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INSTITUTE OF BIOTECHNOLOGY

Characterization of Wild Indigenous Yeasts from Molasses and Other Sugary Substrates and Their Potential for Bioethanol Production

M.Sc. Thesis

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By

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THESIS APPROVAL SHEET

We certify that Sisay Degu Tawneh’s M.Sc. Thesis entitled “**Characterization of Wild Indigenous Yeasts from Molasses and Other Sugary Substrates and Their Potential for Bioethanol Production’’** has been conducted under our direct supervision. Therefore, we kindly request the Institute of Biotechnology of Addis Ababa University to final approval and acceptance of the thesis.

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Abbreviations

AAFCO	Association of American Feed Control Officials
ABN	African Biodiversity Network
DM	Dry Matter
FE	Fermentation Efficiency
FS	Fermentable Sugars
OD	Optical Density
RS	Residual Sugars
TRS	Total Reducing Sugars
TS	Total Sugars
TY	Theoretical Yield
YP	Yeast Extract and Peptone
YMPD	Yeast Malt Extract Peptone Dextrose

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ABSTRACT

The increasing demand of energy has been supplied through the combustion of petroleum throughout the world. Due to escalating cost of petroleum, its contribution to global warming and non-renewable nature of this oil, there is collectively a need for renewable and ecofriendly energy sources such as bioethanol. Thus, the objective of this study was aimed at isolating, characterizing and evaluating the potent wild yeasts under different stress conditions from locally available resources for bioethanol production to minimize the utility of fossil fuel. In this study, a total of 35 samples of sugary substrates were collected from Metehara Sugar Factory for wild yeasts isolation following the standard protocols. A total of 305 yeast isolates were retrieved and screened using physiological and osmotic stress tolerance tests. Fermentative and potent wild yeasts were identified to species level using morphological and biolog-based biochemical methods. Out of 305 yeast isolates, 20 (6.56%) and 7 (2.29%) of them were found to be tolerant to 18 and 20% of ethanol, respectively. Out of these 20 ethanol tolerant yeast isolates, 17 tolerated the temperature of 45°C for 48 hrs. From 17 ethanol-thermotolerant isolates, 5 (29.41%), 5 (29.41%) and 7 (41.18%) were found producing gas from glucose at 24, 48 and 72 hrs, respectively. Out of the 17 ethanol-thermotolerant yeast isolates, 12 yeast isolates were able to tolerate 35% of glucose. Out of these 12 ethanol-thermo-and-sugar tolerant yeasts, the 7 were found tolerant to pH 2. From the 7 acidic tolerant yeast isolates, 5 yeast isolates were shown tolerant to 7% of NaCl and identified as *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*, respectively. During fermentation, *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa* were able to produce bioethanol with the values of (% v/v) 12.62, 11.61, 10.58, 10.82 and 10.44, respectively at 48 hrs and 12.56, 12.52, 12.08, 11.11 and 11.48, respectively at 72 hrs. The initial inoculum cell density was increased from (1.83 to 3.08, 0.59 to 1.81, 0.54 to 1.36, 0.48 to 1.69 and 0.45 to 1.56) x10^8 cells/ml for *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*, respectively 0 hr to72 hrs. The fermentation efficiency (%) for *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *T. globosa* and *Z. rouxii* was shown as 98.0, 97.8, 93.1, 90.8 and 88.1, respectively. Thus, on the basis of highest stress tolerant and higher fermentation efficiency features, *K. lodderae* and *P. guilliemodii* B wild yeasts were considered to be the best bioethanol producers. Molecular characterization and optimization of fermentation parameters is recommended to utilized these potent yeasts.

Key Words/Phrases: Bioethanol, fermentation efficiency, substrate, stress tolerance and yeasts

1. INTRODUCTION

1.1. Background of the study

The development of societies is based on consumption of energy for diverse human activities. Accordingly, fossil fuel is one of the most important sources of energy in many countries in the world. The increasing demand for energy has been supplied through the combustion of charcoal, oil and natural gases (Molla Asmare and Nigus Gabbiye, 2014). Some of these are non-renewable energy sources that will be depleted in short period of time (Sreenivas *et al.*, 2011). As a result, renewable energy is now a focus of worldwide headline news because of declining supplies of fossil fuels. Moreover, the sharp increase of world population size and industrialization are prompting for the growing demands of renewable fuel sources (MWIE, 2014). Globally, governments have been encouraging the use of alternative sources of energy in order to minimize energy crisis (Ahuja and Tatsutani, 2009; Naik *et al.*, 2010). The higher price of fossil fuels and their environmental concerns have been the major reasons for giving due attention to renewable energy sources that include bioethanol and biodiesel across the globe (Ingale *et al*., 2014).

The United States of America and Brazil are the largest producers of bioethanol that accounts over 80 percent of world's total production of bioethanol (Carriquiry *et al.*, 2011). In Ethiopia, about 95% of total energy consumption is met by utilizing traditional biomass fuels and about 5% coming from electricity and sun light energy (Hilawe Lakew and Yohannes Shiferaw, 2008; MWIE, 2014). The biomass is mainly obtained from firewood, wood charcoal, dung and crop residues that mainly depend on the surrounding forest resources and agricultural residues (Meskir Tesfaye, 2007). On the other hand, Ethiopia imports its entire petroleum fuel requirement by spending over 80% of its foreign currency earnings annually to purchase fossil fuel (MME, 2007). Consequently, bioethanol has been emerged as a potential alternative source of energy because it is eco-friendly with minimum pollution to the environment and can be blended with gasoline in the current combustion engine systems in order to minimize fossil fuel consumption. Hence, bioethanol can be produced in Sugar Factories by utilizing byproducts from these industries (Hansen *et al*., 2005).

Bioenergy derived from biomasses is efficient source of energy and eco-friendly to the nature (Babarinde *et al*., 2016), which are generated from biological materials like carbohydrate and cellulosic plant materials (Baskar *et al*., 2016). One of the most important biomasses is cane molasses (byproduct of sugar factories) that efficiently utilized by yeasts in order to produce bioethanol for different purposes. Currently, in Ethiopia, there are three functional sugar factories i.e., Wonji-Shoa, Metehara and Fincha'a. But only two Plants, Fincha'a and Metehara sugar factories have involved in the production of cane molasses for bioethanol accordingly to the two factories the yield is very low because of low fermentative capacity of yeasts. This problem calls for urgent action to screen indigenous wild yeasts from the surroundings of sugar factories for better performance because of that the yeast well adapted the factories environmental condition.

Yeast selection for bioethanol production over the past two decades especially in most bioethanol related researches in developing tropical countries focusing primarily on isolation of local *Saccharomyces* yeasts (Abdel Fattah *et al.*, 2000). The major type of yeast species that has been widely used in the conversion of sugar and starch-based substrate into bioethanol is *S. cerevisiae* (Canilha *et al.*, 2012). Generally, *S. cerevisiae* is able to produce high amount of bioethanol from monomer substrate but it may be affected with inhibitory compounds (Balat *et al*., 2008). Most strains of *S. cerevisiae* prefer glucose in molasses compared to fructose for their energy source. Sometimes bioethanol production using *S. cerevisiae* with high concentration of substrate (molasses) containing sucrose might not completely converted into products. This incomplete conversion of sugar into bioethanol leads to lower bioethanol yield. In order to solve this problem, it is necessary to pre-treat substrate using preferably hot-acid method and screen well adapted wild yeast isolates to stress conditions (high substrate, temperature and ethanol concentration) in order to increase bioethanol yield (Guillaume *et al.*, 2007; Berthels *et al.*, 2008; Wu *et al.*, 2010). Recently, recombinant *S. cerevisiae* strains can be capable of fermenting pentose (5-C) sugars and well stressed tolerant after pre-treatment. However, pre-treatment of biomass results hydrolysates with high osmolarity and high concentrations of inhibitors. These hydrolysates can be negatively affecting the fermentation process. Therefore, robust yeast species with high stress tolerance are required including *S. cerevisiae*. Up to now, more than 2000 yeast species have been described and some of these could provide a solution to these limitations because of their high tolerance to the most predominant stress conditions (Radecka *et al.*, 2015). Beyond *S. cerevisiae*, some of the non-conventional yeasts such as *Zygosaccharomyces rouxii*, *Kluyveromyces marxianus*, *Ogataea polymorpha*, *Zygosaccharomyces bailii* and *Issatchenkia orientalis* which are stress tolerant and produce better bioethanol from molasses (Martorell *et al.*, 2007; Mukherjee *et al.*, 2014).

