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SCHOOL OF GRADUATE STUDIES
Institute of Biotechnology

ISOLATION AND CHARACTERIZATION OF SOME POTENTIAL BIOETHANOL PRODUCING INDIGENOUS WILD YEASTS FROM LOCAL ALCOHOLIC RAW MATERIALS (*AREKE DIFDIF AND TENSES*)

MSc Thesis:

Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology (Industrial Biotechnology).

By:

Alene Admas

Advisors: Diriba Muleta (PhD)

Anteneh Tesfaye (PhD)

December, 2020

Addis Ababa, Ethiopia

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EXAMINERS'APPROVAL SHEET

This is to certify that the Thesis prepared by Alene Admas, entitled: **"Isolation and Characterization of Potential Bioethanol Producing Indigenous Wild Yeasts from Local Alcoholic Raw Materials (*Areke Difdif and Tenses)"*** is submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the standard with respect to originality and quality signed by the examining committee:

Signed by the Examining Committee:

Dr. Driba Muleta (Advisor) _____ _____

Signature Date

We, the undersigned, members of the board of examiners of the final open defense by Alene Admas have read and evaluated her Thesis entitle **"Isolation and Characterization of Potential Bioethanol Producing Indigenous Wild Yeasts from Local Alcoholic Raw Materials (*Areke Difdif and Tenses)"*** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirement for the degree Mater of science in Biotechnology.

Dr. Addis Simachew (Examiner) _____ _____

Signature Date

Dr. Asnake Dessalegn (Examiner) _____ _____

Signature Date

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List of abbreviation/acronyms

ADH	Alcohol Dehydrogenase
HMF	Hydroxyl methyl Formaldehyde
PEG	Polyethylene Glycol
DDG	Distiller Dried Grain
DDGS	Distiller Dried Grain with soluble
DNS	Dinitrosalisalic Acid
MW	Mega Watt
CFU/ml	Colony Forming Unit per milliliter
Dd	Dembecha difdif
Dbd	Debre Berhan difdif
Dt	Dembecha tinses
Md	Mother difdif
dNTP	Dideoxy nucleotide triphosphate
ddTTP	Dideoxy thymine triphosphate
ddCTP	Dideoxy cytosine triphosphate
ddGTP	Dideoxy guanine triphosphate
ddATP	Dideoxy adenine triphosphate
NCBI	National Center for Biotechnology Information
CTAB	Acetyl Trimethylammonium Bromide
BLAST	Basic Local Alignment Search Tool
ITS	Internal transcriber spacer
EDTA	Ethylene diamine tetra acetic acid
OD	Optical Density
ANOVA	Analysis of Variance
vvm	Volume of air in volume of medium per minute

Abstract

Shortage of fossil fuel supplies has increased a great deal of interest in worldwide production of renewable energy. Bioethanol produced from renewable biomass like molasses has received considerable attention as an alternative biofuel in recent years. Thus, the main purpose of this research work was to isolate, screen and characterize ethanol tolerant wild indigenous yeasts with high bioethanol yield from areke difdif and tinses samples collected from different areke producing sites. The wild yeast isolates were retrieved from the alcoholic beverages following standard methods. From a total of 270 isolates, ten (3.7%) of the yeast isolates tolerated 22% and four (1.5%) isolates from the same 10 isolates tolerated 23% ethanol concentration, and were selected for further characterization. Based on morphological appearance of vegetative cells under microscope, colony characters and molecular analysis, the isolates were identified as Saccharomyces cervicae and Kluyvermyces marixianus. Ten (Mda, Mdb, Mdd, D5f, D5b, D5d, D5e, Dt1e, Dt1c and Dtlj) isolates were found thermotolerant (45^{o}C and Mda able to tolerate 47 ^{o}C) and also all ten isolates grow at low pH (>=2.5). Ethanol production from sugar cane molasses by the yeast isolates was determined by using Hall method and was crosschecked with Ebulliometer. From 10 isolates, two isolates (Mda and Dt1e) showed the highest bioethanol production capacity of 14.3% v/v and 13.2% v/v, respectively at pH 4.39 and temperature 35^{o}C in 30 degree brix molasses concentration at 150 rpm shaking condition. During this study potentially useful and excellent ethanol producing indigenous yeasts with high ethanol tolerant features from areki difdif and tenses were isolated, identified and characterized. This study exhibited the potential of local fermentation processes and products (areke difdif and tenses) as possible sources of industrially useful microbial isolates.

Keywords: *Yeasts, Areki Difdif/Tenses, Tolerance, Bioethanol, Molasses*

1. INTRODUCTION

Bioethanol is an ecofriendly and most promising biofuel that can be used in unmodified petrol engines and produced from diverse agro-industrial wastes such as molasses. The combustion of bioethanol results in relatively low emission of volatile organic compounds with low evaporation, carbon monoxide and nitrogen oxides and has high biodegradable properties. The emission and toxicity of ethanol are lower than those of fossil fuels such as petroleum and diesel (Hansen *et al*., 2005) that makes it the most preferred fuel in various sectors.

Molasses is the most preferable substrate for fermentation by yeasts to produce bioethanol because it is rich in nutrients, salts and contains easily consumable quantity of sugars 40 to 50% w/v (Ayhan, 2008). It is the final byproduct of sugar factories that is easily available and economically low in price for using it as a raw material for ethanol production (Khoja *et al*., 2015). In Ethiopia, currently, the only raw material for bioethanol production at industrial level is molasses. Molasses is used as an alternative feed in animal fattening and also used as raw material for production of yeast biomass and diverse organic acids. It is stated that from 2014 to 2020 the production of molasses increased from 0.5 million tons to 1.5 million tons and is estimated to be 3 million tons by 2030 (Ethiopian Sugar Corporation, 2020). Generally, conversion of one ton of molasses by yeast fermentation yields 250 liters of green energy (bioethanol).

Therefore, bioethanol produced from renewable biomass like molasses has received considerable attention in current years. Ethanol can be produced by bacteria and yeasts. However, yeasts have some advantages over bacteria in having efficient bioconversion ability of sugars into bioethanol with some important industrial characteristics (low nutrient requirements, ethanol resistance and tolerance to pH).

The energy crisis in Ethiopia prompts the study and discovery of new processes of producing renewable bioenergy such as bioethanol from sugar cane molasses using potent

fermentative wild indigenous yeasts. In many respects, bioethanol is investigated as renewable energy and functionally it is comparable to gasoline fuel. A large number of research activities have been done so far on isolation and characterization of different species of wild fermentative yeasts for production of ethanol from different substrates. Most of these studies are focused on isolation and characterization of wild yeast from diverse sources such as fruit, teff, molasses, bagasse, sugar cane juices, tej, tella, borde and so on.

Accordingly, Ethiopian local *areke difdif* contains large varieties of microbes to initiate the fermentation process and can serve as potential source of wild yeasts isolation. Fermentation of *tinses* and *difdif* for *areke* production is one of the promising sources for isolation and characterization of potential ethanol tolerant indigenous yeasts for bioethanol production. During ethanol fermentation, yeasts are exposed to various stresses such as ethanol they produce and which is considered to be the major stress that decreases the final yield. In addition, heat stress which is generated during fermentation process can greatly affect ethanol production by decreasing the growth rate of yeast strains. However, ethanol production at high temperatures has gained much interest due to several advantages that include a reduced risk of contaminations and theromtolerant isolates can able to adapt to heat and ethanol generated during fermentation (Techaparin *et al*., 2017). Hence, the ability of stresses tolerance of the yeasts becomes one of the most influencing factors in ethanol fermentation using fermentative microbes (Fakruddin *et al*., 2013).

Therefore, major purpose of this study was to isolate and characterize potential ethanol tolerant indigenous wild yeast from some local beverages and to examine the proper fermentation processes for bioethanol production from cheap substrates.

2. Objectives

2.1. General objective

The general objective of this study was to screen stress tolerant indigenous wild yeasts isolated from some traditionally fermented beverages in order to enhance bioethanol production from cheap substrates generated from sugar industries.

2.2. Specific objectives

The specific objectives of the present study were to:

- Isolate and screen wild indigenous yeasts from local *areke difdif* and *tensis* based on their ethanol tolerance.
- Characterize the selected stresses tolerant yeasts using morphological and molecular methods
- Determine bioethanol yield from molasses using potential ethanol, temperature and pH tolerant wild yeasts.

3.LITERATURE REVIEW

3.1. Ethiopian traditional beverages

Ethiopia is a country rich in ethnic diversity that made the possibility of producing varieties of traditionally fermented foods and beverages. Thus the varieties of foods and beverages processed and consumed among the various ethnic groups are manifestations of this diversity. 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 *tella*, *tej*, *brode*, *areke*, *keribo*, *korefe*, etc are very common. Fermentation of *tej*, *tella*, *areki*, and *korefe* like other traditionally fermented alcoholic beverages depends on the microorganisms present in the substrates and fermentation vats (Getachew Tafere, 2015).

The preparation of many indigenous or traditional fermented beverages in Ethiopia is still a household art. Although some data were generated on the economic and nutritional implications of the indigenous fermented traditional alcoholic beverages in the 1970s, the involvement of Ethiopian researchers in studying the microbiology of traditional fermented beverages started only in the 1980's and quite a number of publications have been produced during the last two decades (Mehari and Ashenafi, 1999). Considering the rich diversity in fermented beverage types in the country, however, the microbiology of a variety of Ethiopian beverages still remains to be studied.

Fermented beverages constitute a major part of the diet of traditional African homes serving the fermented beverages are consumed in different occasions such as marriage, naming and rain making ceremonies (Zvauya *et al*., 1997), at festivals and social gatherings, at burial ceremonies and settling disputes (Getachew Tafere, 2015). They are also used as medicines for fever and other sicknesses by adding barks or stems of certain plants to them (Okafor, 1972). Various groups of microorganisms are involved in the fermentation of indigenous beverages in different parts of the world including Ethiopia.

The sources of the microorganisms are usually the ingredients and the instruments used for fermentation processes to produce many traditional fermented beverages (Blandino *et al*., 2003). Therefore, a wide variety of microorganisms especially yeast species and lactic acid bacteria are involved in fermentation but not all microflora could effectively ferment to produce the targeted traditional products (Girum *et al*., 2005b). It may, thus be said that the initiation of fermentation of most traditional fermented beverages may be undertaken by different groups of microorganisms as far as sufficient fermentable sugars are available in the substrate. As the fermentation proceeds and the environment becomes more and more acidic, yeasts and lactic acid bacteria dominate the fermentation. These two groups of microorganisms usually determine the alcohol content and flavor of the final traditional fermented product (Girum *et al*., 2005a).

3.2. Areki fermentation in Ethiopia

Areki is a colorless, clear, traditional alcoholic beverage which is commonly produced in many parts of Ethiopia especially Debrebrhan, Dembecha and Arsi Negele. It is distilled from fermentation products prepared in almost the same way as tella except that the fermentation mass in this case is more concentrated (Fite *et al*., 1991). Areki is usually brewed in rural and semi-urban areas and is used more commonly by farmers and semi-urban dwellers than by people who live in the cities. In cities, those who drink areki are predominantly lower class people or those who have become dependent on alcohol and cannot afford to buy industrially produced alcohol (WHO, 2004).

Traditionally areki is classified into two: Terra-areki and Dagim-areki. The term dagim in Amharic refers to ‘second time’ and, indicates that it is distilled second time, whereas the term ‘terra’ indicates that the fermentation is done once in Amharic refers to ‘ordinary’. The alcohol content of terra-areki was reported to be 34.09% (v/v) (Desta, 1977), and varies between 22.0 – 28.0% (v/v) (Selinus R., 1971). Dagim areki is a stronger type of terra-areki, which is prepared in the same way as terra-areki, except that the distillation process is allowed to remain for a shorter period of time, or three volumes of terra-areki are redistilled to give about one volume of dagim-areki. The redistilled areki therefore has

higher alcohol content. The average alcohol content of dagim areki is around 45% (v/v) (Selinus, 1971). It was also reported to have a mean value of 46.6% (v/v) ethanol content (Desta, 1977).

3.3. The fermentation and microbiology of *tinsis* and *difidif*

3.3.1. Areki tensis

Areki Tensis is prepared by mixing powdered Gesho leaves and powdered bikil (1:2 ratios) with water to give a mixture of free flowing consistency, and which is then put aside to ferment for about five days (Desta, 1977).

3.3.2. Areki difdif

Areki difdif can be prepared based on fermentation time of areki tensis. Depending on the locality some amount of cakes of flour Dagussa (Elusine coracann) or Maize or Wheat roughly equivalent to four times that of the bikil, is added into tensis after being kneaded with water to make dough and baking. The hot cakes are broken into pieces and added to the first mixture and with more water, well mixed to make difdif, which is believed to have strong and efficient fermenting yeasts. Then difdif is again left aside to ferment for about four days. Portions of the second mixture are transferred to the traditional distillation apparatus and distilled to give what is known as terra areki (Getachew Tafere, 2015).

3.4. Yeasts

Yeasts are unicellular fungi that reproduce by budding or fission and form spores which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). They are classified into two phylogenetic groups such as teleomorphic and anamorphic Ascomycetous or teleomorphic and anamorphic Basidiomycetous). They are first classified based on their sexuality (Ascomycotina or Basidiomycotina) or lack of sexual phase in the life cycle (Deuteromycotina). The lower taxonomic subdivisions (families, subfamilies, genera, species and strain) are determined by their morphological, physiological and genetic characteristics including sexual reproduction (Kurtzman and Fell, 1998). At the genus level yeasts can be classified as *Schizosaccharomyces, Zygosaccharomyces, Zygopichia,*

Debaryomyces, Nadsonia, *Nematospora* with haploid vegetative cells and *Saccharomyces, Saccharomycodes, Hansenula* with diploid vegetative cells (Kurtzman, 1994).

Since thousands of years ago, yeasts have been used in alcohol production especially in the brewery and wine industries. It keeps the distillation cost low as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration (Kasavi *et al*., 2012). Now a day, yeasts are used to generate fuel ethanol from renewable energy sources (Kosaric and Velikonja, 1995). Certain yeast strains such as *Pichia stipites* (NRRL-Y-7124), *S. cerevisiae* (RL-11) and *Kluyveromyces fagilis*(Kf1) were reported as good ethanol producers from different types of sugars (Mussato *et al*., 2012). Baker's yeast was traditionally used as a starter culture in ethanol production due to its low cost and easy availability. Stressful conditions like an increase in ethanol concentration, temperature, osmotic stress and bacterial contamination are the reasons why the yeast cannot survive during the fermentation (Basso *et al*., 2008). Flocculent yeasts are also used during biological fermentation for ethanol production as it facilitates downstream processing, allows operation at high cell density and gives higher overall productivity (Domingues *et al*., 2000) and also reduces the cost of cells recovery as it separates easily from the fermentation medium without centrifugation (Choi *et al*., 2010).

The yeast species *Saccharomyces cerevisiae* under the genus of *Saccharomyces* has been used in baking and fermentation to produce alcoholic beverages by converting sugars into ethanol for thousands of years. The art of bread making needs the carbon dioxide produced by yeast in order for dough to rise. It is also extremely important as a model organism in modern cell biology research, and is one of the most thoroughly researched eukaryotic microorganisms. Researchers have used it to gather information about the biology of the eukaryotic cell and ultimately human biology (Ostergaard, *et al*., 2000). Other species of yeast, such as *Candida albicans*, are opportunistic pathogens and can cause infections in humans.

Yeasts like *Saccharomyces cervisiae* after genetically engineered have been used to generate electricity in microbial fuel cells and produce bioethanol for the biofuel industry

(Madhavan *et al.*, 2012). In the making of wine and beer, the yeasts' produce alcohol which is desired and necessary for the final product and carbon dioxide is what makes beer and champagne sparkling. To multiply and grow, all yeasts need is the right environment, which includes moisture, food (in the form of sugar or starch) and a warm, nurturing temperature (21°C to 30°C) is best (Neiman, 2005).

3.4.1. Sources of yeast and ecological diversity

The ecological diversity of yeasts is relatively well known compared to the other microorganisms. Yeasts, including *Candida albicans*, *Rhodotorula rubra*, *Torulopsis* and *Trichosporon cutaneum*, have been found in between people's toes as part of their skin flora. Yeasts are also present in the gut of mammals as normal flora and some insects and even deep-sea environments host several yeast species (Bass *et al*., 2007). The number of discovered yeasts has been increasing from year to year. More than 2500 yeast species were published so far. It is assumed that only 1% of yeast species is currently known which represents approximately 1500 species. The total numbers of yeast species on earth are expected to reach 150,000 (Barriga *et al*., 2011). The diversity of yeast species in particular niches is determined by its capability of utilizing different sugar rich raw materials and its nutritional selectivity as it exhibits great specialization for habitat (Phaff and Starmer, 1987). Yeasts can be isolated from the terrestrial, aquatic and aerial environment. Plant is the preferred habitat of yeasts community. A few species are found to have commensalism or parasitic relationships with animals. Extreme environments like low water potential (high sugar or salt concentration) and low temperature can inhibit the growth of yeasts (Walker, 2009).

