0·53	0·9940	3·29	4·14	3·27
0·9989	0·59	0·74	0·58	0·9939	3·35	4·21	3·32
0·9988	0·64	0·81	0·64	0·9938	3·41	4·28	3·38
0·9987	0·69	0·87	0·69	0·9937	3·47	4·36	3·44
0·9986	0·75	0·94	0·74	0·9936	3·52	4·43	3·49
0·9985	0·80	1·01	0·80	0·9935	3·58	4·50	3·55
0·9984	0·85	1·08	0·85	0·9934	3·64	4·57	3·61
0·9983	0·91	1·14	0·90	0·9933	3·70	4·65	3·67
0·9982	0·96	1·21	0·96	0·9932	3·76	4·72	3·72
0·9981	1·01	1·28	1·01	0·9931	3·82	4·79	3·78
0·9980	1·07	1·35	1·06	0·9930	3·87	4·87	3·84
0·9979	1·12	1·42	1·12	0·9929	3·93	4·94	3·90
0·9978	1·18	1·48	1·17	0·9928	3·99	5·01	3·96
0·9977	1·23	1·55	1·23	0·9927	4·05	5·09	4·02
0·9976	1·28	1·62	1·28	0·9926	4·11	5·16	4·07
0·9975	1·34	1·69	1·33	0·9925	4·17	5·23	4·13
0·9974	1·39	1·76	1·39	0·9924	4·23	5·31	4·19
0·9973	1·45	1·83	1·44	0·9923	4·29	5·38	4·25
0·9972	1·50	1·89	1·50	0·9922	4·35	5·46	4·31
0·9971	1·56	1·96	1·55	0·9921	4·41	5·53	4·37
0·9970	1·61	2·03	1·60	0·9920	4·47	5·61	4·43
0·9969	1·67	2·10	1·66	0·9919	4·53	5·68	4·49
0·9968	1·72	2·17	1·71	0·9918	4·59	5·76	4·55
0·9967	1·78	2·24	1·77	0·9917	4·65	5·83	4·61
0·9966	1·83	2·31	1·82	0·9916	4·71	5·91	4·67
0·9965	1·89	2·38	1·88	0·9915	4·77	5·99	4·73
0·9964	1·94	2·45	1·93	0·9914	4·84	6·06	4·79
0·9963	2·00	2·52	1·99	0·9913	4·90	6·14	4·85
0·9962	2·05	2·58	2·04	0·9912	4·96	6·22	4·91
0·9961	2·11	2·65	2·10	0·9911	5·02	6·29	4·97
0·9960	2·16	2·72	2·15	0·9910	5·08	6·37	5·03
0·9959	2·22	2·79	2·21	0·9909	5·15	6·45	5·09
0·9958	2·27	2·86	2·26	0·9908	5·21	6·53	5·15
0·9957	2·33	2·93	2·32	0·9907	5·27	6·60	5·21
0·9956	2·39	3·00	2·37	0·9906	5·33	6·68	5·27
0·9955	2·44	3·07	2·43	0·9905	5·40	6·76	5·34
0·9954	2·50	3·14	2·48	0·9904	5·46	6·84	5·40
0·9953	2·55	3·21	2·54	0·9903	5·52	6·92	5·46
0·9952	2·61	3·28	2·59	0·9902	5·59	7·00	5·52
0·9951	2·67	3·35	2·65	0·9901	5·65	7·07	5·58

0·9900	5·71	7·15	5·65	0·9850	9·04	11·26	8·89
0·9899	5·78	7·23	5·71	0·9849	9·11	11·34	8·95
0·9898	5·84	7·31	5·77	0·9848	9·17	11·43	9·02
0·9897	5·90	7·39	5·83	0·9847	9·24	11·51	9·09
0·9896	5·97	7·47	5·90	0·9846	9·31	11·59	9·15
0·9895	6·03	7·55	5·96	0·9845	9·38	11·68	9·22
0·9894	6·10	7·63	6·02	0·9844	9·45	11·76	9·29
0·9893	6·16	7·71	6·09	0·9843	9·52	11·85	9·35
0·9892	6·23	7·79	6·15	0·9842	9·59	11·93	9·42
0·9891	6·29	7·87	6·21	0·9841	9·66	12·02	9·49
0·9890	6·36	7·95	6·28	0·9840	9·73	12·10	9·55
0·9889	6·42	8·03	6·34	0·9839	9·80	12·19	9·62
0·9888	6·49	8·11	6·40	0·9838	9·87	12·28	9·69