The present study gives due emphasis to screening using different parameters and selecting the potent indigenous wild yeast species with high efficiency of converting sugars in cane molasses and thereby increasing the bioethanol yield.

1.2. Statement of problem

In Ethiopia, currently, three sugar factories are fully operational and others are under establishment. When all sugar factories start their production, excess byproducts will be generated as waste and requiring proper management options. The byproduct such as molasses can be stored for about 20 years, some of it could also be damped into the environment and reduce benefits of the sugar industries. Sometimes the byproducts may be sold with a cheap price for animal feeds and this calls for urgent measure in designing innovative strategies of utilizing byproducts for other purposes such as bioethanol production. At present, cane molasses is largely used as the best substrate for bioethanol production (Mukherjee *et al.*, 2014) in many countries including Ethiopia.

On the other hand, the price of petroleum is being kept on increasing. The current (February, 2021 GC) price of petroleum for engines is around 25.82 Birr per liter (MTI, 2021). According to reporter news sourced from Ethiopian Petroleum Supply Enterprise (EPSE, 2017), Ethiopia spent more than 2.8 billion dollars to import petroleum fuel annually for transportation and other industrial purposes. The foreign currency expenditure for buying petroleum is souring. About 87% of export earnings goes for fuel purchase (EAPIC, 2007). Emission from fossil fuels also contributes highly to climate change and not friendly for environment. Transport is one of the most important sectors that consumes majority of imported petroleum and contributed to greenhouse gas especially road transport accounts 22% of greenhouse gas emissions (www.foodfen.org.uk).

Globally, biofuels such as bioethanol is considered as an opportunity for enhancing and meeting the growing demand of energy. Moreover, blending gasoline with bioethanol has already started with ratio of 90:10 in practice and there was a plan to increase this combination to 25% (MWIE, 2014) but was not successful so far. The total cost of petroleum can be reduced significantly when bioethanol is blending with petroleum. Accordingly, there is a great push by the government of Ethiopia to facilitate adequate production of bioethanol from home-grown resources such as molasses in order to substitute partial imported petroleum (MWIE, 2014). Currently, both at Metehara and Fincha'a bioethanol Plants, the reduction of bioethanol (5-8% v/v) yield has been reported that could be attributed to contamination or mutation of commercial yeast strains and that necessitate to screening indigenous wild yeasts from sugary substrates from the ecology of Sugar Factories. The yield is becoming very low and it needs immediate attention to keep its production above 10%. According to Arshad *et al.* (2008) and Goswami *et al.* (2015) indicated that the production of bioethanol can be maximized to 10-14% (v/v) from dilute molasses using local yeast isolates and 14-16% (v/v) using genetically engineered *S. cerevisiae*. Therefore, it is very important to isolate and screen the best wild yeast isolates from different environmental sources for the production of bioethanol. This study was done in isolating and screen indigenous wild yeasts from sugary substrates (molasses) abased on stresses tolerance and high ethanol yield.

1.3. Objectives of the study

1.3.1. General objective

The main objective of this study was to characterize indigenous wild yeasts isolated from different local sugary substrates for the production of bioethanol using high stress tolerant yeasts.

1.3.2. Specific objectives

The specific objectives of this study were:

- To isolate and purify wild indigenous yeasts from locally available resources (molasses, sugar juice, leakages lines, drainage sites and other wastes) of Metehara Sugar Factory.
- To evaluate ethanol, temperature, pH and salt tolerances of purified wild yeast isolates.
- To screen yeast isolates for utilization of sugars, ultimate gas production and select the best stress tolerant isolates.
- To produce bioethanol using molasses as fermenting medium.
- To identify the best and selected wild yeast isolates those with higher potential of producing bioethanol from molasses at species level.

2. LITERATURE REVIEW

2.1. Biofuels

Biofuels are defined as a renewable clean burning and efficient fuels derived from biomass through fermentation (Mansoori, 2016) while bioethanol is defined as an alcohol manufactured by microbial conversion of biomass materials through fermentation. It is an organic matter taken from plants as a source and both in the form of cellulose or carbohydrate (EASAC, 2012).

Bioethanol or grain alcohol is a flammable, colorless, far less in toxicity, biodegradable, distinctive perfume like odor and little cause of environmental pollution than petroleum (Gasmalla *et al.*, 2012). Bioethanol, an alternative to fossil fuels, is mainly produced by yeast fermentation from different feedstocks. It is a high-octane fuel and used as blending to gasoline; it burns more completely and that reduces the polluting emissions and to minimize the spending of money for it (Busic *et al.*, 2018).

The sources comprise mainly of wood, agricultural crops and products, aquatic plants, forestry products and animal wastes and residues. In general, biofuel is all type of solid, gaseous and liquid fuels that can be derived from biomass (Richard, 2007). Biofuel include methanol, ethanol, plant oils and methyl esters produced from oils (biodiesel). The most common biofuels are bioethanol and biodiesel in which bioethanol is made from fermented sugars while biodiesel is made from oils of certain plants (Bryn, 2009). In order to produce biofuels from low-cost biomass such as agricultural wastes and byproducts (including crop residues, sugar cane waste, wood, grass and wastewater from food processing industries) are important materials to make them competitive with other fuels. Moreover, biofuels derived from only agriculture wastes show low environmental effects such as reduction of greenhouse gas (GHG) emission to minimize environmental impacts (Kisielewska, *et al.*, 2015).

Some developed countries like USA have been using corn whereas Brazil is using sugar cane molasses for first generation bioethanol production with simple fermentation using *S. cerevisiae* (Muktham *et al.*, 2016). Because, it is better to use bioenergy to mitigate environmental problems that is derived by utilizing petroleum as energy source. The growing demand for energy is worldwide especially it has been increasing by many magnitudes in many rapid developing countries like China and India (Darzins *et al.*, 2010). Ethiopia entirely uses imported petroleum for most of its energy demands and this makes the transportation cost expensive.

2.2. Local raw materials for bioethanol production

There are many types of raw materials can be used as potential substrates for bioethanol production. Bioethanol may be classified under categories of first, second and third generation. First-generation biofuels are made from carbohydrates or food grains; second-generation are derived from lignocellulosic materials and third-generation biofuels are obtained from microbial sources like algae (Naik *et al*., 2010). According to Mussatto *et al.* (2010), raw materials used for the production of bioethanol are divided into three categories based on their carbohydrate sources: (i) sugar containing, (ii) starch containing and (iii) lignocellulosic raw materials (Table 1) (Rutz *et al.*, 2008; Kang *et al*., 2014).

The major raw materials used for bioethanol production can be classified as follows: - sugars such as sugarcane, sugar beet, sweet sorghum, whey and molasses (Laopaiboon *et al*., 2007; Limtong *et al*., 2007; Laopaiboon *et al*., 2009); starchy crops such as corn, wheat, cassava and potato (Choi *et al*., 2010; Watanabe *et al.*, 2010; Yuangsaard *et al*., 2013) and lignocellulosic feedstocks such as woody materials, agricultural wastes and crop residues (Mussatto *et al.*, 2010; Singh *et al*., 2013). High sugar concentration is more preferred in industrial bioethanol production to increase the amount of bioethanol produced at the end of fermentation. However, when high sugar concentration is used, yeast cells are exposed to stress by a substrate and this may also affect fermentation performance (Dhaliwal *et al*., 2011).

Table 1: Summary of raw materials for bioethanol production.

Types of substrate	**Source**	**Examples**
Sugar	Root crop	Sugar beets
	Stalk crop	Sugar cane, sweet sorghum
Starch	Cereals	Corn, barley, rye, wheat, sorghum grain
	Root crops	Potatoes, cassava
Cellulose	Forest residues	
	Energy crops	Popular, willows, switch grass
	Agriculture wastes	Corn stover, bagasse, straw
	Paper wastes	

Source: Rutz *et al.* (2008).

2.2.1. First generation bioethanol

First-generation biofuel is directly sourced from edible crops like sugar cane and maize or from sugar and starch containing sources (Rutz *et al.*, 2008; Busic *et al.*, 2018). These feedstocks present the problem of affecting the food security (IEA, 2011). Sugar cane and sugar beet are the most important sugar producing plants in the world. About two-third of the world sugar production is from sugar cane and one-third is from sugar beet (Linoj *et al.*, 2006). The byproduct of these sugar producing plants is mostly molasses and used for the production of first-generation bioethanol.