There are a broad diversity of yeast cells including in their sizes, shapes and colors (Walker *et al*., 2002). Cell sizes of yeasts are influenced by its species and growth condition. The length of some yeast cells are only 2–3 µm while the other species may reach the length of 20–50 µm (Phaff and Starmer, 1987). The width of most yeasts ranges from 1–10 µm. Generally, the sizes of brewing strains of *S. cerevisiae* are larger than laboratory strains

(Hough *et al*., 1982). Many yeast species including *Saccharomyces spp*. are ellipsoidal or ovoid in shape and have creamy color colonies (Walker, 2009).

3.4.2. Yeast and fermentation

Fermentation is a metabolic process that produces chemical changes in organic substrates such as sugars through the action of microbial enzymes especially yeast to produce important bio-products like bioethanol in the absence of oxygen (Tortora *et al.*, 2010). Sugars, which are preferable yeast food, must be broken down into smaller molecules to become a source of energy and building blocks for the synthesis of important by products through fermentation. The major initial process for oxidizing sugars is glycolysis which is a series of ten reactions breaking down a glucose molecule into two molecules of pyruvate (Palaez *et al*. 2010). The first step in fermentation of sugars to produce ethanol is conversion of pyruvate into acetyl CoA which is catalyzed by the pyruvate dehydrogenase complex in the mitochondria (Figure 1). However, in many types of yeast, cytoplasmic pyruvate enters into the alcoholic fermentation pathway. Yeasts, depending on conditions, can use sugars by fermentation (Flores *et al.* 2000). Since respiration of sugars is energetically more favorable than fermentation, most organisms use fermentation only when respiration is impaired, for example when oxygen availability decreases. However, in several yeast species, like *S. cerevisiae*, the metabolic destiny of pyruvate formed at a high rate is largely switched from respiration to fermentation even when oxygen is abundant. In other words, *S. cerevisiae* may ferment sugars also under aerobic conditions, showing the so-called ‘‘Crabtree positive’’ phenotype (Piskur *et al.* 2006).

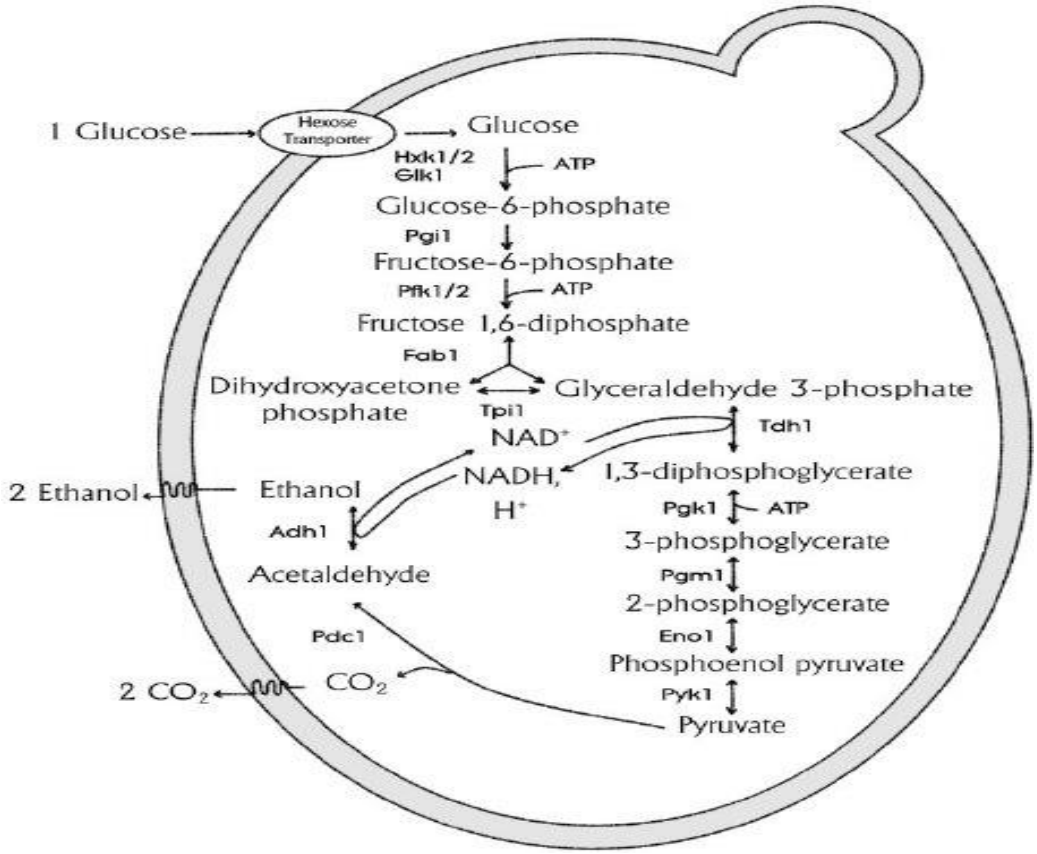

Figure 1. Alcoholic fermentation enzymatic steps on *S. cerevisiae* (adopted from Pretorius, 2000)

Hundreds of specialty strains have been commercialized for ethanol or CO_2 production, including specific strains for baking, wine, beer, distilled beverages, and fuel ethanol. In practice, more than 90% of the glucose from starch and other sugar sources are converted to ethanol and carbon dioxide by the yeast cell in fermentation mode (Behera *et al*., 2010).

3.4.3. The genetics of alcohol production by yeasts

Yeasts are able to produce a variety of reduced organic molecules and have good potential to play a central role in the development of bioethanol from any carbon sources (Nielsen *et al*., 2013). The production of bioethanol depends on the ability of yeasts to catabolize six carbon molecules especially glucose into two carbon component, ethanol.

In the production of ethanol from pyruvate the first step is the cytosolic decarboxylation to acetaldehyde and CO_2 by the enzyme pyruvate decarboxylase (PDC). In *S. cerevisiae* three

genes (PDC1, PDC5 and PDC6) encode this enzymatic activity. PDC2 encodes a positive regulator of the transcription of PDC1 and PDC5 (Hohmann 1993). So that, the process of bioethanol production through fermentative metabolism and the catabolism shift is dependent upon the enzyme alcohol dehydrogenase (ADH1). ADH1 catalyzes the reduction of acetaldehyde to ethanol during the fermentation of glucose leading to the cytosolic re-oxidation of glycolytic NADH and it can also catalyze the reverse reaction which is the conversion of ethanol into acetaldehyde (Bennetzen and Hall, 1982). In *S. cerevisiae* five genes (Adh1p, Adh2p, Adh3p, Adh4p and Adh5p) encode alcohol dehydrogenases involved in ethanol metabolism (Thomson *et al*. 2005). Four of these enzymes, Adh1p, Adh3p, Adh4p, and Adh5p, preferentially reduce acetaldehyde to ethanol during glucose fermentation, whereas Adh2p preferentially catalyzes the reverse reaction of oxidizing ethanol to acetaldehyde (Compago *et al.*, 2014).

The substrate for the enzyme ADH2 is ethanol (Raj *et al*., 2014). The expression of ADH2 gene is governed by transcription factors and genome sequencing and transcriptome analysis has revealed the structure and DNA binding elements of these regulatory proteins (Alper *et al.*, 2006). Recent advances in synthetic biology have focused on reengineering of the ADH gene for greater substrate specificity and improvement of catalytic activity as well as engineering the yeast genome with protein coding genes which improve tolerance to ethanol and catalysis of a wide range of carbon sources (Matsushika *et al*., 2009). Molecular biologists are actively seeking novel genes encoding ADHs using metagenomic approaches, and this had yielded a number of unique variants (Wexler *et al*., 2005).

3.4.4. Important yeast strains in bioethanol production

The selection of potential strains for bioethanol production is made by considering their productivity, tolerance to ethanol, fermentation inhibitors and severe pH and temperature conditions (Cao *et al.*, 2014). Some widely used and most dominant high alcohol producing strains are *Saccharomyces cerevisiae, S. uvarum* (formerly *S. carlsbergensis*), *Zymomonas mobilis, S. candidautilis, S. anamensis* and *Schizosaccharomyces pombe* (Mohd Azhar *et al.*, 2017). *Kluyveromyces* species, which ferment lactose, are good producers of ethanol

from whey. Ethanol production by yeast is characterized by high selectivity, low accumulation of byproducts, high ethanol yield, high fermentation rate, good tolerance toward both increased ethanol and substrate concentrations, and lower pH value (Kasemetes *et al*., 2007). Viability and genetic stability of yeast cells under process conditions and at high temperature are also desirable. *S. cerevisiae* is the most commonly employed potential yeast in industrial bioethanol production with uniform, rapid fermentation and high ethanol yields as a result they are tolerant to wide ranges of, temperature, pH levels, and high ethanol concentrations which makes the fermentation process less susceptible to infection (Lin *et al.*, 2012). Yeasts are facultative organisms which mean that they can live with or without oxygen, so in a normal fermentation cycle they use oxygen at the start, and then continue to thrive once it has all been used up.

3.5. Bioethanol

Bioethanol is a liquid biofuel produced by microbial fermentation mostly from carbohydrates produced in sugar bearing plants (Andre *et al.,* 2016). Main bioethanol feed stocks can be obtained from a corn, sugar cane, bagasse, molasses, sugar beet, sorghum, switch grass, barley, hemp, potatoes, sunflower, wheat straw, woodchips, paper, straw, cotton and most recently microalgae (Yaser *et al*., 2019). Bioethanol from biomass sources is the principal fuel used as a petrol substitute for road transport vehicles. Renewable biomass fuels reduce greenhouse gas emissions from fossil fuels (Ibeto and Okpara, 2010). Due to expensive price of crude oil biofuels production becomes attractive (Bryner and Scott, 2006). Although, bioethanol is mainly used in blends with gasoline as E10 and E20 (10 and 20% of ethanol mixed with 90 and 80% gasoline respectively), the demand has increased. For instance, consumption of bioethanol in most countries of the European Union is far greater than the quantity produced in those countries.

3.5.1. Process in bioethanol production

The biological process of ethanol production depends on the types of feed stocks used. Monosaccharide sugar containing substrates such as molasses needs only simple treatment whereas lignocellulosic biomass needs pretreatment as the yeasts cannot able to utilize

them directly. Generally, there are three major steps in ethanol production such as pretreatment of lignocellulosic substrate and hydrolysis into fermentable sugars, converting sugars to ethanol by fermentation, and ethanol separation and purification (Behera *et al*., 2010). Feed stocks are usually pretreated in order to reduce its size and facilitate subsequent processes. Then, the hemicellulose and cellulose will be hydrolyzed to fermentable sugars. Yeasts are given the responsibility to ferment these sugars into ethanol. Separation technologies are used to recover ethanol before it can be used as fuel (Wyman, 1996).

3.5.1.1. Pretreatment

The pretreatment step in the process of bioethanol production is the stage at which the lignocellulosic biomass becomes more readily available for yeasts to produce bioethanol through fermentation (Karolina *et al.*, 2018). Pretreatment has a significant effect on the overall process which makes the hydrolysis easier and produces higher amount of fermentable sugars which influences the amount of ethanol yield and production cost (Srichuwong *et al*., 2009). Different stages of pretreatment in bioethanol production include physical, chemical, physiochemical and biological pretreatments (Farrukh *et al.*, 2017). Physical pretreatment is the reduction of the size of biopolymer particles by means of fragmentation, grinding, and milling, hacking and rolling (Sinha and Pandey, 2011). This stage also includes microwave radiation, sonication, spray drying and pyrolysis (Nomanbhay *et al.*, 2013). Chemical pretreatment stage enables the decomposition of lignocellulosic biomass in to simple compounds by acid base treatment, oxidation, ozonolysis and dissolution in aqueous liquids or organic solvents (Wang *et al*., 2009) and organosolysis based process (Zhao *et al*., 2009). Physiochemical pretreatment stage uses combination oxidation and thermal such as steam explosion and or hot water, carbon dioxide explosion and ammonia fiber explosion to decompose lignocellulosic biomasses (Prasad *et al*., 2007).

Microbial enzymes are involved in biological pretreatment of lignocellulosic biomasses. Microbial pretreatment is based on wood-decomposing fungi especially white rot fungi

which produce enzymes which are able to depolymerize or cleave lignin, cellulose, and hemicellulose (Beukes *et al*., 2008). Enzymes produced by white rot includes cellulolytic enzymes (endo-1,4-β-glucanase, exo-1, 4-β-gluconase and glucohydrolases), hemicellulases (i.e., endo-1,4-β-xylanases, β-xylosidases, galactoglucomannazes or galactosides), lignin degrading enzymes such as lignin peroxidase, dioxygenases, peroxydismutases and glyoxal oxidases, and enzymes specifically interacting with lignosaccharides (glucose oxidase, piranose oxidase, oxyreductase and cellobiase) (Rodrigues *et al.,* 2008).

During pretreatment release of furan compounds like 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde induce the inhibition of cell growth and reduce ethanol productivity (Taherzadeh and Karimi, 2008). Yeasts fermentation is inhibited by the weak acid stress induced from lignocellulosic materials. However, the low concentration of weak acids can increase ethanol production by cellular division. It was reported that the presence of weak acids can improve glucose utilization, ethanol production and tolerance to HMF and furfural in *S. cerevisiae* (Greetham *et al.,* 2016).

3.5.1.2. Hydrolysis

Hydrolysis process takes place after pretreatment to break down the feed stocks into fermentable sugar for bioethanol production. The two most commonly used hydrolysis methods are acidic and enzymatic. Acid hydrolysis is considered as the oldest and most commonly used method (Jeffries and Jin, 2000). Acidic hydrolysis can be divided into two types namely dilute and concentrated. Dilute acid hydrolysis is performed at higher temperature using low acid concentration while concentrated acid hydrolysis is carried out at lower temperature using high acid concentration (Hongzhang, 2015). Dilute acid hydrolysis is the most commonly used process. However, it generates large amount of inhibitors compared to concentrated acid hydrolysis.

Acid hydrolysis of lignocellulosic biomass is conducted in two-stage process as the pentose sugars degrade more rapidly compared to hexose sugars. Hemicellulose is hydrolyzed in the first stage using dilute acid while cellulose is hydrolyzed in the second stage using concentrated acid. Concentrated acid process generates high sugar recovery (90%) in

shorter period of time (Joshi *et al*., 2011). The disadvantages of acid hydrolysis are the difficulty of performing acid recovery and recycling process which increases the production cost. Enzymatic hydrolysis requires enzymes to hydrolyze the feed stocks into fermentable sugars. Three types of enzymes that are commonly used for cellulose breakdown such as endo-β—1,4-glucanases, cello-biohydrolases and β-glucosidases. The activity of cellulase enzyme is influenced by the concentration and source of the enzyme. Cellulose will be degraded into reducing sugars under mild reaction conditions (pH: 4.8–5.0, temperature: 45–50 °C). Moreover, it does not cause corrosion problem in the reactors which can result in high sugar yields. The efficiency of enzymatic hydrolysis is influenced by optimized conditions such temperature, time, pH, enzyme loading and substrate concentration (Canilha *et al*., 2012). The amount of fermentable sugar obtained increases as the enzyme load increases while cellulose load decreases.

Enzymatic saccharification of cellulose can be enhanced by using surfactants which function to block lignin. The efficiency of cellulose hydrolysis can be improved by adding Polyethylene glycol (PEG) or Tween 20 to increase enzymatic saccharification and reduce the adsorption of cellulase on lignin (Joshi *et al*., 2011). The limitation of using enzymes in hydrolysis is because they are too expensive for the economical production of ethanol from biomass.

3.6. Bioethanol production from different sources

Bioethanol is produced from various biomass sources. Feed stocks for bioethanol are generally classified as starchy materials which include potato, corn, barley and wheat; sucrose containing feed stocks such as sugar beet, sweet sorghum and sugarcane (Dalla Marta *et al.,* 2014) and lignocellulosic biomass like wood straw and grasses (Balat, 2009). Besides that several attempts on producing bioethanol from forest residues (Hu *et al*., 2008), industrial wastes (Kadar, 2004) and corn strove (Kazi *et al.,* 2010) were reported.

3.6.1. Starch

Starchy materials such as cereal grains, potato, sweet potato and cassava are commonly used in bioethanol production after breaking starch into simple glucose molecules using bacterial enzymes (α-amylase). Starchy materials require hydrolysis to break down the starch into fermentable sugars (scarification). Typically, hydrolysis is performed by mixing the starch with water to form slurry which is then stirred and heated to rupture the starch. During the heating cycle specific enzymes such as α amylase, saccharifying α amylase, βamylase, gluco-amylase and pullulanase are added at different stages to break dawn the starch in to different components of carbohydrates (Chaves and Borges, 2016).

Bioethanol production from starch follows two major processes, dry grind (67%) and wet mill (33%) by using yeasts that are capable of metabolizing starch hydrolysates (Khanal, 2008). Dry milling is often used for bioethanol production in the USA due to its lower capital and operating costs. In this process, the whole corn is milled (hammer or roller mill) and mixed with water to obtain a mash.

A few modifications of dry-grind process have been introduced in order to recover corn germ or both germ and fiber before fermentation (Huang *et al*., 2008). Wet milling process produces various value-added co-products (e.g. fiber, germ, starch and gluten) before fermentation, which makes this process more economically feasible and energy efficient (Bothast and Schlicher, 2005).