0·9887	6·55	8·19	6·47	0·9837	9·94	12·36	9·76
0·9886	6·62	8·27	6·53	0·9836	10·01	12·45	9·83
0·9885	6·68	8·35	6·59	0·9835	10·08	12·53	9·89
0·9884	6·75	8·44	6·66	0·9834	10·15	12·62	9·99
0·9883	6·81	8·52	6·72	0·9833	10·22	12·71	10·03
0·9882	6·88	8·60	6·79	0·9832	10·29	12·80	10·10
0·9881	6·95	8·68	6·85	0·9831	10·36	12·88	10·17
0·9880	7·01	8·76	6·92	0·9830	10·43	12·97	10·24
0·9879	7·08	8·84	6·98	0·9829	10·50	13·06	10·31
0·9878	7·15	8·93	7·05	0·9828	10·58	13·14	10·37
0·9877	7·21	9·01	7·11	0·9827	10·65	13·23	10·44
0·9876	7·28	9·09	7·18	0·9826	10·72	13·32	10·51
0·9875	7·35	9·17	7·24	0·9825	10·79	13·41	10·58
0·9874	7·41	9·26	7·31	0·9824	10·86	13·49	10·65
0·9873	7·48	9·34	7·37	0·9823	10·93	13·58	10·72
0·9872	7·55	9·42	7·44	0·9822	11·01	13·67	10·79
0·9871	7·61	9·51	7·50	0·9821	11·08	13·76	10·86
0·9870	7·68	9·59	7·57	0·9820	11·15	13·85	10·93
0·9869	7·75	9·67	7·63	0·9819	11·22	13·94	11·00
0·9868	7·82	9·75	7·70	0·9818	11·30	14·02	11·07
0·9867	7·88	9·84	7·76	0·9817	11·37	14·11	11·14
0·9866	7·95	9·92	7·83	0·9816	11·44	14·20	11·21
0·9865	8·02	10·00	7·90	0·9815	11·51	14·29	11·28
0·9864	8·09	10·09	7·96	0·9814	11·59	14·38	11·35
0·9863	8·15	10·17	8·03	0·9813	11·66	14·47	11·42
0·9862	8·22	10·25	8·09	0·9812	11·73	14·56	11·49
0·9861	8·29	10·34	8·16	0·9811	11·81	14·65	11·56
0·9860	8·36	10·42	8·22	0·9810	11·88	14·74	11·63
0·9859	8·42	10·50	8·29	0·9809	11·95	14·83	11·70
0·9858	8·49	10·59	8·36	0·9808	12·03	14·92	11·77
0·9857	8·56	10·67	8·42	0·9807	12·10	15·01	11·85
0·9856	8·63	10·75	8·49	0·9806	12·17	15·10	11·92
0·9855	8·69	10·84	8·55	0·9805	12·25	15·19	11·99
0·9854	8·76	10·92	8·62	0·9804	12·32	15·28	12·06
0·9853	8·83	11·00	8·69	0·9803	12·39	15·37	12·13
0·9852	8·90	11·09	8·75	0·9802	12·47	15·46	12·20
0·9851	8·97	11·17	8·82	0·9801	12·54	15·54	12·27

0·9800	12·62	15·64	12·34	0·9750	16·47	20·30	16·03
0·9799	12·69	15·73	12·41	0·9749	16·55	20·40	16·10
0·9798	12·76	15·82	12·48	0·9748	16·62	20·49	16·18
0·9797	12·84	15·91	12·56	0·9747	16·70	20·59	16·25
0·9796	12·91	16·00	12·63	0·9746	16·78	20·68	16·32
0·9795	12·99	16·09	12·70	0·9745	16·86	20·77	16·40
0·9794	13·06	16·18	12·77	0·9744	16·93	20·87	16·47
0·9793	13·14	16·27	12·84	0·9743	17·01	20·96	16·54
0·9792	13·21	16·36	12·91	0·9742	17·09	21·05	16·62
0·9791	13·29	16·45	12·99	0·9741	17·17	21·15	16·69
0·9790	13·36	16·54	13·06	0·9740	17·24	21·24	16·76
0·9789	13·44	16·64	13·13	0·9739	17·32	21·33	16·84