2.2.2. Second generation bioethanol

Second-generation bioethanol feedstocks are mainly agricultural wastes such as corn stover, sugarcane bagasse and wood, grasses or non-edible parts of plants (IEA, 2011). The huge source of lignocellulose is available from non-food wild plants that grow in non-cultivated lands. Second-generation ethanol feedstocks overcome the bottlenecks of first-generation bioethanol: adverse effects on food prices and influencing their supply (Mussatto *et al.*, 2010). Cellulosic bioethanol can be produced from wood, grasses or non-edible parts of plants. Lignocellulose is mainly composed of cellulose, hemicellulose and lignin (IEA, 2011).

Bioethanol produced from lignocellulose has an advantage because of abundant and diverse raw materials as compared to maize but it requires lots of energy for process to convert into monomer sugars and to be accessible for microorganisms especially for yeasts (Tomás-Pejó *et al.*, 2011). Developing countries have been using lignocellulosic plant materials instead of food materials as second-generation biofuel with heat treatment (Abadi Birhanu and Shimels Ayalew, 2017). The most common molasses substrates used as source for the production of biofuels is indicated in Table 2 (AAFCO, 1982).

Table 2: Different sources of molasses for the production of bioethanol with their respective brix.

Substrate as byproduct	Sources	°Brix	Total sugars (%)
Cane molasses	Sugar cane	>79.5	>46
Beet molasses	Sugar beet	>79.5	>48
Citrus molasses	Dried citrus	>71.0	>45
Hemicellulose extract	Pressed wood	Variable as a source	>55
Starch molasses	Dextrose manufacture from grains	Variable as a source	>73

Source: AAFCO (1982).

2.2.3. Third generation bioethanol

Third-generation biofuel, early stage of developments, are produced from algae (Maity *et al.*, 2014). Algal oils can be used to produce ethanol, butanol, biodiesel, jet fuel, bio-oil and fertilizer (USDOE, 2016). The same author indicated that alga encompasses a diverse group of organisms that including microalgae, macroalgae (seaweeds) and cyanobacteria (blue-green algae). There are 800,000 different species of algae which occur either in the form of microalgae or seaweeds (Bowyer *et al.*, 2018). The vast majority occur is fresh, waste or salt water and interested with fast growing and harvesting in days rather than months (Maity *et al.*, 2014).

2.3. Chemical composition of molasses for bioethanol production using yeasts

Molasses is commonly used as a feedstock for bioethanol production. Molasses obtained after cane processing contains about 60% (w/w) sucrose and 40% (w/w) other components. The non-sucrose substances in molasses are including inorganic salt, raffinose, organic acids and nitrogen containing compounds (Patrascu, 2009; Palmonari *et al.*, 2020). The residue of sugar factory is viscous liquid with approximately 80°Brix (Bx) which is called cane molasses. It has a high nutrient and sugar content of about 45-60% sucrose and 5-20% glucose and fructose. During fermentation process, cane juice and cane molasses are mixed and must be diluted to 18-22% (w/w) of total reducing sugars. The compositions of sugar cane molasses used as fermentation medium are water content (18.2%), soluble solids (81.8%), total sugars (54.6%), total nitrogen (0.5%) and mineral substance (6.2%) (Eliodório *et al*., 2019). According to Patrascu *et al.* (2009), the optimum amount of diluted molasses is about 180-200g/l in order to minimize the effect of substrate on yeast during fermentation to produce bioethanol. Molasses obtained from Sugar Factories have the compositions that used as a fermentation medium presented in Table 3.

Table 3: Chemical composition of cane molasses (% w/w of dry matter).

Item	% of average	Item	%of average	Item	% of average
DM	76.8	Fructose	8.07	Starch	0.33
CP	6.65	Raffinose	0.03	Levans	0.89
Total sugars	62.3	Galactose	0.04	Dextrans	0.79
Sucrose	48.8	Arabinose	0.01	Arabans	0.20
Glucose	5.29	Xylose	ND[1]	Acontic acids	1.42
Lactic acid	6.10	Acetic acid	0.44	Sulfates	2.09

Malic acid	0.10	Ash	13.1	Sulfur[2]	0.69
Citric acid	0.13	Ca	1.39	Phosphates	2.03
Pyrocarbonic acid	0.34	Mg	0.43	Nitrates, mg/kg	464
Oxalic acid	0.06	Na	0.08	Chlorides	60
Glycolic acid	0.00	K	1.82	DCAD[3]	7

DM= dry matter, CP= crude protein, ND[1]= non detectable. [2]sulfur obtained from sulfates considering their respective molecular weight. [3]calculated as DCDA, mEq/100g- [K (% of DM/0.039+Na (% of DM)/0.023]- [Cl (% of DM)/0.0355+S (% of DM)/0.016].

Source: Palmonari *et al.* (2020).

2.4. Treatment methods for production of bioethanol from diverse substrates

There are different methods of pre-treatment mechanisms that to prepare substrates and making accessible for fermenting microbes. These are mechanical treatment [size reduction (53-149µm) through chopping, milling or grinding can lead to significant improvement of enzymatic hydrolysis or microbial digestion (Saida *et al.*, 1982)], chemical treatment [acid such as concentrated H_2SO_4 to remove hemicellulose (Talebnia *et al.*, 2010), alkaline such as 0.5N NaOH to remove lignin (Rivers *et al.*, 1984)] and steam heat explosion (rapidly heated by high pressure steam for a certain period of time) (Zandersons *et al.*, 2004). Microbial treatment also applied for a certain material such as fungi (*Trichoderma reesei* and *Coniochaeta ligniaria*) (Palmqvist *et al.*, 1997; López *et al.*, 2004). Microbial conversion of lignocellulose using microbial fermentation is a typical preceded by an acidic thermochemical treatment step designed to enable enzymatic hydrolysis of cellulose (Jönsson *et al.*, 2013). The fungal treatment is less energy intensive than others and environmentally friendly but loss of high amount of cellulose and hemicellulose during pre-treatment and slow rate of hydrolysis reaction and that reduce its overall attractiveness. Steam pretreatment or hot-acid pretreatment methods are more effective than others due to lower reaction time, minimum use of chemicals and cost effective (Al-Haj Ibrahim, 2012).

2.5. Bioethanol production

There are different methods to produce bioethanol from different sources as indicated below. These are biological method that commonly used in most laboratories and industries (fermentation using yeast), enzymatic method (production of enzymes and using them as catalyst especially alcohol dehydrogenase) and biotechnological method (manipulation of essential genes of yeast to maximize bioethanol yield through minimizing inhibitors).

2.5.1. Biological method

Bioethanol is produced commercially by fermentation which is the oldest technology from cereal grains, molasses or other materials with high starch or sugar contents in the presence of fermenting organisms (Ishmayana *et al.*, 2011). Yeast isolates have been used for the production of bioethanol along with fermentation process. Fermentation process involves conversion of sugars to alcohol and carbon dioxide with the presence of yeast. The principle of fermentation involves the use of biological agent to catalyze alcoholic fermentation for production of bioethanol. Mostly in industries, *S. cerevisiae* is used in the fermentation of molasses to utilize sugars and produce bioethanol (Ishmayana *et al.*, 2011). Another important yeast is *Schizosaccharomyce pombe* that can be grown under both aerobic and anaerobic conditions which characterized as colorless, clear, bright and free from turbidity that indicates its high specification quality and used in fermentation for the production of bioethanol (Bakhiet and Mahmoud, 2015).