3.6.2. Lignocellulose

Lignocellulose is a basic component of plant cell wall which is mainly composed of three fractions, lignin 7-25%, hemicellulose 14-40% and cellulose 30-60% of its dry weight depending on different types of raw materials (Table 1). (Mussatto and Teixeira, 2010). These polymers are associated with the each other by hetero matrix and their relative composition is different depending on type, species and source of biomass (Qiankang *et al.*, 2014). Cellulose is an unbranched crystalline structured mono-polymer composing the cell walls of plants as well as bacteria, fungi, and algae whereas hemicellulose is a branched

heteropolymer composed of hexoses (D-galactose, L-galactose, D-mannose, L-fructose), pentoses (L-rhamnose, arabinose, xylose), D-glucuronic acid and acetylated sugars (Karolina *et al.,* 2018). Lignin is an amorphous, water-insoluble heteropolymer which is a product of condensation of three monomeric phenol alcohols: trans-p-cumarylic, trans-p-coniferylic, and trans-p-sinapylic. Lignin is a component of a cell wall which forms an impermeable structure that protects a plant from an invasion of microbes (Karimi *et al.,* 2006).

Lignocellulosic biomass sources can be categorized as primary sources such as crops, sugar cane and short rotation plantations, secondary sources which include residues from production process like bagasse, husks and straws, and tertiary sources such as municipal solid waste (MSW), sewage treatment sludge and wood trimmings (Fischer and Schrattenholzer, 2001; Qiankang *et al.*, 2014).

Table 1. Average composition of lignocellulose components in different raw materials

Raw materials	Hemicelluloses (%w/w)	Cellulose (%w/w)	Lignin (%w/w)
Agricultural residues	25–50	37–50	5–15
Hardwood	25–40	45–47	20–25
Softwood	25–29	40–45	30–60
Grasses	35–50	25–40	0
Waste papers	12–20	50–70	6–10
Newspaper	25–40	40–55	18–30
Switch grass	30–35	40–45	12
Sugar cane bagasse	40	27	10
Corn cobs	35	16.8	7

Sources:Lukajtis *et al*., 2018, Mussatto and Teixeira, 2010, Swart *et al*., 2008 and Harel, 2009.

Bioethanol production from lignocellulose biomass sources usually needs pretreatment and hydrolysis (Shah and Rehan, 2014). Pretreatment involves in delignification of lignocellulosic feedstocks in order to make cellulose more readily accessible in hydrolysis steps (Sung and Cheng, 2002). In hydrolysis stage, cellulolytic enzymes convert cellulose

released during pretreatment in to fermentable sugar, glucose, which is easily consumable by yeasts to ferment and produce bioethanol (Zhang *et al.*, 2015).

3.6.3. Molasses

Sugarcane molasses is a viscous, dark and sugar rich byproduct of sugar industry which is used for bioethanol production to contribute a huge flux in transportation sector as well as in energy sector. Molasses is classified into two categories based on crop source: cane molasses (sourced from sugarcane) and beet molasses (from sugar beet). Cane molasses is further classified as either refinery molasses, final molasses or blackstrap molasses in accordance to the purity of the sugarcane juice used in sugar production. Blackstrap molasses is available commercially, whereas refinery and final molasses are used for animal feed, binder and it is recycled into bio-plastic production (Martel *et al*., 2011; Tripathi *et al.,* 2012).

Molasses contains about 62% of carbohydrates in the form of 30% uncrystallized sucrose and about 32% of invert sugar which is a mixture of glucose and fructose (Hemamalini *et al*., 2012). It also contains about 40% other non-sucrose components including inorganic salts, raffinose, kestose, organic acids and nitrogen containing compounds and ash content of around 5-15% (Asif *et al.,* 2018). Average approximate composition of sugar cane molasses is shown in Table 2. The utilization of sugar cane molasses for fermentation process is one of the oldest chemical processes known to human and its high simple sugar content and easily availability makes the molasses suitable substrate for rum and bioethanol production (Fedal *et al*., 2016 and Sheoran *et al*., 1998). In recent years, however many of the products are synthesized chemically from petroleum feedstock including bioethanol often cost effectively It depends upon the behavior of the microorganism during fermentation and to get the useful products like bioethanol (Kennea *et al.,* 2016). From last three decades, studies were carried out to minimize the issue in the fermentation technology for efficient bioethanol production (Asif *et al.,* 2018).

Molasses can be employed using baker's yeast for production of bioethanol; citric acid, lactic acid and gluconic acid production besides glycerol, butanol and acetone production,

as an ingredient of mixed feeds or in the production of amino acids (Satyanarayana *et al*., 2009). The sugar, which is converted into molasses, is adjusted to 14-16%, which allows alcohol content of 8 - 10 volume percentages in the fermented worts (Hemamalini *et al*., 2012).

Table 2. Average approximate composition of sugar cane molasses

Components	Normal Range (%)
Water	17-25
Sucrose	30-40
Glucose	4-9
Fructose	5-12
Total reducing substances	10-25
Ash	5-15
Vitamin (B6)	8
Wax and sterols	0.1-1
Crude protein	2.5-4.5
True protein	0.5-15
Amino acids	0.3-0.5
Salt	2-8
pH	5-7

Source: Clarke, 2003.

3.7. World bioethanol production status

Biofuels, mainly produced from biomass, are a broad range of fuels in the form of solid biomass (bio-char), liquid fuels (bioethanol, biodiesel, and vegetable oil), and various biogases (biogas, bio-syngas, and bio hydrogen) (Demirbas and Ayhan, 2009). Biofuels have attracted much attention globally due to concerns on climate change, energy security, and dependency on import encumber of petroleum products. They are increasingly premeditated by many countries as much as practicable to substitute the fossil fuel source in

the transport sector. At present bioethanol and biodiesel are the main biofuels for transport as both can be used in blended or neat form, although neat usage requires engine modification. Bioethanol, derived from starch crops like sugarcane, sugar beets, corn, wheat, and sorghum is utilized blended with petroleum based gasoline, and biodiesel (Alan *et al.*, 2005). On the other hand, bioethanol derived from oil crops like rapeseed, palm oil, jatropha, sunflower, and soy is utilized blended with petroleum based diesel (Dufey and Annie, 2006).

The highest bioethanol producing countries in the world so far are United States and Brazil (Budny and Sotero, 2011). These countries collectively dominate the world's ethanol by 87% and collectively producing almost 21.8 billion gallons (82.5 billion liters) ethanol production in 2011 (Renewable Fuels Association, 2013). United States was the major producer among the two representing 13.3 billion gallons (50.3 billion liters) of the world's ethanol production in 2011 using corn as the main feedstock accounting for the vast majority of the input. Brazil was the second major producer uses sugarcane for its 5.6 billion gallon (21.2 billion liters) ethanol production in 2011. The annual ethanol production of top countries is shown in Table 3. However, global ethanol production dropped to some extent in 2011 due to severe drought and economic distress, it has shown a considerable increase steadily through 2010 (Renewable Fuels Association (RFA), 2002 to 2012).

Table 3. Annual fuel ethanol production by world top country from 2014-2018

Rank of 2019	Country/Region	Ethanol in millions of gallons/year				
		2018	2017	2016	2015	2014
1	United States	16,061	15,936	15,413	14,807	14,313
2	Brazil	7,920	6,860	6,760	7,200	6,760
3	European Union	1,430	1,400	1377	1387	1445
4	China	1,050	860	845	1387	635
5	Canada	480	470	436	436	510
6	India	400	210	275	195	85
7	Thailand	390	370	322	334	310
8	Argentina	290	290	264	211	160
	Rest of World	549	414	490	391	865

	World total	28,570	26,810	26,182	25,774	25,083

Source: RFA, 2017; 2019

3.8. Bioethanol production in Ethiopia

Ethiopia is a country with a total land mass of 1.2 million square kilometer and is said to have an estimated potential of about 25 million hectare land which is suitable for production of renewable energy (Biofuel) feedstock (Gebremeskel and Tesfaye, 2008). Besides, Ethiopia is the most suitable country in Africa for trapping renewable sources of energy and working to enhance its energy capacity. Ethiopian government's biofuel strategy is a manifestation of this effort by encouraging domestic biofuel production with an objective of reducing dependence on high cost fossil fuel (MoME, 2007). As part of the planned large scale expansion in the sugar industry that is incorporated in Ethiopian first national Growth and Transformation Plan (GTP I) the country planned to produce 181,604 cubic meters of ethanol in 2010/2011-2014/2015 (MoFED, 2010).

Total sugar production in Ethiopia from 2012-2015 was 2,250,000 tons from three sugar factories Fincha, Methara and Wonji and was estimated to be 4268000 tons in 2020 (Ethiopian Sugar Corporation, 2013). Ethiopia uses molasses as the only bioethanol production feedstock with the production of 115,000 tons of molasses per year, thus one tone of molasses yields around 250 liters of bioethanol (Zenebe Gebreegziabher, *et al.*, 2017). The three largest bioethanol producing sugar factories in Ethiopia are Fincha, Methara and Wonji (Muluken Wondwossen, 2010). The first bioethanol plant was built at Fincha sugar factory in 1999 and had produced 1820 tons of ethanol from molasses (Sugar Corporation, 2005). In 2013 Ethiopia produced 130 million liters of ethanol from three sugar factories, Fincha, Methara and Wonji which was 16 times greater than that of 2010 production which was 8 million liters (Ministry of Mines and Energy, 2014).

Metehara Sugar Factory has the highest production capacity as compared to the other existing factories, average annual production capacity of 136,692 tons of sugar. Its ethanol plant had begun its operation by December, 2011 with a capacity of 12,500 m^3 per year

(60,000 liters per day with annual operation day of 230 days). Through its expansion project the factory would build an ethanol plant with a capacity of producing 25,500 m^3 of ethanol. Whereas the third factory in Ethiopia is Wonji sugar factory with the production capacity of 75000 tons of sugars with the annual ethanol production of 10,299 cubic meter (Sugar Corporation, 2013).

The primary factor to determine the economic benefits of bioethanol production from molasses is the price of molasses for alternative use or products and comparable price of ethanol with alternate fuels (Ferede *et al.* 2013). Considerable amount of molasses can be generated from the sugar factories and this amount is in excess for the domestic cattle feed and beverage alcohol production need. The selling price of molasses is not attractive; even significant amount of molasses was spoiled during storage or sold at dumping cost until a supply contract for hydrous bioethanol was signed by Fincha sugar factory (Girma, 2012). Thus, the current movement to operate bioethanol plants at higher capacity leads to utilize the available molasses and production of bioethanol for transport fuel is an opportunity to utilize the excess molasses from the supply side (Zenebe *et al.*, 2017).

3.9. Factors affecting bioethanol production by yeast

There are common challenges to yeasts during sugar fermentation like, rise in temperature (35–45 °C) and ethanol concentration (over 20%) (Tofighi *et al.*, 2014). Yeast growth rate and metabolism increases as the temperature increases until it reaches the optimum value. Increase in ethanol concentration during fermentation can cause inhibition to the growth of microorganism and their viability (Alexandre and Charpentier, 1998). Inability of *S. cerevisiae* to grow in media containing high level of alcohol leads to the inhibition of ethanol production (Fiedurek *et al*., 2011). The other problems in bioethanol fermentation by yeast are the ability to ferment pentose sugars. *Saccharomyces cerevisiaeis* is the most commonly used in bioethanol production, can only ferment hexoses but not pentoses (Kumar *et al*., 2009). Only some yeast from genera *Pichia*, *Candida*, *Schizosaccharomyces* and *Pachysolen* are capable of fermenting pentoses to ethanol (Mussato *et al*., 2012). Generally the common factors which influence the production of bioethanol are

temperature, sugar concentration, pH, fermentation time, shaking condition and inoculum size (Lin *et al*., 2012)

3.9.1. Temperature

Temperature changes have thoughtful effects upon living things. Enzyme catalyzed reactions like fermentation are especially sensitive to small changes in temperature. The growth rate of the microorganisms is also directly affected by the temperature (Charoenchai *et al*., 1998). High temperature which is harsh for cell growth becomes a main factor for growth of microorganisms including yeast. Yeasts contain a very complex heat resistance mechanism that changes multiple physiological processes which are tightly connected with heat shock protein synthesis and response (Marelne Cot *et al*., 2007). The ideal temperature range for industrial fermentation for bioethanol production is between 32 and 35°C. Free cells of *S. cerevisiae* have an optimum growth temperature near 30 °C whereas immobilized cells have slightly higher optimum temperature due to its ability to transfer heat from particle surface to inside the cells (Liu and Shen, 2008). If yeast can efficiently work at more than 36°C high temperature, then the fermentation period would be shortened with the increase of fermentation rate (Liu *et al*., 2019). Furthermore, during industrial bioethanol production, yeast enzymes which regulate metabolic processes during fermentation are sensitive to high temperature which can inactivate and denature the 3D structure of enzymes (Phisalaphong *et al*., 2006). Thus, temperature is carefully regulated throughout the fermentation process.

3.9.2. Sugar concentration

The sugar concentration, especially of the simple sugar concentration in a fermentation medium is one of the factors that generally affect the rate of bioethanol production by yeasts (Phisalaphong *et al*., 2006). Higher substrate concentration could achieve higher ethanol production, but a longer incubation time is required for initial glucose concentrations (Lin *et al*., 2012). However, the use of excessive sugar concentration will cause steady fermentation rate. The initial sugar concentration also has been considered as an important factor in ethanol production. Generally, high ethanol productivity and yield in

batch fermentation can be obtained by using higher initial sugar concentration. However, it needs longer fermentation time and higher recovery cost (Zabed *et al*., 2014).

3.9.3. pH

Power of Hydrogen (pH) is a scale, which is used to specify the acidity or basicity of fermentation and affects the activity of fermenting microbes by disrupting the shape of enzymes involved in metabolic process (Erfle *et al.*, 1982; Arroyo-Lopez *et al*., 2009). Ethanol production is influenced by pH of the broth as it affects bacterial contamination, yeast growth, fermentation rate and byproduct formation (Kasemets and Nisamedtinov, 2007). The permeability of some essential nutrients into the cells is influenced by the concentration of H^+ in the fermentation broth. Additionally, the survival and growth of yeasts is influenced by the pH in the range of 2.75–4.25 (Fleet and Heard, 1993). During the course of fermentation for bioethanol production, the optimum pH range of *S.cerevisiae* is 4.0–5.0 (Lin, *et al*., 2012). When the pH is lower than 4.0, a longer incubation period is required but the ethanol concentration is not reduced significantly. However, when the pH is greater than 5.0, the concentration of ethanol was reduced substantially (Staniszewski, *et al*., 2007).

3.9.4. Fermentation time

Fermentation time affect the growth of microorganisms. Shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms (Silva and Malcata, 2000). On the other hand, longer fermentation time, more than 72 hours has toxic effect on microbial growth especially in batch mode for those species of yeasts which are not able to resist ethanol concentration. This is due to accumulation of high concentration of ethanol and depletion of sugar in the fermented broth. It has been reported that complete fermentation can be achieved at lower temperature by using longer fermentation time which results in lowest ethanol yield (Zabed, *et al*., 2014). Production of high ethanol yields in a short fermentation time is very recommendable and economically relevant factor for industrial ethanol production. However, this is dependent on several factors such as yeast strain, process type (batch or fed-batch), cell density, temperature, sugar concentration and

enrichment of the medium with the proper nutrients, along with other factors that influence the microbial activity (Laluce, *et al.*, 2009).

3.9.5.Agitation/shaking conditions

In anaerobic condition, agitation rate controls the permeability of nutrients from the fermentation broth to inside the cells, equal distribution of heat and substrates to the yeast cells, and removal of ethanol from the cell to the fermentation broth. The greater the agitation rate is the higher the amount of ethanol produced. Besides, it increases the amount of sugar consumption and reduces the inhibition of ethanol on cells. The common agitation rate for fermentation by yeast cells is 150–200 rpm. Excess agitation rate is not suitable for smooth ethanol production as it limits to the metabolic activities of the cells (Zabed, *et al*., 2014).

3.9.6.Yeast inoculum concentration

Initial inoculum concentration is an important factor that greatly affects industrial fermentation by influencing the lag phase duration and specific growth rate of fermenting microbe, so that yield, quality and rate of ethanol production is affected (Wanderely *et al.*, 2014). Inoculum concentration does not give significant effects on the final ethanol concentration but it affects the consumption rate of sugar and ethanol productivity (Laopaiboon *et al*., 2007). Yeast inoculum size has significant effect on bioethanol production. Optimum inoculum size with high ethanol concentration and production rate was 3-10% (Tahir *et al*., 2010; Izmirlioglu and Demirci, 2012; Wanderely *et al*., 2009)

3.9.7. Ethanol concentration

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Birch and Walker, 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu, *et al*., 2007).

The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and endoplasmic reticulum. Meaden *et al.,* (1999) stated that during ethanol stress yeasts vacuole morphology is altered from segregated structures to a single, large organelle besides this, exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity (Stanley, *et al.*, 2010).

Yeast survival and growth under all stress conditions is achieved through a series of stress responses that depend on a complex network of sensing and signal transduction pathways leading to adaptations in cell cycle (Hohmann and Mager, 2003). The response of yeast to ethanol stress is associated with general stress response mechanisms; but Takemura *et al.,* (2004) identified the novel ethanol- specific responses; he observed that the ethanol stress as well as heat shock, causes selective mRNA export, bulk poly (A+) mRNA accumulates in the yeast nucleus, whereas mRNA of Heat Shock Proteins (HSPs) is exported under such conditions. The nuclear localization of DEAD box protein Rat8 changed rapidly and reversibly in response to ethanol stress (Betz *et al.,* 2004; Takemura *et al*., 2004).