0·9788	13·51	16·73	13·20	0·9738	17·40	21·42	16·91
0·9787	13·59	16·82	13·28	0·9737	17·47	21·52	16·98
0·9786	13·67	16·91	13·35	0·9736	17·55	21·61	17·06
0·9785	13·74	17·00	13·42	0·9735	17·63	21·70	17·13
0·9784	13·82	17·10	13·50	0·9734	17·70	21·79	17·20
0·9783	13·89	17·19	13·57	0·9733	17·78	21·89	17·28
0·9782	13·97	17·28	13·64	0·9732	17·86	21·98	17·35
0·9781	14·05	17·38	13·72	0·9731	17·93	22·07	17·42
0·9780	14·12	17·47	13·79	0·9730	18·01	22·16	17·49
0·9779	14·20	17·56	13·86	0·9729	18·09	22·25	17·56
0·9778	14·28	17·66	13·94	0·9728	18·16	22·34	17·64
0·9777	14·35	17·75	14·01	0·9727	18·24	22·43	17·71
0·9776	14·43	17·84	14·08	0·9726	18·31	22·52	17·78
0·9775	14·51	17·94	14·16	0·9725	18·39	22·62	17·85
0·9774	14·59	18·03	14·23	0·9724	18·46	22·71	17·92
0·9773	14·66	18·12	14·31	0·9723	18·54	22·80	17·99
0·9772	14·74	18·22	14·38	0·9722	18·61	22·89	18·07
0·9771	14·82	18·31	14·45	0·9721	18·69	22·98	18·14
0·9770	14·90	18·41	14·53	0·9720	18·77	23·07	18·21
0·9769	14·98	18·50	14·60	0·9719	18·84	23·16	18·28
0·9768	15·05	18·60	14·68	0·9718	18·92	23·25	18·35
0·9767	15·13	18·69	14·75	0·9717	18·99	23·34	18·42
0·9766	15·21	18·79	14·83	0·9716	19·07	23·43	18·49
0·9765	15·29	18·88	14·90	0·9715	19·14	23·52	18·56
0·9764	15·37	18·98	14·98	0·9714	19·22	23·61	18·64
0·9763	15·45	19·07	15·05	0·9713	19·29	23·70	18·71
0·9762	15·53	19·17	15·13	0·9712	19·37	23·79	18·78
0·9761	15·60	19·26	15·21	0·9711	19·44	23·88	18·85
0·9760	15·68	19·36	15·28	0·9710	19·52	23·97	18·92
0·9759	15·76	19·45	15·36	0·9709	19·59	24·06	18·99
0·9758	15·84	19·55	15·43	0·9708	19·67	24·15	19·06
0·9757	15·92	19·64	15·50	0·9707	19·74	24·24	19·13
0·9756	16·00	19·74	15·58	0·9706	19·82	24·33	19·20
0·9755	16·08	19·83	15·65	0·9705	19·89	24·42	19·27
0·9754	16·15	19·93	15·73	0·9704	19·97	24·51	19·34
0·9753	16·23	20·02	15·80	0·9703	20·04	24·59	19·41
0·9752	16·31	20·12	15·88	0·9702	20·12	24·68	19·48
0·9751	16·39	20·21	15·95	0·9701	20·19	24·77	19·55

0·9700	20·27	24·86	19·62	0·9650	23·88	29·14	23·00
0·9699	20·34	24·95	19·69	0·9649	23·95	29·22	23·07
0·9698	20·41	25·04	19·76	0·9648	24·02	29·31	23·13
0·9697	20·49	25·12	19·83	0·9647	24·09	29·39	23·20
0·9696	20·56	25·21	19·90	0·9646	24·16	29·47	23·26
0·9695	20·64	25·30	19·97	0·9645	24·23	29·55	23·33
0·9694	20·71	25·39	20·04	0·9644	24·30	29·64	23·39
0·9693	20·78	25·48	20·11	0·9643	24·37	29·72	23·46
0·9692	20·86	25·56	20·18	0·9642	24·44	29·80	23·52