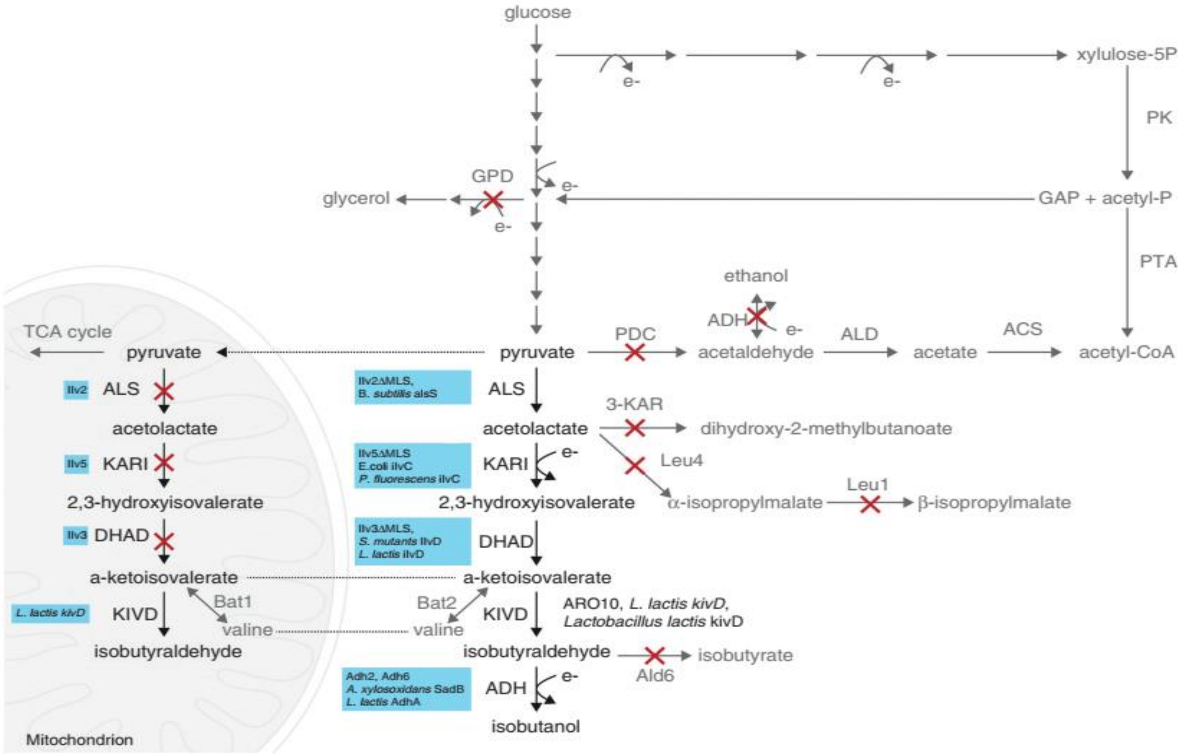
Zymomonas mobilis, a gram-negative bacterium, is extensively studied over the last three decades in fuel ethanol production from grains, raw sugar, sugarcane juice and syrup due to its ethanol tolerance and higher glucose uptake as well as good ethanol production capability (Lee and Huang, 2000). The *Z. mobilis* produce bioethanol via aerobic fermentation with the maximum production of 7.9% (v/v) at pH 5 (Khoja *et al.*, 2018). The same author indicated that the optimal condition of

Z. mobilis to produce 9.3% (v/v) bioethanol with 92.5% of fermentation efficiency through anaerobic fermentation was 16g/100ml sugar concentration, pH 5 and temperature of 34°C. Even if some bacteria involved in the fermentation process for the production of bioethanol, due to their low pH in-tolerance, difficulty in recycling and their lower capability of carbohydrates utilization that they contributed to low bioethanol yield (Khoja *et al.*, 2015). However, yeast species were found better tolerant to low pH that resulted during the course of fermentation, easy to recycling and utilization of sugars than bacteria and as a result they are commonly used for industrial bioethanol production including this study.

2.5.1.1. Enzymatic method

Enzymes are purified from different microbes used for the production of bioethanol (Láine *et al.*, 2019). Enzymes like Glycerol-3-phosphate dehydrogenase, acetolactate synthase, aldehyde dehydrogenase and alcohol dehydrogenase are common enzymes applied for bioethanol production in metabolic pathway starting from glucose to bioethanol. The alcohol dehydrogenase can be produced from *S. cerevisiae* during fermentation used to convert sugar in to ethanol. Glucose in molasses converted to pyruvic acid through glycolysis. Pyruvic acid then converted to acetaldehyde and carbon dioxide then acetaldehyde further converted to ethanol by alcohol dehydrogenase (Fig. 1). During fermentation, sugar is used by yeast not only for the growth and proliferation but also it is important to produce ethanol and carbon dioxide (Buglass, 2011; Buijs *et al.*, 2013).

Bioethanol produced from lignocellulosic materials have been getting challenges because of lignin. Lignin is found closely bound to cellulose and not easily accessible for cellulases. The cost of getting effective strains and enzyme purification is very difficult. Enzyme are credible agents for lignin degradation and certainly act between macromolecular substrate of cleavage and polymerization (Chen *et al*., 2012). Lignin degradation acted by peroxidases where lignin peroxidase and manganese peroxidase are cooperating together (Binod *et al.*, 2010) but the cost of purification of those enzymes are very expensive.



Where GPD = glycerol-3-phosphate dehydrogenase, TCA = tricarboxylic acid, ALS = acetolactate synthase, KARI = ketoacid reductoisomerase, DHAD = dihydroxyacid dehydrogenase, KIVD = ketoisovalerate dehydrogenase, ADH = alcohol dehydrogenase, PDC = pyruvate decarboxylase, 3-KAR = 3-ketoacid reductase, ALD = aldehyde dehydrogenase, ACS = acetyl-CoA synthase, PK = phosphoketolase and PTA = phosphotransacetylase. Red crosses: enzymatic steps eliminated by gene deletion.

Figure 1: Current enzymatic production of bioethanol from molasses.

Source: Buijs *et al.* (2013).

The production of ethanol using free yeast cells is still inefficient due to its higher cost of cell cycling, high risk of contamination and susceptibility to the environmental variations (Kumar *et al.*, 2011). Besides, free cells cause substrate and/or product inhibition for direct contact between cells and medium. Most of the problems occurred in free-cell systems are reduced by the immobilization method. Immobilized technology is commonly applied in fermentation process (Duarte *et al.*, 2013).

The benefits of immobilized cells/enzymes over free cells/enzymes include higher cell density per volume of reactor, easier separation from the medium, higher substrate conversion, less inhibition byproducts, shorter reaction time and control of cell replication (Vucurovic *et al*., 2009).

2.5.2. Biotechnological method

The *S. cerevisiae* genome has been fully sequenced and it is possible to easily identify the gene that can be manipulated/genetically modified in order to maximize the yield of bioethanol and minimizing inhibitory effects (Patrascu *et al*., 2009a). The same author indicated that *S. cerevisiae* is extensively studied to overcome the stress problem of ethanol using biotechnological techniques by modifying its metabolic activities. The factors triggering the expression of genes may be biotic or abiotic. Biotic factors induce changes in the gene expression and synthesis of specific compounds that generate the resistance to organism (Edgardo *et al.*, 2008). The abiotic factors can be temperature, osmotic stress, anaerobic conditions, heavy metals, ultraviolet radiation, inhibitors and pH that affects the chemical reaction during fermentation (Chen and Chen, 2004; Patrascu *et al*., 2009b). Some of the factors that inhibit extensive activities of *S. cerevisiae* can be minimized through manipulation of genes (genetic modification) such as ADH gene that encoded for alcohol dehydrogenase for complete conversion of all acetaldehydes to ethanol and maximized the yield (Buglass, 2011).

2.6. Fermentation types and processes for bioethanol production

Fermentation is breakdown of organic compounds by metabolic process through microorganisms. Fermentation may be classified into Solid-state fermentation (SSF) and Submerged fermentation (SMF) that mainly based on the type of substrate used during fermentation. Solid-state fermentation utilizes solid substrates e.g., bran and bagasse. In this fermentation type, substrates are utilized very slowly and gradually that fermentation takes long time with the same substrate. The SSF is the best fermentation techniques when fungi and other microorganisms are involving and that requires less moisture content. However, it cannot support microbes that requires extra water such as bacteria and yeast. The product recovering requires quite less energy (Subramaniyam and Vimala, 2012). Submerged fermentation utilizes free flowing liquid substrates such as molasses and broths. The products are secreted into the fermentation broth. The substrates are utilized quite rapidly and it requires constant supplement of nutrients. The SMF is suitable for microorganisms such as bacteria and yeast those require high moisture content in their medium. Its advantage includes the suitability and easiness for purification of the products from the bulk but it requires lots of energy (Bušić *et al*., 2018).