3.9.8. Combined effects of different factors

Generally, the ideal temperature for bioethanol production depends on the ideal temperature of the yeasts. Most of the fermenting medium used for bioethanol production has pH in the range of 4.5–5.5 with various sugar concentrations (Staniszewski *et al*., 2007). Fermentation process is commonly performed at 24 and 72 hours with rotation at 150 - 200 rpm. The common inoculum size employed in bioethanol production is 5% and 10% (Izmirlioglu and demirci, 2012; Wanderely, *et al*., 2009). The highest ethanol concentration (128.5g/L) and ethanol productivity (4.76g/L/h) is probably due to favorable conditions for the yeast (Zhang, *et al*., 2011). The lowest ethanol concentration (9.5g/L) and ethanol productivity (0.31g/L/h) was produced from water hyacinth due to its low sugar concentration which limits substrate for bioethanol production (Yan, *et al*., 2015).

A large amount of ethanol must be produced in order to fulfill the increasing worldwide demand. However, the production of ethanol using free yeast cells is still inefficient due to its higher cost of cell cycling, greater contamination risk, limitation of the dilution rate and

susceptibility to environmental variations (Kumar *et al*., 2011). Moreover, free cells cause substrate or product inhibition from direct contact between the cells and medium. Most of the problems occurred in free-cell systems are reduced by the immobilization method (Liu and Shen, 2008).

3.10. Maintenance methods of yeast strains

After isolation of from nature, strains may become unstable and change on continued maintenance in artificial media and conditions. Strains containing plasmids and genetically engineered microorganisms containing extra chromosomal DNA and manipulated genes may also change. Successful preservation of such cells is major problem because loss in viability and stability of such microbes may cause serious disruption to industrial processes (Matoulkova and Sigler, 2011). So, it is important to preserve reference strains with one of the more permanent preservation and maintenance techniques as soon as possible after attainment. Laboratory investigations of the suitability of yeast storage methods have concentrated on the effects of different cooling rates, intracellular trehalose levels and type and concentration of the cryoprotective component on cell post freezing viability (Hubalek , 2003), and the role of growth phase and ethanol and aquaporin in freeze-thaw stress resistance (Seki *et al*., 2009; Tanghe et al., 2004). The most suitable maintenance and preservation techniques are lyophilization (freeze drying) (Tang and Pikal, 2004), L-drying, in liquid nitrogen at -196^{o}C and vacuum drying (Manzanera *et al*., 2004), besides that, for long time maintenance of strains; use of immobilization, encapsulation, storing in sterile double distilled water and mineral oil is effective (Polyak *et al.*, 2010).

4.MATERIALS AND METHODS

4.1. Description of the sample collection sites

Areke difdif and *tinses* were collected from Debre Birhan, Dembecha Amhara Regional State and Arsi Negele, Oromia Regional State. All the samples were collected by using sterilized bottles in ice box and transported to Bioengineering laboratory, Addis Ababa Institute of Technology, Addis Ababa University. Debre Berhan is located in the North Shewa Zone of the Amhara Region, about 120 kilometers North East of Addis Ababa on the paved highway to Dessie City. The town has a latitude and longitude of 9°41′N 39°32′E and 9°41′N 39°32′E coordinates with an elevation of 2,840 meters above sea level. Dembecha is a town in North Western Ethiopia 350 km North of Addis Ababa and located in the West Gojjam Zone of the Amhara Region. This town has a latitude and longitude of 10°33′N 37°29′E and 10°33′N 37°29′E coordinates with an elevation of 2083 meters above sea level. Arsi Negele is a town in southeastern Ethiopia and located in the West Arsi Zone of the Oromia Regional State on the paved highway North of Shashamane. This town has a longitude and latitude of 7°21′N 38°42′E and an elevation of 2043 meters above sea level.

4.2. Sampling

Sampling sites were selected based on their high areke production capacity and quality. A total of 26 samples; 10 from Debre Berhan, 10 from Arsi Negelle and 6 from Dembecha were collected using sterile 100 ml bottles and were transported to Addis Ababa University Institute of Technology in ice box and stored in bioengineering laboratory refrigerator for further work.

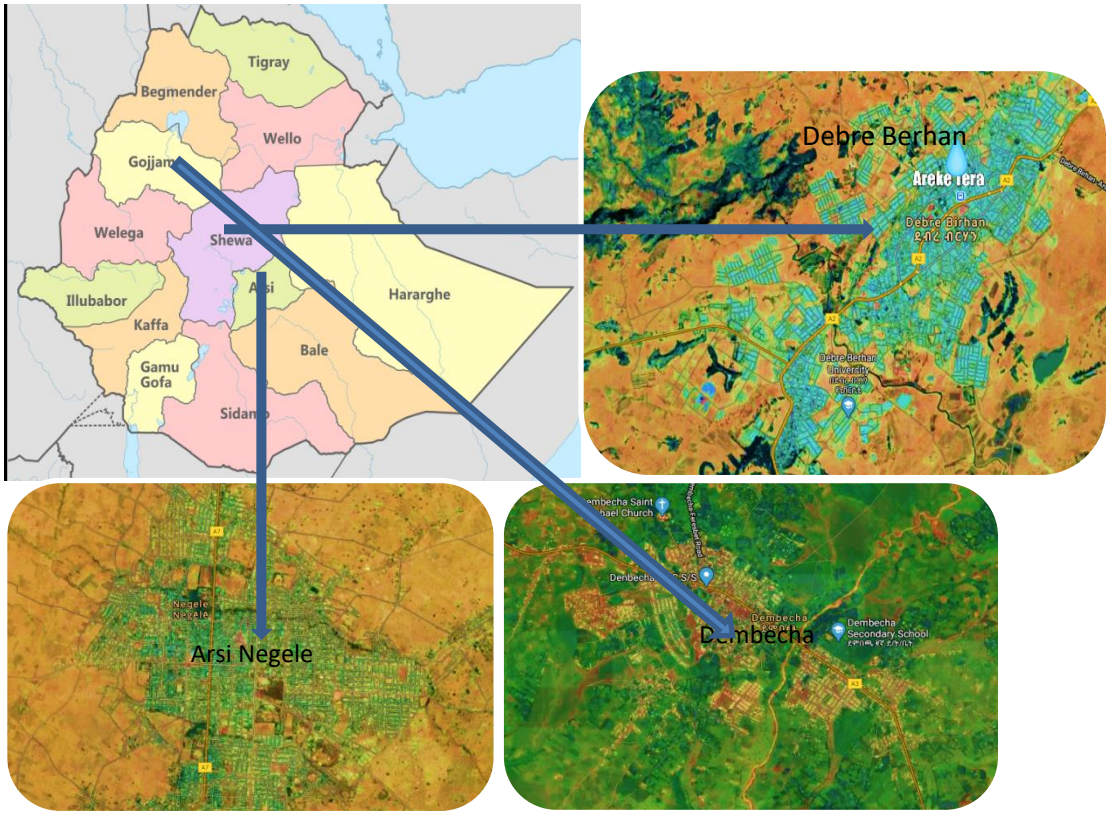

Figure 2. Map description of sample collection sites (source: Google satellite map).

4.3. Isolation of indigenous wild yeasts

Yeast extract peptone dextrose (YPD) agar with the composition of Yeast Extract 10.0 g/l, Peptone 20.0 g/l, Dextrose 20.0 g/l and Agar 15.0 g/l was used to isolate and screen the yeasts from the collected samples. The pH of the medium was adjusted to 4.5 and after sterilization; chloramphenicol was added to inhibit the growth of other bacteria. One ml of *difdif* and *tinses* were separately added into separate tubes containing 9 ml sterile distilled water**.** A ten-fold serial dilution (10^{-1} to 10^{-6}) was made with sterile distilled water. From appropriate serial dilution a 0.1 ml was plated onto modified YPDA medium and incubated for 48-72 h at 30°C to isolate morphologically well-formed discrete colonies (Qureshi *et al*., 2007). The yeast isolates were further sub-cultured (streak plated) on the respective medium to establish pure cultures.

4.4. Characterization of isolated yeasts

4.4.1.Morphological characterization

Yeast Peptone Dextrose (YPD) solid medium was after autoclaving for yeasts isolation and purification (Kreger-van Rij, 1984; Kurtzman and Fell, 1997). After cooling, the plates were inoculated by 48 hours old yeast isolates and incubated at 30°C for 48 hours. The colonies on YPD medium were determined for colony morphology (color, shape, texture). Cellular morphology was examined under compound microscope (Brand and origin) using high power (40x) and oil immersion (100x) objective lens.

4.4.2. Screening of the yeast isolates

4.3.2.1. Ethanol tolerance

YPD liquid medium (6 g/L yeast extract, 10 g/L peptone, 6 g/L malt extract) was used for detecting yeasts for ethanol tolerance. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. Various concentrations of absolute ethanol (99.8% v/v) as 10, 15, 20 and 25% (v/v) were prepared as a stock solution and then all the isolates were tested with 10% ethanol. The isolates that tolerated 10% were tested at 15% ethanol and then tested with 20, 22, 23, and 24% ethanol until they stop growth. All the screening tests were done by using 15 ml test tubes which contained the same amount of YPD broth medium. The initial optical density of each test tube was read on spectrophotometer at 600 nm against the medium as blank. All the cultures were incubated at 30°C for 24 to 48 hours. The increase in optical density in test tubes was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts. Viability was measured following 48 hours of incubation at 30^{o}C by striking with sterile inoculation loop onto plates containing YPD **(**Chiranjeevi *et al*., 2013**).**

4.4.3. Growth at different pH

YPD liquid medium (broth) was used for detecting the ability of potent yeast isolates to grow at different pH. The medium was autoclaved at 121°C for 15 minutes and cooled. YPD broth was prepared at pH 2, 2.5, 3, 3.5 and 4 in 250 ml flasks. Each flask contained 150 ml of YPD medium with different pH and blank medium without yeast cell was used as a control. Then each flask was inoculated with half loop-full of top ten ethanol tolerant yeast isolates which tolerated 22% and 23% to measure the initial optical density at 600 nm after incubating at 30°C for 24 to 48 hours. After 24 to 48 hours, cell density was recorded at 600 nm for growth and the growth at different pH was tested using solid YPD medium (Armanul, 2014).

4.4.4. Growth at different temperature

YPD liquid medium was autoclaved at 121 °C for 15 min. and cooled. A portion of the medium (150 ml) was distributed into a flask with 250 ml capacity. The flasks were inoculated with half loop-full of 48 hours old top ten ethanol tolerant yeast isolates. The initial optical density of each culture in flask was recorded using spectrophotometer (Brand and origin) at 600 nm against the medium as blank. All the inoculated flasks were incubated at different temperature starting from 25, 30, 35, 40 and 45°C for 48 hours to observe thermotolerance of yeast isolates. The increase in optical density of the yeast cultures in a flask was recorded as evidence of growth and was tested using solid YPD medium to check their growth at different temprature (Armanul, 2014).

4.4.5.Carbohydrate assimilation and fermentation test

To test the aerobic assimilation of carbon source by yeast, Phenol red broth (peptone 10 g, Sodium Chloride [NaCl] 5 g, Beef extract 1 g, Phenol red [7.2 ml of 0.25% phenol red solution]: 0.018 g, Carbohydrate source: 100 g [10% sugar] per liter of distilled water) medium was used. A 20 ml of phenol red carbohydrate broth was filled in to 25 ml test tube and sterile Durham tube was inserted to detect gas production. The prepared test medium

was autoclaved at 121°C for 15 minutes to sterilize. Active yeast which was 24 hours old was inoculated aseptically in to each test tube using an inoculating loop and incubated at 30-33°C for 24-72 hours. Longer incubation periods may be required to confirm a negative result. Capability of fermentation was assessed by looking for the formation of gas (CO_2) in Durham tube and color change of the fermentation medium, deep pink to yellow due to the formation of acids and gas (Warren and Shadomy, 1991). The sugars used were Sucrose, glucose, lactose, maltose, mannose and fructose.

4.5. Clarification of molasses for ethanol production

Molasses was collected from Methara, Fincha and Wonji Sugar Factory. In this study, 1 ml concentrated sulfuric acid and 0.40 g urea was used in one liter (one to one diluted 500 ml molasses for the clarification). Then molasses, sulfuric acid and urea were mixed and boiled in 1000 ml beaker at 100^{o}C for 30 minutes. Then it was allowed to stay for 24 hours to cool and decant. The supernatant was used for propagation of yeasts and fermentation to produce ethanol (Atiyeh and Duvnjak, 2003).

4.6. Fermentation medium preparation

The clarified molasses was used as a fermentation medium. In all the experiments, initial sugar concentration was measured by using Refractometer. Fermentation medium was diluted until the molasses concentration of 40 degree brix using distilled water and autoclaved at 121° C for 15 minutes then stored as stock for propagation and fermentation (Armanul, 2014).

4.7. Preparation of yeast of suspension/propagation

One milliliter of 48 hours old broth culture of the ten potential yeast isolates was inoculated in to each of the ten 500 ml flasks containing 100 ml treated and sterilized molasses. The flasks were incubated at 30^{o}C with 150 rpm shaking condition for 24 hours, and then after 24 hours l00 ml sterilized fresh molasses was added to each flask and incubated for 24 hours in order to boost the cell number. The initial (0 hours) and final (48 hours) OD of the propagated culture was measured by using UV-Vis Spectrometer (UVD-3200,

LABOMED.INC, USD) at 600nm. Finally, 200 ml molasses medium which contained 48 hours propagated yeast cultures was ready as inoculum for fermentation to produce ethanol.

4.8. Fermentation of molasses

Submerged fermentation was carried out in Erlenmeyer conical flasks. A 300 ml treated and sterilized molasses was added into ten 1000 ml Erlenmeyer flasks and each flask was inoculated with 48 hours propagated yeast cells. The flask was cotton plugged to make partial aerobic condition for 12 hours and incubated at 35^{o}C for fermentation with shaking at 150 rpm, then became anaerobic after 12 hours and the fermentation continues for 60 hours to make total fermentation time 72 hours (12 hours aerobic plus 60 hours anaerobic). The pH, brix of the fermentation medium and ethanol production was recorded with in 24, 48 and 72 hours as indicated in Armanul (2014).

4.9. Estimation of ethanol concentration by volume

The ethanol concentration (ABV%) from the fermented medium was calculated by using a method which is based on specific gravity (SG) of the substrate as indicated in Hall (1995) by considering molasses without yeast culture as initial SG and fermented medium as final SG. As Hall (1995) indicated that alcohol percentage of the final fermented medium would be directly proportional to the difference in the original and final (real) extract values. After all, the chemical equation for the conversion of a monosaccharide to ethanol is:

$$C_6H_{12}O_6 \Longrightarrow 2C_2H_5OH + 2CO_2$$

So the weight of the sugar molecule (180 amu) should be converted into the weight of two ethanol molecules (92 amu) and two carbon dioxide molecules (88 amu). This would give us an equation for the alcohol percent by weight (A%w) of:

$$A\%w = (OE - RE)\frac{92}{180} = \frac{(OE - RE)}{1.9565,}$$

Where, OE is original extract and RE is real extract.

Then alcohol content as a function of the original and final specific gravities can be derived as:

$$A\%w = \frac{76.08\ (OG - FG)}{1.775 - OG}$$

Where OG is Original specific gravity and FG is Final specific gravity.

This equation can be converted to alcohol percent by volume (A%v) by the following formula:

$$A\%v = A\%w\ (FG / 0.794),$$

Where 0.794 is the specific gravity of ethanol.

Original specific gravity and Final specific gravity was measured using Digital Density Meter (DMA 4100M, UK)

4.10.Molecular analysis

4.10.1. DNA extraction from yeast isolates

From potent yeast isolates, DNA was extracted following the modified CTAB method (Shahzadi*et al*., 2010). Overnight grown yeast cultures in YPD broth was centrifuged at maximum speed 13,000 rpm. Ten mg of yeast cells, 1.4M NaCl, 2% CTAB, 20 mM EDTA (pH 8.0), 0.2% ethanol and 100 mM Tris HCl (pH 8.0) were mixed well and incubated at 65°C for 20 minutes in water bath. After incubation, all the tubes were cooled for 5 minutes and 200 µl of Chloroform: Isoamyl alcohol, 24:1 was added and mixed thoroughly and centrifuged at 13,000 rpm for 10 minutes at room temperature. Aqueous phase (supernatant) was taken in to each eppendorf tube separately and 3 M sodium acetate (1/10) was added in to each eppendorf tube along with equal volume of cold isopropanol and was mixed gently and then placed on ice for 10 minutes. All the eppendorf tubes after incubation were centrifuged separately at 13,000 rpm at 4°C for 15 minutes and the supernatants were removed. A 500 µl of cold 70% ethanol was used for washing pellet and then centrifuged at 13,000 rpm at 4°C for 2 minutes. The pellet was air dried after discarding supernatant from each tube. The pellet was re-suspended in 50 µl double deionized water or TE-buffer and was stored at −20°C for amplification.