0·9691	20·93	25·65	20·25	0·9641	24·51	29·88	23·59
0·9690	21·00	25·74	20·32	0·9640	24·58	29·96	23·65
0·9689	21·08	25·83	20·39	0·9639	24·65	30·04	23·71
0·9688	21·15	25·91	20·45	0·9638	24·71	30·12	23·78
0·9687	21·22	26·00	20·52	0·9637	24·78	30·20	23·84
0·9686	21·30	26·09	20·59	0·9636	24·85	30·29	23·91
0·9685	21·37	26·17	20·66	0·9635	24·92	30·37	23·97
0·9684	21·44	26·26	20·73	0·9634	24·99	30·45	24·03
0·9683	21·52	26·35	20·80	0·9633	25·06	30·53	24·10
0·9682	21·59	26·43	20·86	0·9632	25·13	30·61	24·16
0·9681	21·66	26·52	20·93	0·9631	25·19	30·69	24·22
0·9680	21·73	26·61	21·00	0·9630	25·26	30·77	24·28
0·9679	21·81	26·69	21·07	0·9629	25·33	30·85	24·35
0·9678	21·88	26·78	21·14	0·9628	25·40	30·92	24·41
0·9677	21·95	26·86	21·20	0·9627	25·47	31·00	24·47
0·9676	22·02	26·95	21·27	0·9626	25·53	31·08	24·54
0·9675	22·10	27·04	21·34	0·9625	25·60	31·16	24·60
0·9674	22·17	27·12	21·41	0·9624	25·67	31·24	24·66
0·9673	22·24	27·21	21·47	0·9623	25·74	31·32	24·72
0·9672	22·31	27·29	21·54	0·9622	25·80	31·40	24·78
0·9671	22·38	27·38	21·61	0·9621	25·87	31·47	24·84
0·9670	22·46	27·46	21·68	0·9620	25·94	31·55	24·91
0·9669	22·53	27·55	21·74	0·9619	26·00	31·63	24·97
0·9668	22·60	27·63	21·81	0·9618	26·07	31·71	25·03
0·9667	22·67	27·72	21·88	0·9617	26·13	31·78	25·09
0·9666	22·74	27·80	21·94	0·9616	26·20	31·86	25·15
0·9665	22·81	27·89	22·01	0·9615	26·27	31·94	25·21
0·9664	22·89	27·97	22·08	0·9614	26·33	32·01	25·27
0·9663	22·96	28·05	22·14	0·9613	26·40	32·09	25·33
0·9662	23·03	28·14	22·21	0·9612	26·46	32·17	25·39
0·9661	23·10	28·22	22·28	0·9611	26·53	32·24	25·45
0·9660	23·17	28·31	22·34	0·9610	26·59	32·32	25·51
0·9659	23·24	28·39	22·41	0·9609	26·66	32·39	25·57
0·9658	23·31	28·47	22·48	0·9608	26·72	32·47	25·63
0·9657	23·38	28·56	22·54	0·9607	26·79	32·54	25·69
0·9656	23·46	28·64	22·61	0·9606	26·85	32·62	25·75
0·9655	23·53	28·73	22·67	0·9605	26·92	32·69	25·81
0·9654	23·60	28·81	22·74	0·9604	26·98	32·77	25·87
0·9653	23·67	28·89	22·81	0·9603	27·05	32·84	25·93
0·9652	23·74	28·98	22·87	0·9602	27·11	32·92	25·98
0·9651	23·81	29·06	22·94	0·9601	27·17	32·99	26·04

0·9600	27·24	33·07	26·10	0·9550	30·30	36·60	28·89
0·9599	27·30	33·14	26·16	0·9549	30·36	36·66	28·94
0·9598	27·37	33·22	26·22	0·9548	30·42	36·73	28·99
0·9597	27·43	33·29	26·28	0·9547	30·48	36·80	29·05
0·9596	27·49	33·36	26·34	0·9546	30·54	36·87	29·10