There are mainly three types of fermentation process such as batch, fed-batch and continuous. In batch fermentation, feedstock is added into fermenter along with microbes at the beginning of the process and the product is recovered and then start new operation for next while fed-batch fermentation is added one or more ingredients at one or two times into the fermenter when fermentation is ongoing (Bušić *et al*., 2018). Continuous fermentation requires continuously added the nutrients/ ingredients in to the fermenter/ vessel and taking out the products from the fermenter (Zabed *et al.*, 2014). Batch fermentation is simple fermentation process that requires low cost, easy to control, sterilize and manage the product and most of ethanol production from juice feedstocks is carried out by batch fermentation (Bai *et al.*, 2008). Fed-batch process is mainly employed in industrial production that accumulating the benefits than both batch and continuous processes. It has some advantages over batch process that in order to: maximize viable cell concentration, extended lifespan of cells, increase product accumulation, minimize inhibitory effects due to maximum substrate concentration and control the physical factors such as pH, temperature and dissolved oxygen at a specific level through feedback activities (Saarela *et al*., 2003). Continuous fermentation offers some advantages over batch fermentation. This type of fermentation process requires less time to clean the vessel, filling and that increased the productivity with lower cost (Brethauer and Wyman, 2010; Zabed *et al.*, 2014). The selection of fermentation type and process is based on the nature of feedstock and the organisms that employed for fermentation.

2.7. Yeasts and other microbes used for bioethanol production

Yeasts are single-celled eukaryotic microorganisms that are classified as members of Fungi Kingdom. Yeasts can be also defined as Ascomycetous or Basidiomycetous fungi that are capable of reproducing by budding or fission and form spores which are not enclosed in a fruiting body. The lower taxonomic subdivisions (families, subfamilies, genera, species and strain) are determined by its morphological, physiological and genetic characteristics including sexual reproduction (Kurtzman and Fell, 1998). Most yeasts are gram positive, microscopically circular or oval shaped and that reproduce asexually by budding through asymmetric division (Oca *et al.*, 2016). These eukaryotic organisms have a capacity to grow on different types of sugars and exhibiting high sugar and ethanol tolerance (Techaparin *et al*., 2017).

According to Anbessa Dabassa *et al*. (2019) reported that the 5 yeast species isolated from 5 sugarcane samples were including *Geotrichum silvicola, K. marxianus, Meyerozyma guilliermondii*, *P. kudriavzevii* and *Yarrowia lipolytica*. The 26 different yeast species were also isolated from 77 sugarcane samples. The most common species were including *Saccharomyces carlsbergensis*, *S. cerevisiae*, *Pichia membranaefaciens*, *Candida krusei*, *Torulopsis stellata*, *Candida guilliermondii*, *Pichia fermentans, Candida intermedia* and *Schizosaccharomyces pombe* (Figueroaa *et al.*, 2018). The ethanol producing yeast genera *Pichia, Candida, Kluyveromyces, Issatchenkia*, *Zygosaccharomyces*, *Clavispora*, *Debaryomyces*, *Metschnikowia*, *Rhodotorula* and *Cryptococcus* were isolated from fruits and tree barks (Rao *et al*., 2008). Some microorganisms such as *S. cerevisiae, Saccharomyces diasticus* (Maruthai *et al*., 2012)*, Kluyveromyces marxianus, Pichia kudriavzevii*, *Escherichia coli* strain KO11 and *Klebsiella oxytoca* and *Z. mobilis* have been studied for bioethanol production from sugar juices (Zabed *et al*., 2014). *Saccharomyces cerevisiae* is the most promising yeast species involved in the conversion of hexoses (monomeric sugar and sucrose) into bioethanol (Yu and Zhang, 2004).

There are a few yeast species which are able to produce ethanol from xylose such as *Scheffersomyces stipitis*, *Pachysolen tannophilus*, *Candida shehatae* and *Candida guilleromondii*. They are only 1% of potential yeasts utilizing xylose to bioethanol (Canilha *et al*., 2012; Kostas *et al*., 2016). Important features of wild indigenous yeasts to be considered for screening and used for bioethanol productions are utilized the given raw materials efficiently and provide high yield, consistent, high productivity and stable (Azhar *et al.*, 2017).

2.8. Factors affecting production of bioethanol during fermentation of molasses using yeast

There are many factors that could affect the rate of yeast fermentation such as temperature, source of carbon and nitrogen, source of carbohydrate, pH, salinity and inoculum size (Naser, 2014). Some of the basic factors are going to be discussed below:

2.8.1. Temperature

Mostly the temperature used for the growth of yeasts is ranging from 25 to 35°C (Dragone *et al*., 2004). Other investigators indicated that the temperature for the growth of yeast is also in the range of 30 to 40°C with most yeasts identified having a maximum peak for bioethanol production at 35°C. For example, the optimum temperature for *S. cerevisiae* was 30-35°C (Zohri *et al.*, 2015) When the temperature is slightly increasing during fermentation, the rate of fermentation was recorded as successfully employed but diminished their yield when the temperature is extremely high (Dragone *et al*., 2004). According to Choudhary *et al.* (2016), when the temperature was elevated above 35°C, the *S. cerevisiae* was shown poor fermentation efficiency and this phenomenon is associated with its mesophilic feature.

According to Irfan *et al.* (2014), the optimum temperature for yeast isolate to produce bioethanol from bagasse was shown as 30°C. *Kluyveromyces marxianus* is also promising yeast for the production of bioethanol at high temperature. Strains of *K. marxianus* are grown well at the temperature ranging from 45-52°C and it was shown that efficiently producing bioethanol from 38-45°C (Hosny *et al*., 2016). The variation of the optimum temperature for production of higher yield of bioethanol by yeasts was found dependent on their differences of species (Table 4; Techaparin *et al.*, 2017).

Table 4: Ethanol production at different temperature with different yeast strains.

Yeast strain	Substrate	T (°C)	Ethanol yield (g/l)	% Theoretical yield
S. cerevisiae ZM1-5	Sugarcane bagasse	40	18.79	82.35
K.marxiamus DBKKU-Y102	Jerusalem artichoke	40	97.46	92.00
K.marxiamus TISTR 5925	Palm sap	40	45.4	92.20
Blastobotrys ademinivorans RCKP2012	Sugarcane bagasse	50	14.05	46.87
P. kudriavzevii HOP-1	Rice straw	45	24.25	82.00
K.marxiamus OT-1	Jerusalem artichoke	40	73.6	90.00
S. cerevisiae JZIC	Jerusalem artichoke	40	65.2	79.70
S. cerevisiae TJ 14	Paper sludge material	42	40	74.00
K.marxiamus IMB3	Kanlow switchgrass	45	22.5	86.00
S. cerevisiae D5A	Switchgrass	37	21.9	92.00

Source: Choudhary *et al.*, (2016).

Thermotolerant yeasts are important to avoid the separate hydrolysis and fermentation processes (SHF) because it is very expensive in terms of energy and material consumptions (Choudhary *et al.*, 2016) and simultaneous saccharification and fermentation (SSF) can be applied if they are extremely thermotolerant and growing at high temperature (Table 5).

Table 5: Bioethanol yield with *S. cerevisiae* and *P. kudriavzevii* strains at 37 and 40°C.

Yeast strain	Temperature (°C)					
	37			40		
	P(g/L)	Qp (g/L/h)	Yp/s	P(g/L)	Qp(g/L/h)	Yp/s
S. cerevisiae TISTR5606	49.44±0.16d	1.03±0.00d	0.42	34.35±0.31a	0.72±0.01a	0.40
P. kudriavzevii (KKU-TH33)	42.60±0.14b	0.89±0.00b	0.40	41.82±0.49c	0.87±0.01c	0.40
P. kudriavzevii (KKU-TH43)	40.64±0.21a	0.75±0.01a	0.39	38.65±0.41b	0.81±0.01b	0.39
S. cerevisiae (KKU-VN8)	49.64±0.36d	1.03±0.01d	0.45	48.51±0.37e	1.01±0.01e	0.43
S. cerevisiae (KKU-VN20)	48.58±0.03c	1.01±0.00c	0.44	47.59±0.49de	0.99±0.01d	0.43
S. cerevisiae (KKU-VN27)	48.09±0.06c	1.00±0.00c	0.44	46.89±0.49d	0.98±0.01d	0.42

Where P= concentration of ethanol (g/L), QP= volumetric ethanol productivity (g/L/h), Yp/s= ethanol yield (g/g) and abcdef=values bearing different superscripts within the same column are significantly different using DMRT at the level of 0.05. The results also expressed as mean ± SD.

Source: Techaparin *et al.* (2017).