4.10.2. Amplification of 5.8S-ITS region by PCR

Amplification of 5.8S-ITS region of yeast rRNA gene was done by using ITS4 (5′-TCCTCCGCTTATTGATATGC) reverse ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) forward universal primers (White *et al.,* 1990) in thermocycler/sure cycler PCR (Aglient, G8800-00, Malysia). The reaction mixture was contained 100 ng DNA, 5 µl of 10 pmol each oligonucleotide primer, 3µl of 25 mM $MgCl_2$, 3µl of 250 mM dNTPs mixture and Taq DNA polymerase (5 units) in a total volume of 50 µl. Polymerase Chain Reaction (PCR) conditions was done as denaturation at 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC, annealing at 55ºC for 45 seconds and final extension at 72ºC for 7 minutes. The amplified product was checked by running on 0.8% agarose gel and visualized by using UV illuminator and was photographed.

4.10.3. DNA sequencing

Gel purified PCR products was sequenced using Next Generation Sequencer machine illuminia, China 2010 with the following modifications to the manufacturer's recommended protocol: ten Pico-moles of Control Primer No. 1 was annealed with 0.1 and 0.5 pmol of PCR product by mixing primer and template with 2 ml of 5´ Sequenase Buffer in a final volume of 12 ml. This mixture was heated for 3 min at 100°C in a thermal cycler and then cooled in an ice water bath for 5 min. A custom dNTP labeling mixture (1.5 mMdGTP, 1.5 mMdCTP) was substituted for the labeling mixture included with the kit. One microliter of a-33 P dATP (ca. 12.5 mCi; 2000 Ci/mmol) (NEN Life Science Products, Boston, MA, USA) was added to each labeling reaction. The omission of deoxythymidine triphosphate and the addition of Mn buffer (a component of the Sequenase Version 2.0 DNA Sequencing Kit) to the labeling reaction eliminated the nonspecific back-ground and pauses sometimes observed in DNA sequencing ladders of PCR products. To prepare the modified ddGTP termination and ddATP termination mixtures, four parts of each stock termination solution was separately added to one part of Sequence Extending Mix (Amersham). For ddTTP and ddCTP, three parts of each stock termination solution was separately added to two parts of Sequence Extending Mix. Labeling reactions was

conducted for 3 min at 20°–25°C and then terminated for 5 min at 37°C. The sequencing reactions were electrophoresed through a 6% Long Ranger Gel (FMC Bio-Products) prepared in 1´ glycerol tolerant buffer (Amer-sham). X-ray film will be exposed to the dried sequencing gels for at least 18 hours (Amersham, 1994). Sequenced data obtained was blasted on NCBI to characterize the yeast to the species level.

4.10.4. BLAST search and phylogenetic analysis

The internal Transcriber Spacer (ITS4 and ITS5) and 5.8s of ribosomal DNA sequences were used for Basic Alignment Search Tools (BLAST). The sequence of the isolates were further aligned and compared to ITS sequences in database using the taxonomy browser of the National Center for Biotechnology Information (NCBI). A total of 20 sequences (13 from the database and 7 from this study) were aligned using ClustalW for phylogenetic tree construction (Larkin *et al*., 2007). Phylogenetic tree was constructed with Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 using a Maximum Likelihood (ML) algorithm character estimation method with bootstrap analysis where 1000 replicates were performed (Kumar *et al*., 2016).

5. RESULTS

5.1. Isolation of indigenous yeasts

From samples collected from all sites, 270 yeast isolates were isolated and purified to be screened on the basis of ethanol tolerance and other parameters.

5.2. Microscopic observation of isolated yeasts

Yeast isolates were found smooth white raised colonies on YEPDA medium. The budding stage of the yeast isolates was observed under oil immersion (100X) objective (Compound light digital, Optika) and confirmed them to be yeast (Figure 3). The cell morphology of wild yeast isolates observed under compound microscope showed an oval and circular shape with creamy/milky color and pair budding cells (Figure 3).

Figure 3. Yeast isolates under the digital compound light digital microscope (B-350, Optika, Italy).

5.3. Screening of ethanol tolerant yeasts

A total of 270 pure culture of yeast isolates were tested for ethanol tolerance as shown in Table 4. Results of the ethanol tolerance testing indicated that all of the yeast isolates were found to be tolerant to 12% Ethanol. From the tested purified yeast isolates 37, 24, 10, and 4 of them were shown to be tolerant to the 15%, 20%, 22% and 23% of ethanol, respectively (Table 5). None of the yeast isolate was found to be tolerant to 24% or more of ethanol (Table 5). From a total of 76 yeast isolates, 10 (those tolerated 22% and 23%) them were considered as the best ethanol tolerant isolates and were subjected to further subsequent evaluations.

5.3.1.Growth on YEPDA

The test of the yeast isolates those shown tolerant to 15%, 20%, 22% and 23% of ethanol concentration were shown on YEPDA (Figure 4 and Table 4 and 5). The colonial feature of these isolates on YPDA was found as white, creamy color with oval and circular shape and has also smooth texture.

Table 4. Total number of isolates that tolerate different ethanol concentration.

Ethanol concentration						
10%	12%	15%	20%	22%	23%	24%
All isolates were tolerated	All isolates were tolerated	37	24	10	4	0

Table **5**. Viability test of 24 isolates grown in liquid media containing 20%, 22%, 23% and 24% ethanol concentration.

	Ethanol Concentration			
	20%	**22%**	**23%**	**24%**
Isolates	**Tolerance**			
Dd5a	+	-	-	-
Dd5b	+	+	-	-
Dd5c	+	-	-	-
Dd5d	+	+	+	-
Dd5e	+	+	-	-
Dd5f	+	+	+	-
Dd5g	+	-	-	-
Dd5h	+	-	-	-
Dd5i	+	-	-	-
Dd5j	+	-	-	-
Dt1b	+	-	-	-
DT1c	+	+	-	-
Dt1d	+	-	-	-
Dt1e	+	-	-	-
Dt1f	+	-	-	-
Dt1g	+	-	-	-
Mda	+	+	+	-
Mdb	+	+	+	-

Mdd	+	+	-	-
Dt1h	+	-	-	-
Dt1j	+	+	-	-
Dd1e	+	+	-	-
Dbd2g	+	-	-	-
Dbd3d	+	-	-	-

Mda=Mother didif a, Mdb=Mother difdif b, Mdd=Mother difdif d, Dd5b= Dembecha difdif 5b, Dd5d= Dembecha difdif, Dd5f= Dembecha difdif, Dd5e= Dembecha difdif, Dt1c= Dembecha difdif, Dt1c= Dembecha difdif, Dt1e=Dembecha Tensis 1e, Dt1j=Dembecha Tensis 1j.

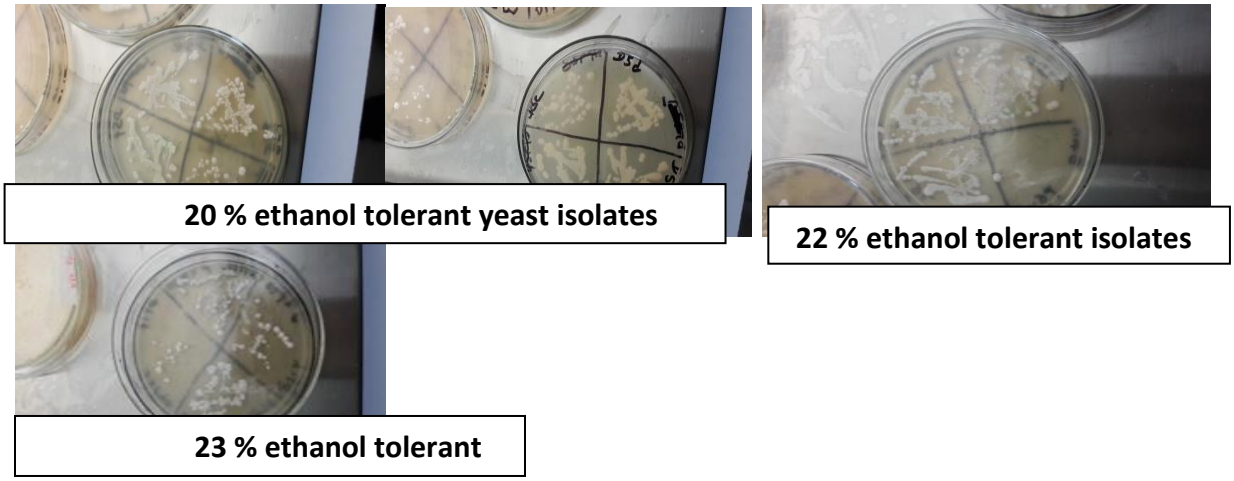

Figure 4. Viability of ethanol tolerant isolates grown in solid YEPDA medium

5.4. Carbohydrates assimilation

The results of the carbohydrate assimilation test following incubation at 30°C for selected carbohydrates along with control in liquid phenol red broth medium for 10 selected ethanol tolerant yeast isolates [ten 22% ethanol tolerant and from those ten isolates, four isolates (Mdb, Mdd, Dd5f and Dd5d) were tolerant to 23% ethanol] is shown in Table 6. The carbohydrates assimilation and gas production profiles for 10 isolates which were best ethanol tolerant are shown in Table 6 and Figure 5. Out of all the carbohydrates assimilated, all the 10 isolates assimilated fructose, glucose, maltose and sucrose, but only isolate Mdb assimilated lactose and 4 isolates assimilated xylose (Table 6 and Figure 5D).

Table 6.Carbohydrate assimilation and gas production test for 10 isolates.

Isolates	Carbohydrate Assimilation						Gas Production					
	Fructose	Glucose	Lactose	Maltose	Sucrose	Zylose	Fructose	Glucose	Lactose	Maltose	Sucrose	Zylose
Mda	+	+	-	+	+	+	**	**	--	*	***	**
Mdb	+	+	-	+	+	-	**	**	*	**	***	--
Mdd	+	+	+	+	+	-	***	***	--	**	**	--
Dd5b	+	+	-	+	+	-	**	**	--	*	**	--
Dd5d	+	+	-	+	+	-	***	***	--	**	**	--
Dd5e	+	+	-	+	+	-	**	***	--	**	***	--
Dd5f	+	+	-	+	+	+	**	***	--	**	***	**
Dt1c	+	+	-	+	+	-	***	***	--	**	***	--
Dt1e	+	+	-	+	+	+	***	***	--	**	**	**
Dt1j	+	+	-	+	+	+	***	***	--	**	**	**

Note: *** (3) Strong gas production, ** (2) Moderate gas production, * (1) Weak gas production and (0) -- /**-ve** no gas production.
Mda=Mother didif a, Mdb=Mother difdif b, Mdd=Mother difdif d, Dd5b= Dembecha difdif 5b, Dd5d= Dembecha difdif, Dd5f= Dembecha difdif, Dd5e= Dembecha difdif, Dt1c= Dembecha difdif, Dt1c= Dembecha difdif, Dt1e=Dembecha Tensis 1e, Dt1j=Dembecha Tensis 1j.

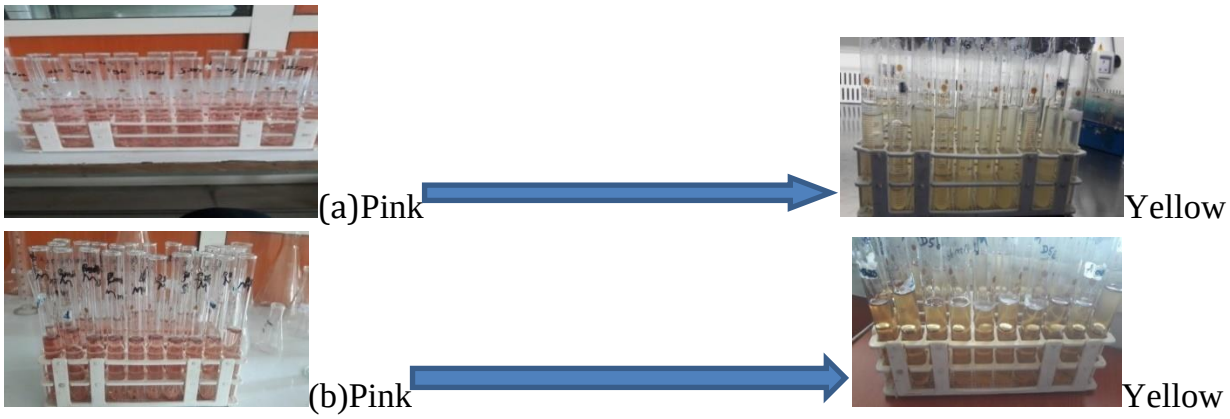

Figure 5. Carbohydrate assimilation (a) sucrose (b) maltose and fructose (c) Glucose (d) Xylose.

5.5. Growth at different temperature

The growth result of the selected 10 (22% and23% ethanol tolerant) yeast isolates at different temperature values (35ºC, 40ºC, 45ºC and 47ºC) is given in Table 7. All the isolates were found to be viable at 35ºC, 40ºC, and 45ºC. Isolate Mda was the only ethanol tolerant yeast isolate that was found to be viable at 47ºC (Table 7).

Table 7. Viability of the best ethanol tolerant yeast isolates growing at different temperature values

Isolates	Viability of isolates at Temperature (ºC)			
	35	40	45	47
Mda	+	+	+	+
Mdb	+	+	+	-
Mdd	+	+	+	-
Dd5b	+	+	+	-
Dd5d	+	+	+	-
Dd5f	+	+	+	-
Dd5e	+	+	+	-
Dt1c	+	+	+	-
Dt1e	+	+	+	-
Dt1j	+	+	+	-

Mda=Mother didif a, Mdb=Mother difdif b, Mdd=Mother difdif d, Dd5b= Dembecha difdif 5b, Dd5d= Dembecha difdif, Dd5f= Dembecha difdif, Dd5e= Dembecha difdif, Dt1c=xx Dembecha difdif, Dt1c=xx Dembecha difdif, Dt1e=Dembecha Tensis 1e, Dt1j=Dembecha Tensis 1j

5.6. Growth at different pH

The top 10 ethanol tolerant isolates were allowed to grow at different pH values and all showed growth at pH>2 but all were not able to grow at pH 2 (Table 8).

Table 8. Viability of the 10 wild yeast isolates growing at different pH values on plates

Isolates	Viability of isolates at pH					
	2	2.5	3	3.5	4	4.5
Mda	-	+	+	+	+	+
Mdb	-	+	+	+	+	+
Mdd	-	+	+	+	+	+
Dd5b	-	+	+	+	+	+
Dd5d	-	+	+	+	+	+
Dd5f	-	+	+	+	+	+
Dd5e	-	+	+	+	+	+
Dt1c	-	+	+	+	+	+
Dt1e	-	+	+	+	+	+
Dt1j	-	+	+	+	+	+

Mda=Mother didif a, Mdb=Mother difdif b, Mdd=Mother difdif d, Dd5b= Dembecha difdif 5b, Dd5d= Dembecha difdif, Dd5f= Dembecha difdif, Dd5e= Dembecha difdif, Dt1c= Dembecha difdif, Dt1c= Dembecha difdif, Dt1e=Dembecha Tensis 1e, Dt1j=Dembecha Tensis 1j

5.7. Propagation

The result of separate propagation of the ten selected yeast isolates in 100ml of molasses at 30ºC for 24 hours then adding additional 100 ml separately after 24 hours in to 250 ml separate flasks is given in Table 9. The result of each isolate has indicated the propagation of each isolate within the formulated molasses and confirmed the boosting of the growth of each isolate by adding 100 ml of molasses after 24 hours. Finally, 200 ml molasses medium with propagated yeast culture was ready as inoculum for fermentation.

Table 9. Propagated yeast cells as indicated by OD (nm) for top ten isolates (Initial OD reading was done immediately after inoculation, one OD =1x10^8 CFU/ml)

Isolates	Initial (0 hours) OD	Final (48 hours) OD
Mda	1.962	3.027
Dd5f	1.966	3.008
Dt1e	1.965	2.964
Dt1j	1.950	2.901
Dd5d	1.940	2.929

Dd5b	1.877	2.756
Dd5e	1.850	2.869
Mdb	1.947	2.611
Mdd	1.908	2.768
Dt1c	1.889	2.905

Mda=Mother didif a, Mdb=Mother difdif b, Mdd=Mother difdif d, Dd5b= Dembecha difdif 5b, Dd5d= Dembecha difdif 5d, Dd5f= Dembecha difdif 5f, Dd5e= Dembecha difdif 5e, Dt1c= Dembecha Tenses 1c, Dt1e=Dembecha Tensis 1e, Dt1j=Dembecha Tensis 1j

5.8. Production of ethanol from molasses

The results of the potential of the ethanol production by the 10 best ethanol tolerant yeast isolates using molasses medium containing 30 degree brix (^{o}B) and shaking at 150 rpm is presented in Table 10. There was a trend of increasing bioethanol yield by all the isolates during the course of the fermentation period (from 0 to 72 hours.). The maximum amount of ethanol (14.3%) was produced by the ethanol tolerant Mda isolate followed by the yeast isolate Dt1e (13.2%), Dt1j (12.2%), Mdd (12%), D5f (12%), and Dt1e (12%) (Table 10). The least ethanol production (9%) was obtained from the isolate D5e (Table 10). In this molasses concentration (30^{o}B), four isolates (4/10, which 1.48% of the total yeast isolates isolated during this study) namely; Mda, Dt1e, Dt1j and Dd5f were shown maximum ethanol production, producing 14.3, 13.2, 12.2 and 12%v/v ethanol, respectively. The pH of fermenting molasses during the production of ethanol by the selected yeasts was observed almost remaining constant, except the case of the yeast isolate named as Mda (Figure 6). The pH of fermenting molasses by Mda was found increased at 48 hours during the course of fermentation (Figure 6). The result of the molasses brix concentration of molasses formulated for the production of ethanol during the course of ethanol production by the yeasts was shown in Figure 7. A pattern of the reduction of the molasses brix concentration of formulated molasses during the production of ethanol by yeasts was observed in Figure 7. Four isolates (Mda, Dt1e, Dt1j and Dd5f) were selected as best ethanol producers considered for further testing. Furthermore, these top four isolates were examined at 25^{o}B and 20^{o}B molasses concentration as well.