0·9595	27·56	33·44	26·39	0·9545	30·60	36·93	29·15
0·9594	27·62	33·51	26·45	0·9544	30·66	37·00	29·21
0·9593	27·68	33·59	26·51	0·9543	30·71	37·07	29·26
0·9592	27·75	33·66	26·57	0·9542	30·77	37·13	29·31
0·9591	27·81	33·73	26·63	0·9541	30·83	37·20	29·36
0·9590	27·87	33·80	26·68	0·9540	30·89	37·27	29·42
0·9589	27·94	33·88	26·74	0·9539	30·95	37·33	29·47
0·9588	28·00	33·95	26·80	0·9538	31·00	37·40	29·52
0·9587	28·06	34·02	26·85	0·9537	31·06	37·46	29·57
0·9586	28·12	34·09	26·91	0·9536	31·12	37·53	29·62
0·9585	28·19	34·16	26·97	0·9535	31·18	37·60	29·68
0·9584	28·25	34·24	27·02	0·9534	31·24	37·66	29·73
0·9583	28·31	34·31	27·08	0·9533	31·29	37·73	29·78
0·9582	28·37	34·38	27·14	0·9532	31·35	37·79	29·83
0·9581	28·43	34·45	27·19	0·9531	31·41	37·86	29·88
0·9580	28·49	34·52	27·25	0·9530	31·47	37·92	29·93
0·9579	28·56	34·59	27·31	0·9529	31·52	37·99	29·99
0·9578	28·62	34·66	27·36	0·9528	31·58	38·05	30·04
0·9577	28·68	34·73	27·42	0·9527	31·64	38·12	30·09
0·9576	28·74	34·80	27·47	0·9526	31·70	38·18	30·14
0·9575	28·80	34·88	27·53	0·9525	31·75	38·25	30·19
0·9574	28·86	34·95	27·58	0·9524	31·81	38·31	30·24
0·9573	28·92	35·02	27·64	0·9523	31·87	38·38	30·29
0·9572	28·98	35·09	27·69	0·9522	31·92	38·44	30·34
0·9571	29·05	35·16	27·75	0·9521	31·98	38·51	30·39
0·9570	29·11	35·23	27·81	0·9520	32·04	38·57	30·45
0·9569	29·17	35·30	27·86	0·9519	32·09	38·63	30·50
0·9568	29·23	35·37	27·92	0·9518	32·15	38·70	30·55
0·9567	29·29	35·43	27·97	0·9517	32·21	38·76	30·60
0·9566	29·35	35·50	28·02	0·9516	32·26	38·83	30·65
0·9565	29·41	35·57	28·08	0·9515	32·32	38·89	30·70
0·9564	29·47	35·64	28·13	0·9514	32·38	38·95	30·75
0·9563	29·53	35·71	28·19	0·9513	32·43	39·02	30·80
0·9562	29·59	35·78	28·24	0·9512	32·49	39·08	30·85
0·9561	29·65	35·85	28·30	0·9511	32·54	39·14	30·90
0·9560	29·71	35·92	28·35	0·9510	32·60	39·21	30·95
0·9559	29·77	35·99	28·41	0·9509	32·66	39·27	31·00
0·9558	29·83	36·05	28·46	0·9508	32·71	39·33	31·05
0·9557	29·89	36·12	28·51	0·9507	32·77	39·40	31·10
0·9556	29·95	36·19	28·57	0·9506	32·82	39·46	31·15
0·9555	30·01	36·26	28·62	0·9505	32·88	39·52	31·20
0·9554	30·07	36·33	28·67	0·9504	32·93	39·58	31·25
0·9553	30·13	36·39	28·73	0·9503	32·99	39·65	31·29
0·9552	30·18	36·46	28·78	0·9502	33·05	39·71	31·34
0·9551	30·24	36·53	28·83	0·9501	33·10	39·77	31·39

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0·8000	96·96	98·09	77·43	0·7950	98·62	99·15	78·26