2.8.2. Carbon and nitrogen sources

In general, carbohydrate fraction of cane molasses is made up of primarily from glucose with small amounts of galactose and pentose fractions (Bušić *et al*., 2018). Pentose fraction is relatively significant as: xylose 5-20% and arabinose 1-5%. Xylose is the second natural abundance sugar next to glucose and it is the most abundant sugar in hemicellulose of hardwoods and crop residues (Aristos *et al*., 2012). According to Nasir *et al*. (2017), the sources of carbohydrate can determine potential of yeasts and varies their performance during fermentation to produce bioethanol. The same author indicated that *Saccharomyces cerevisiae* can utilize different carbohydrates (mostly glucose, sucrose, fructose, maltose, trehalose and xylose) anaerobically and this can be assessed by looking at the formation of gas and color change (from pink to yellow) due to the formation of acid without further treatment (Table 6). According to Basso *et al.* (2008), when cane molasses was used as substrate of fermentation using *S. cerevisiae*, the percent of bioethanol was shown as 8-11%

(v/v) and this was achieved between 6-11 hrs, at temperature of 32-35°C and 10-17% (w/v) of cell density inside the fermenter. According to Zohri *et al*. (2014), ethanol production from treated cane molasses (with 25% sugar concentration) using *S. cerevisiae* EC1118 strain was shown to be of 99.97% of fermentation efficiency at 35°C.

The *Saccharomyces* spp. are the safest and most effective microorganisms for converting sugar (hexose) to bioethanol and traditionally have been used in industry to ferment and convert glucose into bioethanol (Zabed *et al.*, 2014). Although *Saccharomyces* spp. are not able to metabolize xylose, there are yeasts such as *Pichia stipitis* and *Candida shehatae* that ferment xylose to bioethanol and use xylose for aerobic growth but they are characterized by a relative low ethanol tolerance (Aristos *et al.*, 2102). Bioethanol can be produced from corn starch using yeasts such as *S. cerevisiae, S. pastorianus*, *Schizosaccharomyces pombe* and *Kluyveromyces* spp. which are capable of metabolizing starch (Stewart, 2017). In addition to carbon source, the optimum amount of nitrogen source was mostly about 5g/l used for the growth of yeast and fermentation (Khana *et al.*, 2008).

Table 6: Source of carbohydrate and performance of yeast after fermentation.

Carbohydrate	Before fermentation	After fermentation	
	Color of medium	Color of medium	Gas production
Glucose	Pink	Yellow	Yes
Sucrose	Pink	Yellow	Yes
Maltose	Pink	Yellow	Yes
Lactose	Pink	No change	No
Fructose	Pink	Yellow	Yes
Xylose	Pink	Yellow	Yes
Trehalose	Pink	Yellow	Yes

Source: Nasir *et al.* (2017).

2.8.3. Source of substrate

Cane and beet molasses are better than other resources (plant lignocellulosic material, algae and agricultural wastes) for the production of bioethanol in terms of processes cost (Ensinas *et al.*, 2007). The cellulosic materials/bagasse require enzymatic treatment using lignin degrading enzymes in order to be used as one of the sources for the production of bioethanol. For the treatment of lignocellulose material, source of heat and heating materials, and using acid/base hydrolysis are important to degrade compact materials and to make them easily accessible by yeast to produce bioethanol.

2.8.4. pH

The optimum pH for *S. cerevisiae* is found between 4.0-5.0 (Lin and Tanaka, 2006). When the pH was below 4.0, the incubation period was extended and favored the formation of acetic acid. When the pH was above 5.0, the yield of bioethanol was subsequently diminished and favored butyric acid production (Lin *et al.*, 2012).

2.8.5. Salinity

The salinity is important factor in order to increase the conversion of sugars to bioethanol and leads to maximize the production of bioethanol (Choudhary *et al.*, 2016; Techaparin *et al.*, 2017). The *S. cerevisiae* does not tolerate high sugar and high salt concentrations in the medium. Cane molasse has the highest salt concentrations and negatively affect ethanol production. The *Schizosaccharomyces pombe* tolerates higher salt and osmotic pressure (Sánchez and Cardona, 2008).

2.8.6. Agitation and time

Bioethanol yield was also determined by agitation and incubation time. These are major determinant factor to get better quantity of bioethanol and to avoid the production of glycerol and formation of foam. Most yeast cultures during production of bioethanol were agitated at 120-150 revolution per minute (rpm) for 48 hrs. The *S. cerevisiae* strains are suitable for production of bioethanol by fermentation of sugar cane substrates (cane juice and molasses) with proper time of incubation and speed of agitation (Basso *et al.*, 2008).

2.9. Current global status of bioethanol production

The bioethanol production has increased steadily during the last three decades (Walker and Walker, 2018). The United States is the world's largest ethanol producer (from maize) with an estimated total production of 16.1 billion gallons (56%) of the total global production. Brazil is the second largest producer using sugarcane with an estimated amount of 7.95 (28%) billion gallons. Both United States and Brazil are representing 84% of the total bioethanol production in world (Table 7; Renewable Fuels Association, 2019). Globally, United States and Brazil dominating the production of biofuel and exporting to the developing countries. Scientific and technological advances such as sugar cane varieties, agricultural byproduct, fermentation process management and engineering are important to increase the efficiency of bioethanol plants in Brazil (Raghavendran *et al.*, 2017). Developing countries could alleviate the international pressure from developed countries by satisfying their energy demand by producing biofuel, reduce the need of petroleum and thereby solving the global climate crisis (ABN, 2010).

Table 7: Top bioethanol producer countries in 2018.

Country	Total production by billion gallons (%)
United States	16.10 (56)
Brazil	7.95 (28)
European Union	1.43 (5)
China	1.18 (4)
Canada	0.48 (2)
Thailand	0.39(1)
India	0.33(1)
Argentina	0.29(1)
Rest of the world	0.55(2)
Total	28.7

Source: Information from renewable fuels association (2019).

2.10. Opportunities of bioethanol production in Ethiopia

Ethiopia currently assessing the opportunities and benefits of producing biofuel and implementing biofuel strategy is crucial. Because the production and use of biofuel has huge advantages in economic and environmental conditions particularly in developing countries (Goldemberg, 2007). Currently, due to rapid expansion of developmental activities and shortage of fuels, the production of biofuel is mandatory and not a matter of choice. This is because biofuel address the pending of energy shortage and reduce the impact of climate change (Abadi Birhanu and Shimels Ayalew, 2017). In Ethiopia, sugar factories have been growing with the increment of sugar demand and in parallel the production of molasses as a byproduct will increase. Ethiopian government has a plan of constructing more than ten sugar factories throughout the country; the implementation of this plan will produce more byproducts and provide an opportunity for the production of bioethanol in ten folds (FDRE, GTPII plan).

2.11. Status of bioethanol blending with gasoline in Ethiopia

Ethiopia is an energy importer and this shows that the country has limited capacity of producing energy and needs to import energy sources from abroad in order to full fill its requirements (Martha

Negash and Swinnen, 2012). Ethiopia imports petroleum for its fuel requirements to meet its growing economy and expanding infrastructure. On the other hand, the price of petroleum is rapidly increasing (EPA, 2010). Importing fuel has been consuming over 90% of Ethiopian foreign currency and looking alternative source of energy is important to cover domestic fuel needs and to utilize it as a potential export commodity (EPA, 2010). Starting from 2009, Ethiopia has been blending ethanol with gasoline by 5% and later 10% (EPSE, 2017). Totally, 77.38 million liters of ethanol was produced and earned $51.8 million in the last 5 years by blending bioethanol with gasoline (ENA, 2015 and FDRE, GTPII plan, 2016-2020).

3. MATERIALS AND METHODS

3.1. Description of the study area

Metehara Sugar Factory is found in East Shewa Zone, Oromia Regional State, Ethiopia at 200 km distance from Addis Ababa, which is the capital of Ethiopia. It is located with latitude and longitude of 08°54'N 39°55'E with an elevation of 947m above sea level. It has maximum and minimum of annual temperature of 28.1 and 20.9°C, respectively. The annual rainfall for Metehara was 122mm and 2mm with maximum and minimum, respectively. The construction was carried out by H.V.A. Company of Netherlands in 1970. It was formed as a Share Company between Ethiopian government and Netherlands constructing company. Currently, its sugarcane plantation covers more than 10,000 hectares of land. Its average production capacity is 136,692 tons of sugar per year. The Metehara bioethanol Plant was established in 2010 and currently it can produce till 12,500m^3 ethanol per year. The factory has different storage sites for molasses, juice and sugar cane bagasse. Samples were brought from different sites and the study was conducted at General Microbiology laboratory of Addis Ababa University and Industrial Biotechnology laboratories of Addis Ababa Science and Technology University.

3.2. Sample collection

Samples were collected from different sites (molasses storage, sugar juice storage, leakage sites, different drainage sites, bagasse storage, open molasses pit and mill pit) of Metehara Sugar Factory (Table 8). A total of 35 samples of sugary substrate were collected using sterilized bottles, tubes and polyethylene bags kept in ice box and then transported to General Microbiology Laboratory of Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. All the samples were labeled and kept at 4°C till processing.