Table 10. Mean of ethanol concentration (%V/V) produced by 10 isolates at 30 degree brix

Samples	24 hours	48 hours	72 hours
Mda	10	13	14.3
Mdd	9.3	12	12
Dd5d	8.5	11	11.3
Dd5f	10	11	12
Dt1c	9	10	12
Dt1e	10	13	13.2
Dt1j	10	10.6	12.2
Dd5b	9.3	10	10.5
Dd5e	7.6	7.7	9
Mdb	8.2	9	9.5

Mda=Mother *didif* a, Mdb=Mother *difdif* b, Mdd=Mother *difdif* d, Dd5b= Dembecha *difdif* 5b, Dd5d= Dembecha difdif 5d, Dd5f= Dembecha difdif 5f, Dd5e= Dembecha difdif 5e, Dt1c= Dembecha Tenses 1c, Dt1e=Dembecha Tenses 1e, Dt1j=Dembecha *Tenses* 1j

Table 11. Summary of ANOVA and significance value between the isolates

SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
24 hours	30	275.7	9.19	0.9905862	
48 hours	30	318.9	10.63	2.7704483	
72 hours	30	348	11.6	2.6565517	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	88.226	2	44.113	20.62	4.67E-08
Within Groups	186.11	87	2.14		
Total	274.336	89			

- P value is less than 0.05; this implies that there is significant difference between the isolates.
- Where: SS; Sum of square, df; degree of freedom, MS; Mean Square, P value; significance level

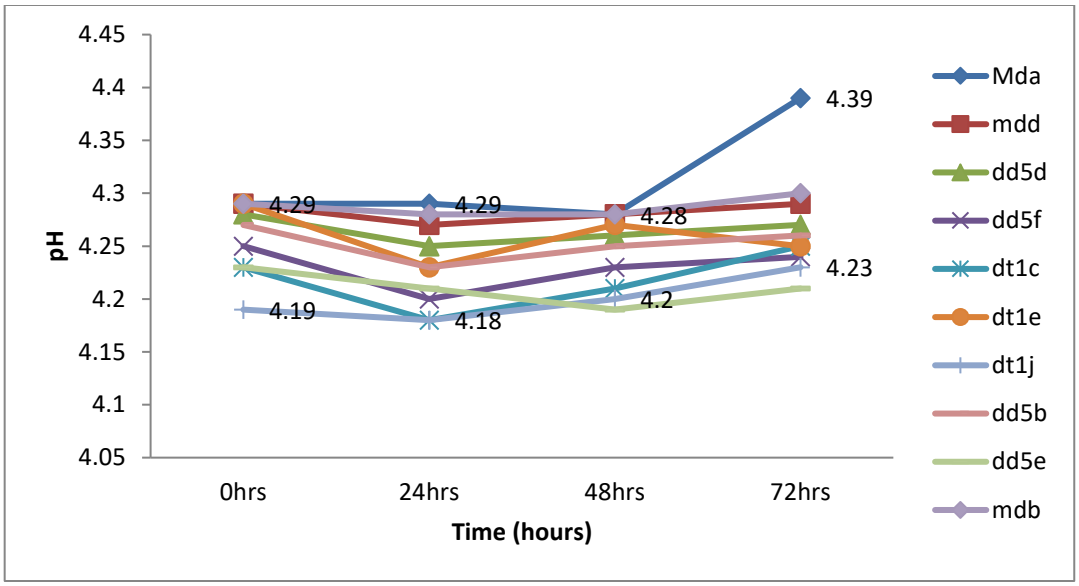

Figure 6. The course of pH during the production of ethanol by the ten isolates using 30 brix molasses concentration at 35ºC.

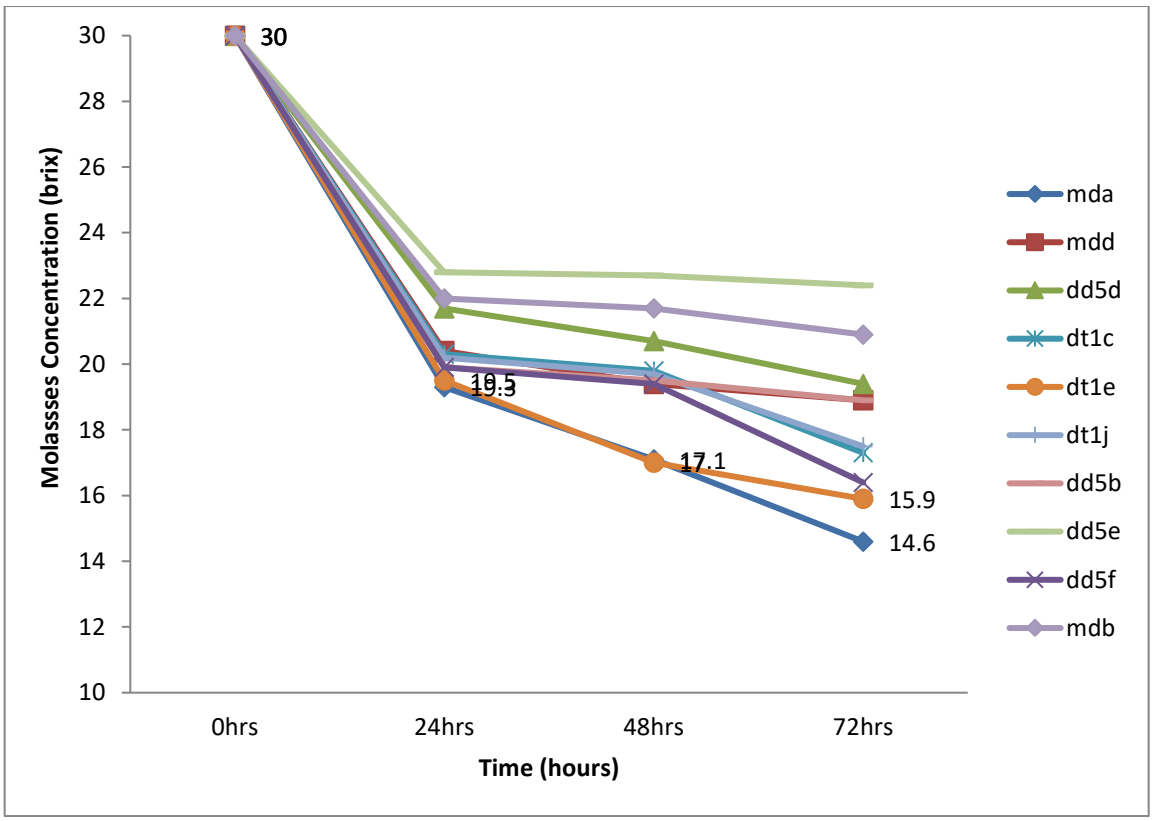

Figure 7. Sugar brix change for ten isolates during ethanol production from molasses

Top four isolates were selected based on their ethanol production performance from 30 ^{o}B were also tested for production of ethanol with 20^{o}B and 25^{o}B molasses concentration (Figure 8). At 20^{o}B the highest ethanol was recorded by isolate Dt1j and Mda producing 8.1 and 7.8%v/v ethanol concentration; respectively at 72hours whereas the lowest yield was recorded by isolate Dt1e with 7.3%. All the four isolates consumed above 50% sugar concentration at 72hours but the ethanol yield is very low comparing with 30^{o}B, whereas the pH was reduced from 5.6 to 4.73 in 72hours (Figure 8, 9 and 10).

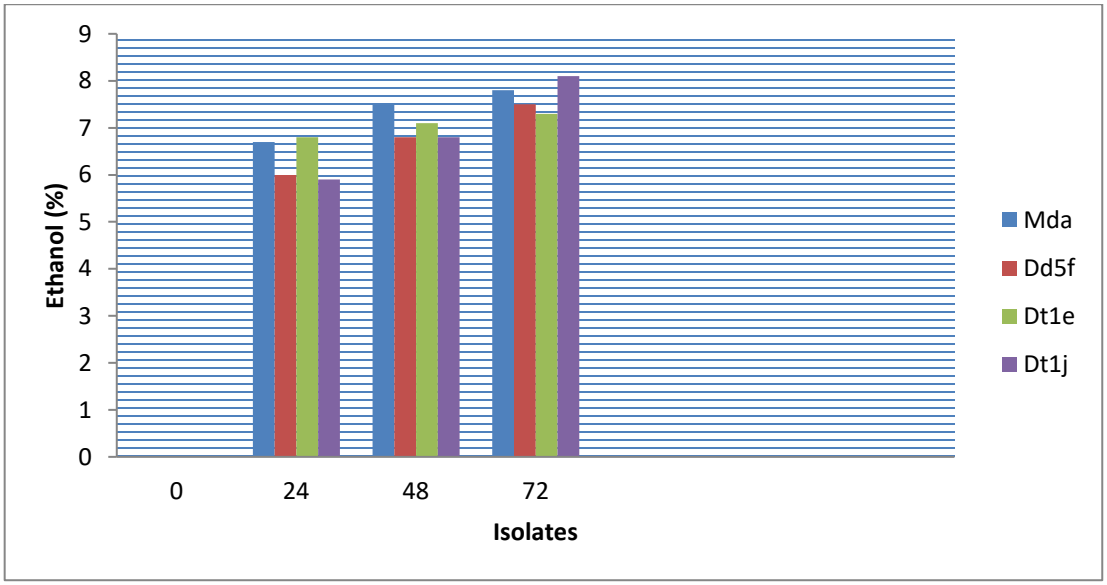

Figure 8. Ethanol production ability of 4 isolates from 20 brix molasses concentration at 35^{o}C

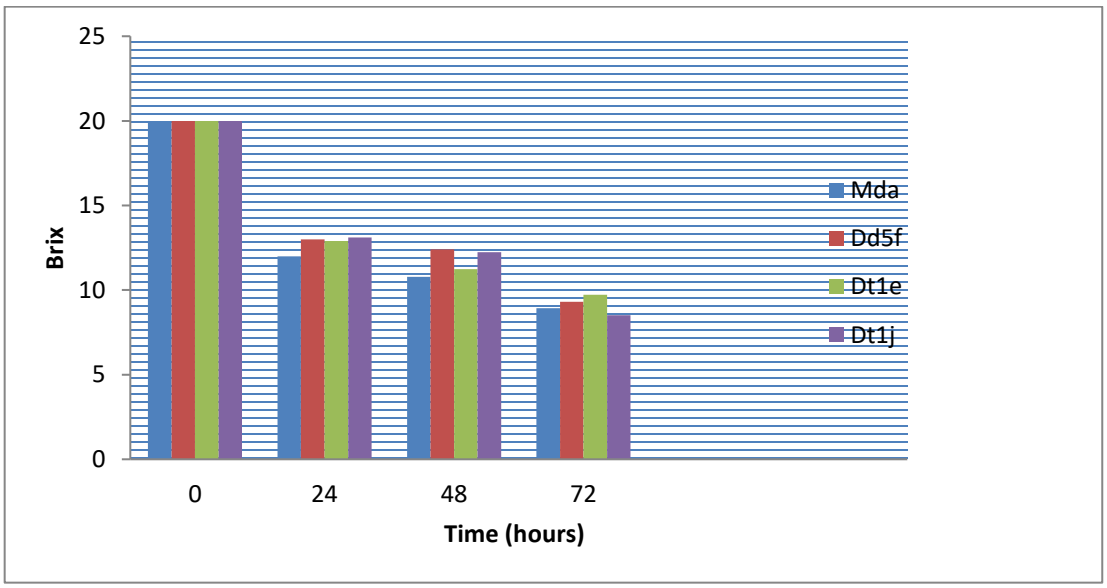

Figure 9. Sugar brix change for 4 top isolates as fermentation time increases

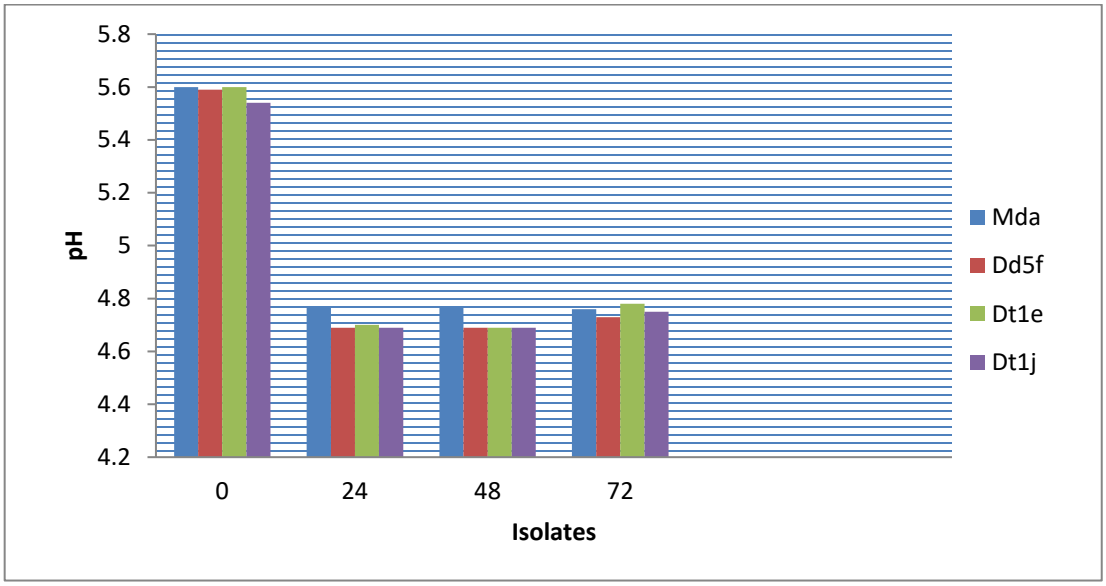

Figure 10. Change in **pH** values during the production of ethanol by the 4 isolates using 20 brix molasses concentration at 35ºC.

With 25ºB molasses concentration, the ethanol yield recorded was almost the same (11%) for all four isolates in 72 hours with small digit differences (Figure 11). The sugar brix was reduced from 25ºB to 9ºB again almost the same for all four isolates in 72 hours (Figure 12) with pH reduction from 5.6 to 4.85 for all four isolates in 72 hours (Figure 13).