0·7999	96·99	98·12	77·45	0·7949	98·65	99·17	78·28
0·7998	97·03	98·14	77·46	0·7948	98·68	99·19	78·30
0·7997	97·06	98·16	77·48	0·7947	98·72	99·21	78·31
0·7996	97·09	98·18	77·50	0·7946	98·75	99·23	78·33
0·7995	97·13	98·20	77·52	0·7945	98·78	99·25	78·34
0·7994	97·16	98·22	77·53	0·7944	98·82	99·27	78·36
0·7993	97·19	98·25	77·55	0·7943	98·85	99·29	78·38
0·7992	97·23	98·27	77·57	0·7942	98·88	99·31	78·39
0·7991	97·26	98·29	77·58	0·7941	98·91	99·33	78·41
0·7990	97·29	98·31	77·60	0·7940	98·95	99·35	78·42
0·7989	97·33	98·33	77·62	0·7939	98·98	99·37	78·44
0·7988	97·36	98·35	77·63	0·7938	99·01	99·39	78·46
0·7987	97·39	98·38	77·65	0·7937	99·04	99·41	78·47
0·7986	97·43	98·40	77·67	0·7936	99·08	99·43	78·49
0·7985	97·46	98·42	77·69	0·7935	99·11	99·45	78·50
0·7984	97·50	98·44	77·70	0·7934	99·14	99·48	78·52
0·7983	97·53	98·46	77·72	0·7933	99·17	99·50	78·54
0·7982	97·56	98·48	77·74	0·7932	99·21	99·52	78·55
0·7981	97·59	98·50	77·75	0·7931	99·24	99·54	78·57
0·7980	97·63	98·52	77·77	0·7930	99·27	99·56	78·58
0·7979	97·66	98·55	77·79	0·7929	99·30	99·58	78·60
0·7978	97·69	98·57	77·80	0·7928	99·34	99·59	78·61
0·7977	97·73	98·59	77·82	0·7927	99·37	99·61	78·63
0·7976	97·76	98·61	77·84	0·7926	99·40	99·63	78·65
0·7975	97·79	98·63	77·85	0·7925	99·43	99·65	78·66
0·7974	97·83	98·65	77·87	0·7924	99·47	99·67	78·68
0·7973	97·86	98·67	77·89	0·7923	99·50	99·69	78·69
0·7972	97·89	98·69	77·90	0·7922	99·53	99·71	78·71
0·7971	97·93	98·71	77·92	0·7921	99·56	99·73	78·72
0·7970	97·96	98·74	77·94	0·7920	99·59	99·75	78·74
0·7969	97·99	98·76	77·95	0·7919	99·63	99·77	78·75
0·7968	98·03	98·78	77·97	0·7918	99·66	99·79	78·77
0·7967	98·06	98·80	77·99	0·7917	99·69	99·81	78·79
0·7966	98·09	98·82	78·00	0·7916	99·72	99·83	78·80
0·7965	98·13	98·84	78·02	0·7915	99·76	99·85	78·82
0·7964	98·16	98·86	78·04	0·7914	99·79	99·87	78·83
0·7963	98·19	98·88	78·05	0·7913	99·82	99·89	78·85
0·7962	98·22	98·90	78·07	0·7912	99·85	99·91	78·86
0·7961	98·26	98·92	78·08	0·7911	99·88	99·93	78·88
0·7960	98·29	98·94	78·10	0·7910	99·92	99·95	78·89
0·7959	98·32	98·97	78·12	0·7909	99·95	99·97	78·91
0·7958	98·36	98·99	78·13	0·7908	99·98	99·99	78·92
0·7957	98·39	99·01	78·15	0·79074	100·00	100·00	78·93
0·7956	98·42	99·03	78·17				
0·7955	98·46	99·05	78·18				
0·7954	98·49	99·07	78·20				
0·7953	98·52	99·09	78·22				
0·7952	98·55	99·11	78·23				
0·7951	98·59	99·13	78·25				