Table 8: Parameters checked during sample collection.

			Season			Meta data	
S.No.	Sample name	Sample source	Summer	Winter	Total	Temp(°C)	pH
1	Mill juice	Mill juice tanker	2	2	4	27	4.9
2	Leakage	Tandem A mill	2	2	4	35	4.8
3	Leakage	Tandem B mill	2	2	4	42	4.7
4	Fine bagasse	Fine bagasse	1	1	2	28	6.7
5	Molasse	Molasses pit	1	1	2	27	6.2
6	Mixed juice	Juice in different tanker	2	2	4	26	4.7
7	Open molasses	Open pit molasses	1	1	2	26	4.8
8	Prepared cane	Crashed cane	1	1	2	27	6.9
9	Storage pit	Storage pit edge	1	1	2	27	4.7
10	Juice drainage	Drainage sites	1	1	2	26	4.5
11	Mill pit	Mill pit tanker	2	2	4	26	9.8
12	Soil sample	Surface soil near molasses storage tanker	1	1	2	26	4.2
13	Mud	Base of big tanker	0	1	1	26	4.3
	Total		17	18	35		

3.3. Medium preparation for yeast isolation

The medium for yeast isolation and propagation was prepared as Yeast Malt Extract Peptone Dextrose (YMPD) agar from (10g/l of yeast extract (Sisco), 10g/l of malt extract (Tmmedia), 20g/l of peptone (Acumix), 20g/l of dextrose (Blulux), 0.1g/l of chloramphenicol and 20g/l of agar (Himedia)) (Techaparin *et al.*, 2017). The pH of the medium for isolation and characterization except for pH tolerance test was adjusted to 4.7 using 1N HCl and 1N NaOH. The yeast malt extract medium (YMPD for broth or YMPDA for solid medium) were prepared by sterilized using autoclave at 121°C for 20 minutes for 15 lbs. pressure. Chloramphenicol (0.1g/l) was added into sterilized and cooled medium (hand touched). The medium was well mixed and poured (about 20ml amount) into sterilized plates. The plates were left overnight at room temperature inside clean safety cabinet to check for any contamination.

3.4. Sample processing, isolation and purification of yeast isolates

For isolation of yeasts from various sites of factory separately, the liquid samples were serially diluted with sterilized distilled water (1:9 ratio) whereas the solid samples were prepared into fine powder form using pestle and mortar. According to Nasir *et al.* (2017), each of the solid sample, 25g from a separate solid sample was mixed with sterile 225ml of broth medium composed of 3g/l of yeast extract, 3g/l of malt extract, 5g/l of peptone, 5g/l of dextrose and 0.1g/l of chloramphenicol in 300ml of flasks in order to enrich and maximize the number of yeasts cells. The culture was incubated in shaker incubator (130 revolution per minute) overnight at 30 °C. Each separate sample was serially diluted and appropriate aliquot (0.1ml) was taken and spread on sterilized pre-dried plates. The inoculated plates were incubated for 48 hrs at 30°C. The colonies grown on YMPDA plate were counted and expressed as log CFU/ml.

Colony counts (CFU/ml) = Number of colonies/ volumes of inoculum*dilution factor.

The colonies (5-10) were taken based on their morphology from each YMPDA plates (sample) depending on the number and appearance of the colonies and transferred to newly prepared solid medium for purification.

3.4.1. Morphological characteristics of yeast isolates

The purified yeast isolates were gram stained and examined using 100x of compound microscope (Ceti, Belgium) using oil immersion. The cultural characteristics (size, surface and color of yeast colonies) were also determined on solid YMPDA plates.

3.5. Stress tolerances of the yeast isolates

3.5.1. Ethanol tolerant yeast isolates

Each of the purified isolate was grown in 5 ml of sterile YMPD broth medium in test tubes as an active culture. The volumes of ethanol used for ethanol tolerance test were 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.05 ml of 99.8% of ethanol. Each separately specified volume was added into newly sterilized YMPD broth (in different volume) in test tubes to form 4, 6, 8, 10, 12, 14, 16, 18, 20 and 21% of ethanol. Finally, the 5ml of ethanol enriched YMPD broth was formed. Each yeast isolate (estimated to $1x10^7$ cells/ml) was inoculated and grown in 5ml of ethanol containing YMPD broth at 30°C for 24 and 48 hrs. After 24 and 48 hrs of incubation at 30°C, each yeast isolate from respective percent of ethanol was separately streaked on fresh YMPDA medium to check the tolerance yeasts with the given ethanol concentration. The survival of yeast isolates (optical density) at 18 and 20% of ethanol was examined using UV-Spectrophotometer (Jenway, UK). According to Fadel *et al*. (2013), the ethanol tolerant yeast isolates were divided into three categories as highly tolerant (>50% survival), moderately tolerant (20-50% survival) and slightly tolerant (<20% survival) at the highest percent of ethanol. The best tolerant (medium to high) yeast isolates at 18% of ethanol were considered for temperature tolerance test. These top tolerant yeast isolates were preserved in 30% glycerol at -18°C.

3.5.2. Thermotolerant yeast isolates

The ethanol tolerant yeast isolates (tolerant at 18%) were taken (0.1ml) for their thermotolerance potential by growing separately in 5ml of sterile YMPD broth medium and incubated at 25, 35, 37, 40, 42, 45, 46.5 and 47.5°C for 48 hrs. After 48 hrs of incubation, each of the separate culture was grown onto fresh medium (YMPDA) to check the tolerance (Techaparin *et al.*, 2017). The yeast isolates grown at 45°C were considered as thermotolerant and were used for further investigations.

3.5.3. Carbohydrate utilization and gas production test for yeast isolates

Each thermotolerant yeast isolate (previously survived at 45°C) was inoculated separately into different carbohydrates and checked their fermenting potentials. The substrates used for carbohydrate utilization test were glucose, fructose and sucrose each at 10% concentration while galactose, maltose, trehalose, dulcitol, starch, lactose and xylose each at 2% concentration. The carbohydrates were prepared 20% (w/v) as stock and 2.5ml and 0.5ml was taken and added into 2.5 and 4.5ml of sterile basal broth to form 5ml of working medium for 10 and 2% (v/v), respectively. The carbohydrates were sterilized separately using membrane filtration (0.45µm pore size). The yeast extract peptone basal medium (YEP) contained 10g/l of yeast extract, 20g/l of peptone and 0.018g/l phenol red and its pH was being adjusted at 4.7. The inverted Durham tubes were incorporated into the basal medium before autoclaving to check for gas production. The overnight yeast culture suspension (0.1ml) was inoculated into basal medium that contained 2 and/or 10% of the aforementioned carbohydrates. After inoculating the yeast suspension, the test tubes were incubated at 30°C for 24, 48 and 72 hrs

3.5.4. Sugar tolerance test for yeast isolates

Based on the dominant abundance of sugars in molasses: sucrose, fructose and glucose were selected and the test was done in different percent using those sugars. The sterile basal medium (yeast extract peptone) contained 10g/l of yeast extract, 20g/l of peptone and 0.018g/l phenol red and the pH was adjusted at 4.7. Each of the ethanol-thermotolerant yeast isolate was separately inoculated into the medium containing different percent of sugars (10, 15, 20, 25, 30, 35 and 40% v/v) from each stock solution (60% w/v) of sucrose, fructose and glucose. The sugars were membrane sterilized (0.45µm pore size) and added into sterile basal medium. The overnight culture (0.1ml) was inoculated into 10ml of medium containing different percent of aforementioned sugars using test tubes. After inoculation, test tubes were incubated at 30°C for 48 hrs and color change and gas production were checked for each isolate (Sundarrajan *et al.*, 2016).

3.5.5. Acid tolerance of yeast isolates

Each ethanol- thermo-sugar tolerant yeast isolates were checked for their acid tolerance in sterile 5ml of YMPD broth and the pH values were adjusted to 1.5, 2, 2.5, 3.0 and 3.5 using 1N HCl and 1N NaOH. The overnight growth (0.1ml) of each yeast isolate was inoculated and incubated at 30°C for 24 hrs separately. After 24 hrs of incubation, a loopful of culture was taken from each test tube and streak plated on fresh pre-dried plates of YMPDA and incubated for 24 hrs at 30°C to check the tolerance (Taye Negera, 2017).