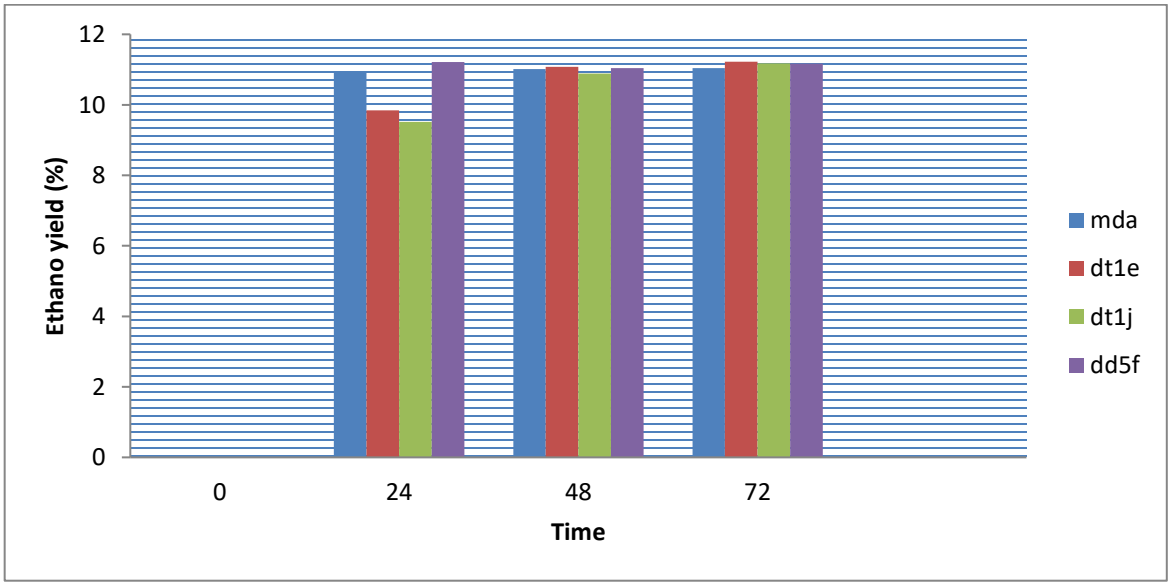

Figure 11. Ethanol production of 4 isolates from 25 brix molasses concentration

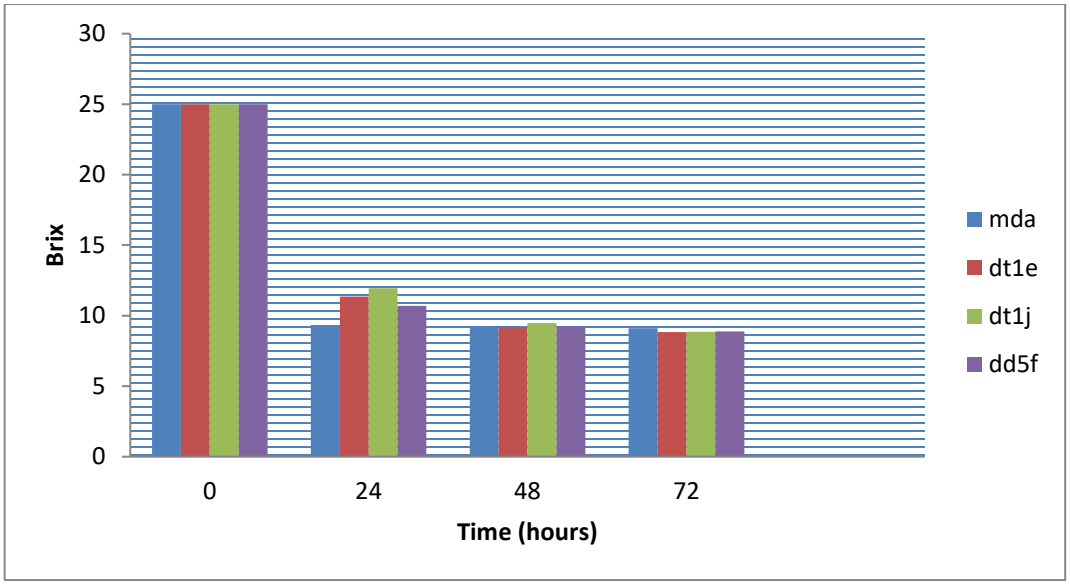

Figure 12. Change in brix (25 degree brix) as time increases

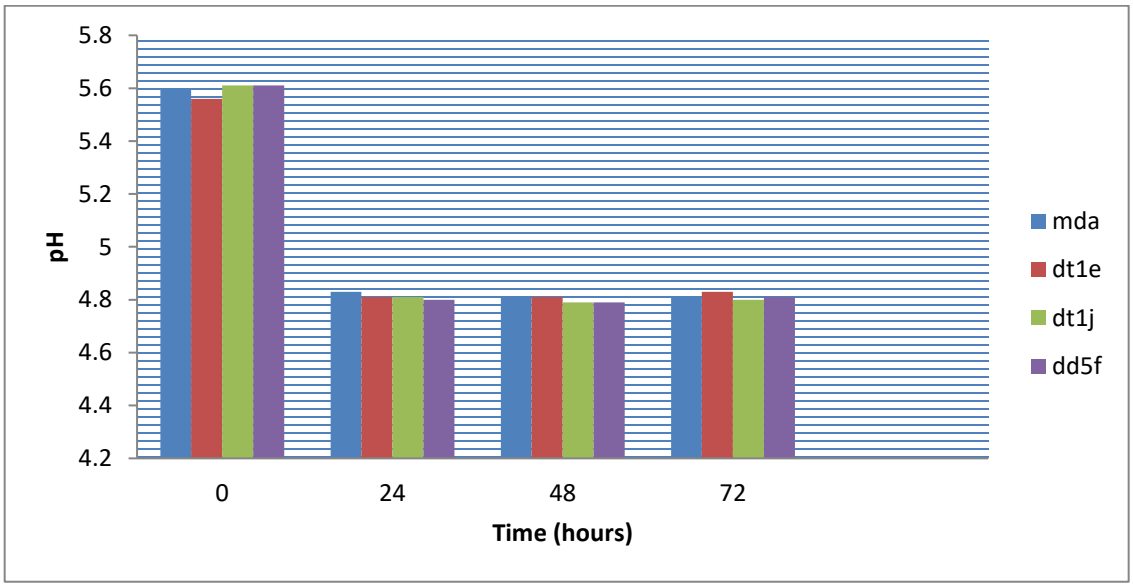

Figure 13. Change in **pH** values during the production of ethanol by the 4 isolates using 25 brix molasses concentration at 35^{o}C

5.8.1.Comparison of ethanol production by four top isolates at 20, 25 and 30 molasses concentration (brix)

As shown in Figure 14, the highest ethanol production was recorded from 30^{o}B in 72 hours., whereas the lowest ethanol was recorded from 20^{o}B in 72 hours.

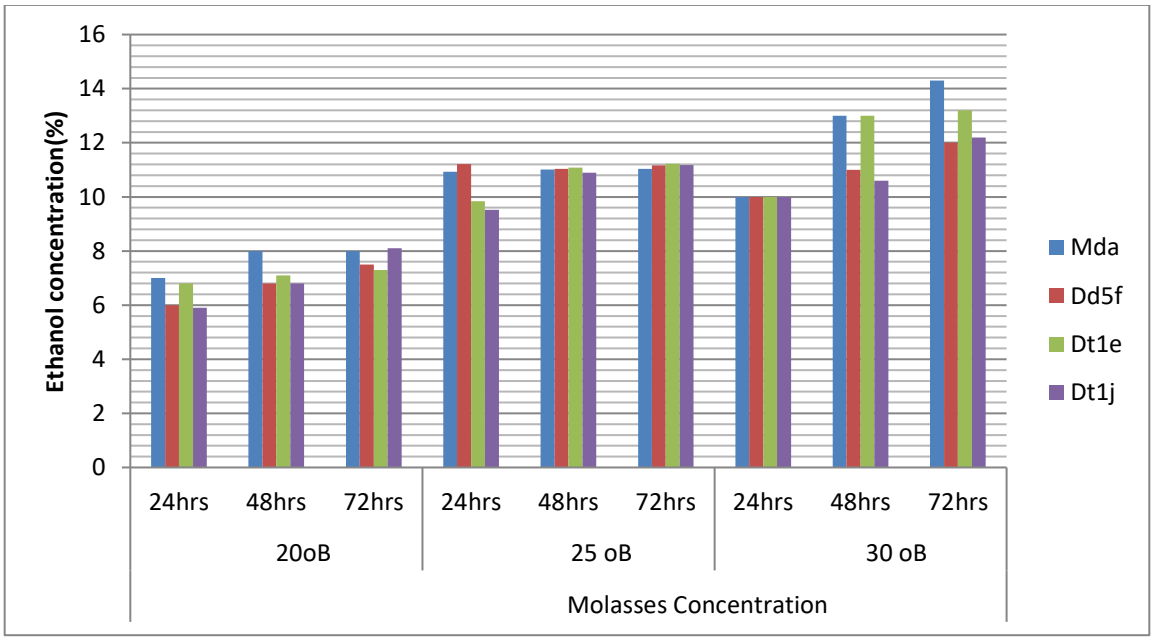

Figure 14. Ethanol yield from top 4 fermenters with different molasses concentration at 24, 48 and 72 hours.

5.9. Molecular analysis

The results of the DNA extracts, PCR amplifications, BLAST search and their phylogenetic tree for DNA sequences of the best ethanol tolerant yeasts are shown in Figure 15, Table 12 and Figure 16 in that order. The amplified PCR products for the 10 isolates are shown in Figure 15. All the amplified PCR products of the isolates were between 830 to 850 bp lengths of gene based on the given ladder (2.5 kb) (Figure 15). Gel purified PCR products of the 10 samples were sequenced using Sanger sequencing machine, ABI 3730 with universal primers ITS4 and ITS5. According to the BLAST result from the ten isolates, seven isolates showed similarity (92-99%) to gene bank data entries, whereas three isolates did not match with any of yeast strains in gene bank. The highest similarity (99%) was shown between Mdb and *Saccharomyces cervisiae* E2598a and Dd5b and *Saccharomyces cervisiae* strain UOA/HCPF5911. Besides, the results of this study indicate that isolate Mdd is 98% similar to *Kluveromyces marixianus* MOA, whereas isolates Mda, Dd5f, Dd5e and Dd5b are 94, 96, 98 and 92% similar to *S. cervicae* strain GP2, *S. cervisiae* isolate E21567, *S. cervisiae* strainUCDFST11-194 and *S. cervisiae* NGL3A, respectively (Table 12). Thus, in this study, the isolates were belonged to two genera *Saccharomyces* and *Kluveromyces*. However, only the isolate Mdd was included under genus *Kluveromyces* and the rest six isolates were included under *Saccharomyces* genus.

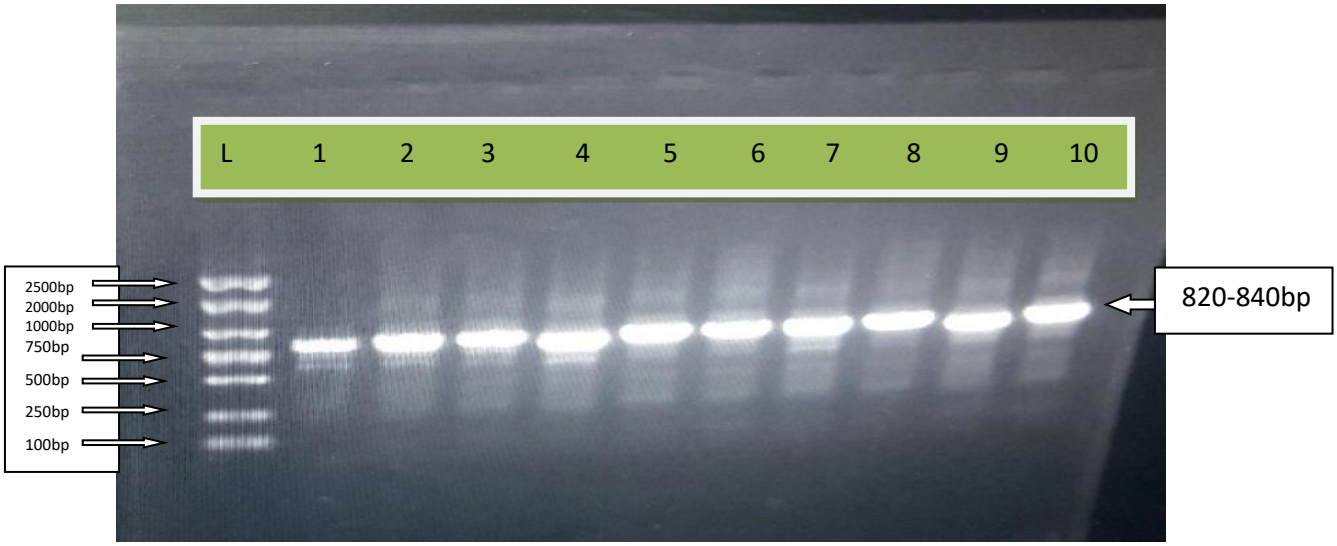

Figure 15. *Products of PCR for ten yeast isolates using primers ITS4 and ITS5. Whereas L: Ladder, 1:Dt1e, 2:Dd5e, 3:Dd5b, 4:Dt1j, 5: Mdd, 6:Dd5f, 7: Mdd, 8: Mda, 9:Dt1c and 10:Dd5d*

Table 12. BLAST similarities of the sequenced seven isolates from NCBI database

Isolates	Species/strain	Accession number	Identity (%)
Mdb	Saccharomyces cervicae strain E25980	MK267784	99
Dd5d	Saccharomyces cerevisiae strain	MT522376	99
Mdd	Kluyvermyces marixianus strain MDA	MK027128	99
Mda	S.cervicae strain GP2	MK680913	94
Dd5f	S. Cervicae strain E21567	MK267684	96
Dd5e	S.Cerevisiae strain UCDFST 11-194	MH595427	98
Dd5b	S. cerevisiae strain NGL3A	MG101823	92

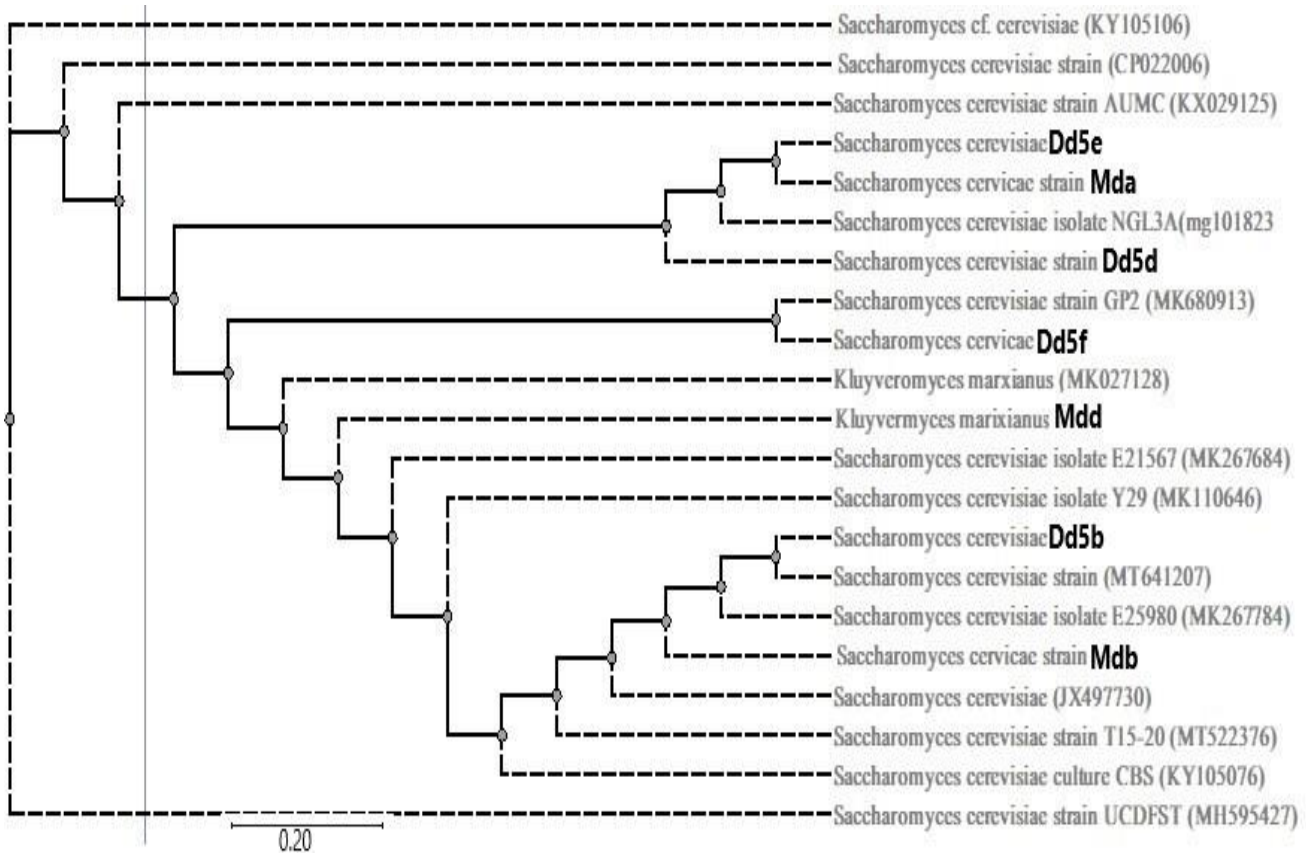

Figure 16. Phylogenetic study of seven isolates from *areki difdif/Tensis* using ITS DNA sequence. Seven isolates were (Mda, Mdb, Mdd, Dd5f, Dd5e, Dd5d, and Dd5b) and accession number of 14 similar strains from gene bank was (MG101823, KY105106, CP022006, KX029125, MK680913, MK027128, MK267784, MK267684, MH595427, MK110646, MT641207, JX497730, KY105076 and MT522376).

6. DISCUSSION

In this study a total of 270 isolates were isolated and allowed to be characterized. Morphological characterization of all isolates using colonial and microscopic observation seemed to indicate that all of the isolates were yeasts that are members of *Saccharomyces* species. Similar conclusion was reached on the basis of growth pattern studies in liquid and solid YPD media; in all cases white and creamy colonies with smooth colony texture were obtained after subculture (Thapa *et al*., 2015). Microscopic observation further confirmed this, as oval and circular shaped individual cells which reproduced by budding were observed in all cases. All growth for isolation of pure culture and screening based on ethanol tolerance were performed in YPD solid and liquid media.

In this study top ethanol tolerant isolates were screened based on their performance of ethanol tolerance. All isolates (270) were subjected to different ethanol concentrations (10 to 24%), thus all isolates were found tolerant to 10% and 12% ethanol concentration; but 37, 25, 10 and 4 isolates were found tolerant to 15%, 20%, 22% and 23% ethanol concentration, respectively (Table 4). Ethanol is the main inhibitor of yeast growth at relatively low concentrations by inhibiting cell division; decreasing cell biomass and specific growth rate; in addition to that high ethanol concentrations, reduces cell vitality and increases cell death also it influences cell metabolism and macromolecular biosynthesis (Stanley *et al*., 2010; Hu *et al.*, 2007). According to Gumaraes *et al*., (2006), three winery *Sacharomyces cervisiae* isolates shown tolerant to 15% ethanol concentration. Techaparin *et al*. (2017) specified the percentage of survivals of thermotolerant yeast isolates in YM medium at 7, 10 and 13% ethanol concentration.

Nwachukwu *et al*. (2006) showed *Saccharomyces cervisiae* isolated from Raffia palm wine (TBY 1 and TGY 2) exhibited resistance to 15.5% and 16% v/v ethanol concentration, respectively. Almost similar tolerance at 16.5% v/v ethanol has been observed for isolate HJ33 followed by the 16% v/v ethanol tolerant yeast isolate HJ12 in the broth medium (Taye Negera, 2016). *Sacchromyces cervisiae* of palm wine origin by protoplast fusion designated by the highest tolerance of 20% v/v ethanol (Nwachukwu *et al.*, 2008). In

present study ten isolates, Mda, Mdb, Mdd, Dd5b, Dd5d, Dd5e, Dd5f, Dt1c, Dt1e and Dt1j were observed tolerating more than 20% ethanol. Interestingly, isolates Mdb, Mda, Dd5d and Dd5f were found tolerant to 23% ethanol concentration and isolates Mdd, Dd5b, Dd5e, Dt1c, Dt1e and Dt1j were found tolerant to 22% ethanol concentration.

In this study the carbohydrate assimilation test was carried out for top ten ethanol tolerant isolates. All ten isolates in this study shows consumption of six carbon sugar. This could be an important indication of isolates to convert the given sugars in to the needed byproducts like ethanol. One isolate, Mdd were shown utilizing lactose and four isolates, Mda, Dd5f, Dt1e and Dt1j were observed utilizing the five carbon sugar, xylose (Table 6), these indicates that the isolates found in this study are broad sugar fermenters and this leads to increase bioethanol productivity. Yeasts especially members of *Saccharomyces* species were shown utilizing glucose, fructose and sucrose and *Pichia stiptis* can able to assimilate 5 carbon sugars (xylose) (Tsegaye *et al.*, 2018).

Thermo tolerance test also specified that all ten isolates of this study grew best at 30, 35, 40, and 45^{o}C within a 24 hours incubation period in YPD solid and liquid medium. It was found that isolate Mda of this study was able to grow at 47^{o}C (Table 7). The optimum growth temperature for members of *Saccharomyces* species was observed at 30^{o}C and optimum temperature for higher ethanol production was 35^{o}C indicated by Shanmugam (2009). According to Mir and Mohammed (2014) strains C2 and TA were shown growing well up to 37^{o}C, but showed weak growth at 40^{o}C and inadequate growth at 42^{o}C. The finding in this study has indicated that the isolates were tolerant to high temperature; which makes them industrially interesting and promising to survive sustainably at temperature stress environment for better ethanol production. It was indicated that isolate Mda were able to grow at 47^{o}C unlike the result given by Mir and Mohammed (2014). Nuttaporn *et al*. (2018) reported the growth of 62 isolates those were obtained from soil and rotten fruits grown at 37^{o}C, whereas 40 isolates grown at 40 and 45^{o}C. All ten isolates from this study could be classified as thermo tolerant yeasts since they were able to grow at 40, 45 and one isolate at 47^{o}C. If yeast can able to grow above maximum temperature of mesophilic yeast,

which ranges from 30 to 40^{o}C it, is considered to be thermo tolerant yeasts (McCrcken and Gong 1982; Sree *et al.* 2000).

Growth at different pH was also performed and all selected top isolates showed growth at pH 2.5, 3, 3.5, 4 and 4.5. However, all were not able to grow at pH 2 and below (Table 8). Shanmugam (2009) observed that isolates those were grown at 2 to 5 pH range produced maximum ethanol at pH 4. In this study it was found that the isolate Mda showed maximum ethanol production at pH 4.4.

During this study the amount of bioethanol production from the fermentation of molasses after 24, 48 and 72 hour time intervals was recorded. Temperature and shaking condition was included as additional parameters, incubation at 35^{o}C and shaking condition was performed at 150 rpm with pH of medium adjusted to 4 to 5.0. In the present study, the amount of bioethanol production of the ten isolates from molasses, with brix of 20, 25, and 30 degree brix (^{o}B) ranged from 8 to 14.3% (v/v) of ethanol yield. Isolate Mda which was able to tolerate high ethanol concentration (23%), produced the highest amount of ethanol yield (14.3% v/v) with 30^{o}B at 35^{o}C, whereas, the lowest ethanol yield was obtained from isolate Dd5e (9% v/v) with the same temperature and sugar concentration with Mda isolate. From the isolates, Mdb, Mdd, Dd5b, Dd5d, Dd5f, Dt1c, Dt1e and Dt1j the ethanol yield obtained were 9.5%, 12%, 10.5%, 11.3%, 12%, 12%, 13.2% and 12.2% v/v, respectively (Table 11). Jayus *et al*. (2016) reported that ethanol concentration produced from alcohol yeast and New Aule Baker's Yeast were 7.48% and 10.29% v/v without initial aeration and 8.7% and 12% v/v with the initial aeration at 38^{o}C, respectively. Aeration in the early stage of fermentation is important for yeast to synthesize cell membrane, especially sterols and unsaturated fatty acids which are essential to assure cell membrane integrity and to vent CO_2 that inhibits yeast cell growth (Khongsay *et al*., 2012). This result was found similar to isolates in this study that produced the average maximum of ethanol yield that ranged from 11% to 14.3% v/v with early stage aeration at 35^{o}C. Alfenore *et al*. (2004) stated that aeration of 0.2 vvm under fed-batch fermentation increased 23% of cell viability. On the average, ethanol yields from sugarcane molasses were found to be within the range of 7.8%

v/v in another similar study (Nofemele *et al.*, 2012). In line to this study, based on molasses concentration at 72 hours, the highest ethanol yield was obtained from 30^{o}B by isolate Mda with the yield of 14.3% v/v, whereas the lowest ethanol yield was obtained from 20^{o}B by isolate Dt1e with the yield of 7.3% v/v. Once more, at 24 hours and 48 hours, the highest ethanol yield obtained from 30^{o}B molasses concentration. Thus, this actually indicates that the selected isolates could produce lowest ethanol yield from 20 and 25^{o}B molasses concentration (Figure 14). Zhang *et al*. (2014) reported that the mutant yeast (YM4) created by applying 266 nm radiation produced 11.9% v/v ethanol at 35^{o}C in 72 hours with 100 rpm shaking condition, which has significant difference from the ethanol produced from isolates of this study which were able to produce above 12% v/v ethanol without any mutational treatment. According to Ethiopian sugar factory data, the commercial industrial ethanol producing yeast in fermentation tank can able to produce 8- 9% v/v ethanol at 32-35^{o}C with pH of 4.8 to 5 in 48 hours from 25-30 brix molasses concentration (Ethiopian sugar factory, 2020). So the isolates in this study showed better performance in ethanol tolerance and production than that of the commercial industrial yeasts used in sugar factory.

The highest sugar consumption was observed by isolate Mda at 72 hours which consumed more than 50% of sugar and reduced the brix from 30 to 14.6^{o}B; high sugar consumption by this isolate in an important indication of high ethanol yield. Whereas the lowest sugar consumption was observed from isolate Dd5d which consumed 35% of sugar with the brix reduction of from 30 to 19.4 at 72 hours as indicted in figure 6, due to lower sugar consumption, this isolate showed low ethanol production comparing to the other isolates.

Molecular level identification of ten isolates in this study was done and all isolates were subjected to total genomic DNA extraction and the internal transcriber spacer (ITS) region for each isolates was amplified using ITS 4 (5′-TCCTCCGCTTATTGATATGC) and ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG) primers giving 820-840 bp of PCR product (Figure 14). The amplified PCR result from each isolate was sequenced and the sequences were analyzed using BLAST at NCBI. Isolate Mdb and Ddfd showed 99% similarity to *S.cervisiae* strain E25980 and *S.cervisiae* strain UOA/HCPF5911, respectively from the

NCBI data base; whereas isolate Dd5f and Dd5e showed 96% and 98% similarity to *S.cervisiae* strain E21567 and *S.cervisiae* strain UCDFST 11-194 respectively; however, isolate Mdd showed 98% similarity to *Kluyvermyces marixianus* isolate MDA (Table 11). This similarity values indicted that it was above the threshold for genus and species identification. Isolate Mda and Dd5b showed 94% and 92% similarity to *S.cervisiae* strain GP2 and *S.cervisiae* isolate NGL3A respectively (Table 11), however, this similarity values are below the threshold for genus and species identification, consequently they could be a new genus or species (Oslan *et al*., 2012). The other three isolates (Dt1c, Dt1e and Dt1j) were did not show any similarities from the NCBI database. Based on assimilation and fermentation test these isolates were found to be yeasts, but this does not assure, so that, the isolates could be the new and it needs to examine at next higher taxonomic rank.

Phylogenetic study for the isolates was plotted and the cladogram tree obtained by using MEGA version 7 (Tamura *et al.,* 2007) was shown in Figure 15. To evaluate the reliability of the inferred tree the option of doing bootstrap analysis was followed as indicated in Oslan *et al*. (2012). The bootstrap value is attached to each branch and this value is a measure of confidence in this branch, so the maximum value was 100 and minimum value was 55 with 0.02 tree distance matrix (Figure 15).

Many wild type yeast strains were subjected to mutagenesis process to create mutant yeasts for better industrial performance. This approach is costly and time consuming, so it is very vial to isolate and characterize efficient, stress tolerant and effective industrially persuasive indigenous yeasts from local and natural sources (Zhang *et al.,* 2012). This study managed to achieve its objective, in contrast to the above studies and by isolating stress tolerant strains from local sources for industrial scale bioethanol production. Physiological studies on the ten isolates and molecular tests on seven isolates yielded the conclusion that isolates were strains of *S.cervisiae* except the isolate Mdd which was found to be member of *Kluyveromyces spp*.

7. CONCLUSION

The experimental results in the present study revealed that the ten isolates showed excellent performance in ethanol tolerance and temprature tolerance as well; they were also found surviving at various pH ranges. The ability to produce ethanol from sugarcane molasses was tested for all top ten isolates. Under all circumstances four isolates (Mda, Dt1e, Dt1j, and Dd5f) showed better results than the other six isolates in terms of productivity. The highest ethanol concentration was obtained from the Mda isolate which was 14.3% v/v.

The evaluation of fermentation conditions for the alcohol produced under different initial sugar concentration revealed that, Mda isolate using 30^{o}B sugar concentration with pH 4.39 under 150 rpm shaking condition at 35^{o}C showed the maximum ethanol production. In addition to isolate Mda that produce better ethanol yield, also isolate Dt1e, Dt1j and Dd5f were top included under top ethanol producer's category. Therefore these four isolates could be used at industrial level for fermentation of various raw materials in order to obtain high bioethanol yield.

8. RECOMMENDATION

Isolation and characterization of indigenous yeasts from locally available sources for better stress tolerance and product performance is promising. These reduce the investment of huge economy to import the commercial yeasts for inside industrial use. A further study on:

- Scale up optimization and production of locally isolated ethanol tolerant isolates.
- Optimization of fermentation kinetics for all ten isolates.
- Molecular identification of ethanol tolerant genes from top ethanol tolerant isolates,
- Immobilization of those isolates to increase their sustainable is needed to be carried out.

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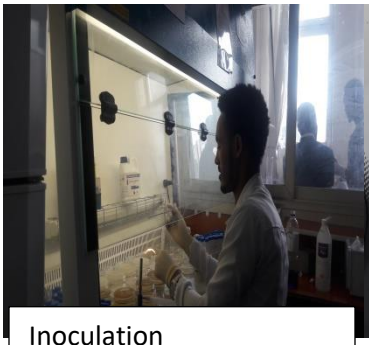
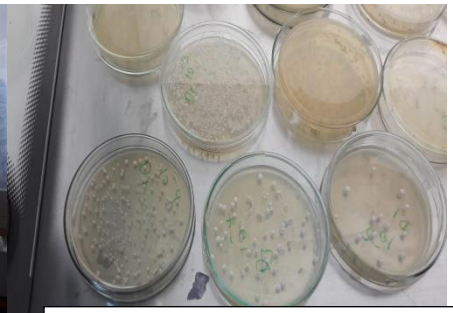
APPENDICES

Appendix 1. Isolation



Inoculation

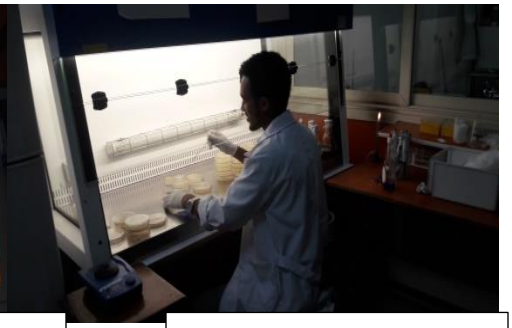


Isolated cultures

Appendix 2. Pure isolates and microscopic observation

Microscopic observation

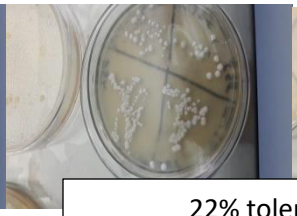
Purified isolates by strike



During Strike plating

Appendix 3. Ethanol tolerance viability

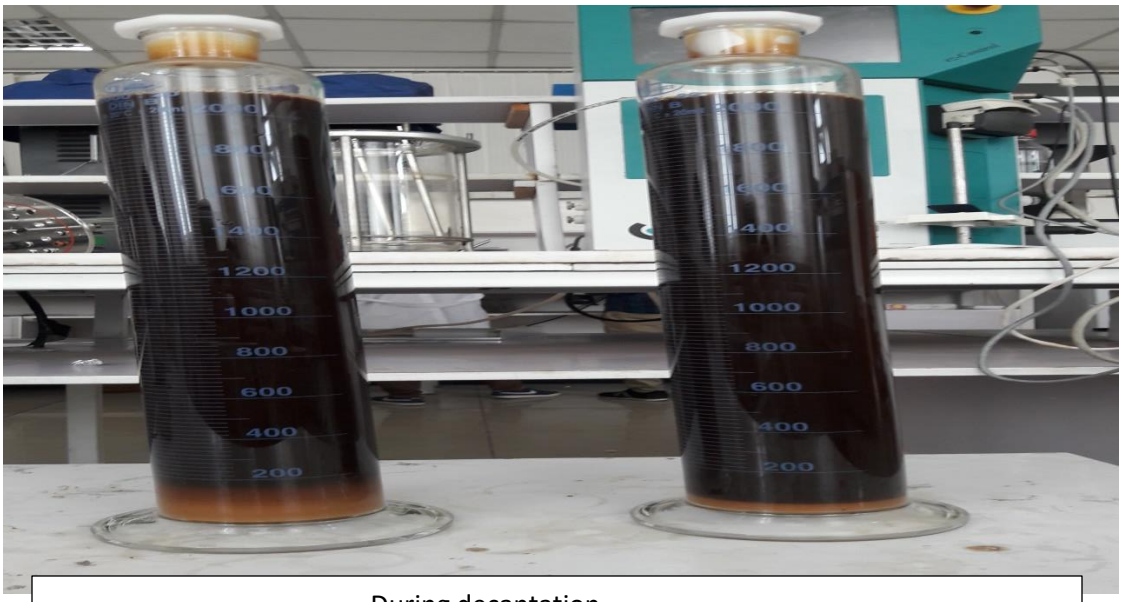
23% tolerant



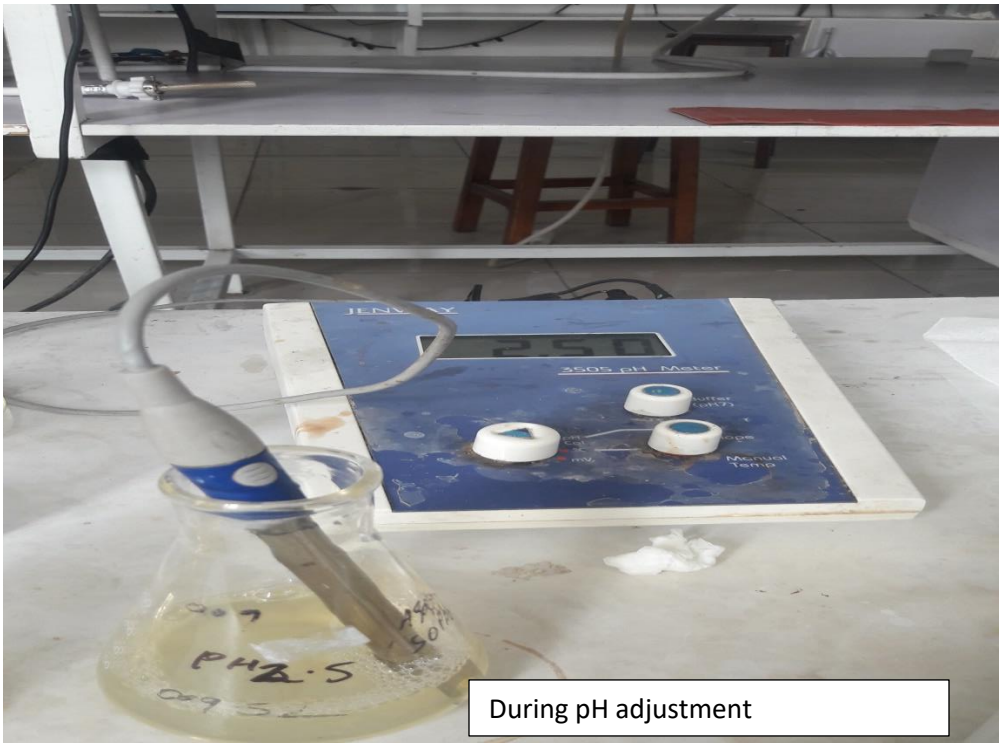
22% tolerant

20% tolerant

Appendix 4. Molasses clarification



During decantation



During pH adjustment