3.5.6. Salt tolerance of yeast isolates

The ability of yeast isolates to tolerate different concentration of NaCl was determined following the protocol indicated in Vinderola and Reinheimer (2003). The sterile YMPD broth (5ml) that contained different concentration of NaCl (0, 4, 7 and 10% w/v) was prepared. The overnight yeast growth (0.1ml) was separately inoculated using test tubes and incubated at 30°C. The optical density was read using UV- Spectrophotometer at 600 nm (Jenway, UK) at 0, 24, 48 and 72 hrs.

3.6. Identification of potent yeast isolates

The potent yeast isolates with higher bioethanol production were identified at species level using morphological and biochemical tests of YT microplates (Biolog Inc., Hayward, CA, USA) supplied with different carbohydrates and other substrates in “Feed and Microbiology laboratory of Animal Products, Veterinary Drug and Feed Quality Assessment Center, Addis Ababa, Ethiopia” following the specific protocol given by the company “BIOLOG Inc.”. The overnight active culture was grown in YPD broth and transferred into solid YPDA plates. The cultures were incubated at 28°C for 48 hrs. The colony of each isolate was transferred into Universal Biolog Yeast Agar medium (UBYA) and incubated for 72 hrs at 28°C. The colony was taken into sterilized distilled water (10ml) and shake to adjust its turbidity against the standard (47%) using Turbidimeter (Biolog Inc., Hayward, CA, USA). The yeast suspension (100µl) was inoculated into substrates of YT microplates (96 wells) using multiple chambers of micropipette, incubated at 28°C for 72 hrs and read each microplate using MicroStation (Biolog Inc., Hayward, CA, USA) with Micro log secure 6.2 software. The similarity index is 0.500 and must be above this to confirm the given species.

3.7. Determination of degree brix and pH of raw molasses

The degree brix of raw molasses (°Bx) was determined by using digital refractometer (ATAGO, Japan) found in Industrial Biotechnology and Fermentation Laboratory, at Department of Biotechnology, Addis Ababa Science and Technology University following the protocol given in Mamudu and Olukanmi (2019). Briefly the protocol can be summarized as in a pre-dried clean 1lites (Ls) capacity beaker, 100ml of molasses sample was measured using measuring cylinder and 100 ml of distilled water was added to make 200ml of total volume (1:1). Thereafter, the mixture was stirred with magnetic stirrer to homogenize completely. The homogenized suspension was taken by micropipette to refractometer (ATAGO, Japan) at 25°C and the brix reading was taken and recorded. The refractometric recorded value was multiplied by the dilution factor (2) to represent the brix of raw molasses. The pH of raw molasses was also determined.

3.8. Pre-treatment of molasses

The pre-treatment of molasses was performed using acid-hot treatment with decantation method following the protocol given in Mamudu and Olukanmi (2019). Briefly, 500ml of molasses was measured and transferred into 2Ls of beaker. Distilled water was added and mixed well till the required brix became adjusted. Thereafter, 5g/l of powder DAP (diammonium phosphate) was added into diluted molasses and again well mixed. Then, the pH of mixed was adjusted to 4.7 using concentrated H_2SO_4 (98%) and 1N NaOH drop by drop. The pre-treated molasses was autoclaved at 121°C for 15 minutes for 15lbs. The sterilized molasses was placed at room temperature in safety cabinet overnight and then decanted into newly sterilized flasks.

3.9. Fermentation of molasses for bioethanol production

3.9.1. Inoculum preparation

Each of the selected yeast isolate (ethanol-thermo-acid- and- salt tolerant and those with better fermenting capability of sugars) was separately activated by growing each isolate overnight in 20ml of sterile YMPD broth with pH adjusted to 4.7 in 50ml of Erlenmeyer flasks and incubated at 30°C on a shaker incubator (150 rpm of agitation) (Wis-10R, Korea). The overnight inoculum yeast culture (1ml) was counted and it was in the range of (2.0 to 3.5) x10^8 cells/ml. The inoculum yeast cells were counted by compound microscope (Labomed, USA) with the help of hematocytometer (Counting Chambers, China). The overnight broth culture (1ml) was taken and added into 1ml of 1% of methylene blue solution and distilled water was added till the final volume became 100ml. After that, the suspension (1ml) was taken and added on quadrantes of hematocytometer (drop by drop) and covered by cover slip to count live cells using 40x of compound microscope (Labomed, USA).

3.9.2. Propagation of yeast isolates

Fed-batch fermentation mode was applied for propagation and experiment was done in duplicate. Each yeast culture (1ml) was transferred into 10°Bx of 100ml of pre-treated diluted molasses in 300ml capacity flasks inside safety cabinet. The pH was adjusted at 4.7 before autoclaved and inoculum transfer. After transfer of the culture, it was incubated on shaker (Wis-10R, Korea) at 30°C (150 rpm agitation) for 24 hrs. After 24 hrs, the fermenting molasses cultures (propagated) were collected through sample valves to analyze pH and °Bx. Then, the remain propagated yeast cultures were transferred into 500ml capacity flasks that contained 100ml of pre-treated and sterile molasses of 15°Bx and again incubated on shaker (Wis-10R, Korea) for 24 hrs at 30°C (150 rpm agitation). Similarly, the fermenting cultures were taken from propagated cultures through sample valves to analyze pH and °Bx. A 200 ml yeast cells were propagated as a final volume with the inoculum size of 13.3%. The final propagated yeast cells were also counted by compound microscope (Labomed, USA) with help of hematocytometer with duplicates as mentioned 3.9.1.

3.9.3. Fermentation process for bioethanol production

Batch fermentation system was employed for production of bioethanol during fermentation in duplicate. The cotton plugged flasks were sterilized using autoclave at 121°C for 15minutes at 15 lbs. The propagated culture (200ml) was transferred into 2Ls of fermentation flasks that contained 1300ml of fresh pre-treated 30°Bx of molasses with 4.7 pH inside safety cabinet and incubated on shaker (Wis-10R, Korea) at 30°C (150 rpm agitation) for 24, 48 and 72 hrs. During fermentation, the total volume was 1500ml in 2Ls of flasks for each yeast isolate and closed using sterilized cotton, aluminum foil and parafilm to be anaerobic.

3.9.3.1. Analytical methods

The fermented mash (sample) was taken to analyze the brix of fermented molasses, percent of alcohol, pH, residual sugars and cell density. Brix of fermented molasses was determined by refractometer (ATAGO, Japan) at 24, 48 and 72 hrs. The pH for each fermented molasses was determined at 24, 48 and 72 hrs. The residual sugar (RS) during fermentation was determined using Dinitrosalycylic acid (DNS) method (Miller *et al*., 1959) for similar fermented samples at 24, 48 and 72 hrs. The cell density was also determined using 40x compound microscope with the help of hematocytometer at 24, 48 and 72 hrs as mentioned 3.9.1. The percent of alcohol (% v/v) that was produced during fermentation was measured using electronic Ebulliometer (EON, USA) at 24, 48 and 72 hrs incubation period. The fermented mash (50ml) inserted into the column of Ebulliometer. After boiling, measure the percent of alcohol content against the boiling of water.

The fermentation efficiency was calculated as:

$C_6H_{12}O_6 \xrightarrow{\hspace{3cm}} C_2H_5OH + 2CO_2 + \text{Heat}$------------------------------------Equation 1.

180 92 88 (Molecular weight)

180g of glucose = 92g of ethyl alcohol and 1g of glucose= 0.511g of ethyl alcohol.

Density = mass of ethanol / volume of ethanol --Equation 2

The specific gravity of ethyl alcohol is 0.7936.

Volume of ethyl alcohol= mass of ethyl alcohol/ Density---------------------------------Equation 3.

Volume of ethyl alcohol=0.511g / 0.7936g/l =0.644 liter of ethanol can be produced.

Theoretical yield (%) = FS (%) * 0.644---Equation 4.

Fermentation efficiency (FE)= actual yield (%) / theoretical yield (%) *100----------Equation 5.

3.10. Data analysis

All experiments were carried out using complete randomized design (CRD). The experiments were done in duplicate. The generated data were analyzed using General linear model of multivariate analysis among the treatment means at 5% of level of significance and means were compared using Duncan multiple range test (DMRT) by SPSS version 29 and presented using tables and figures.

4. RESULTS

4.1. Results

4.1.1. Isolation and purification of yeast isolates

A total of 305 representative yeast isolates were retrieved (Appendix 1). Regarding the load, the total yeast counts of the samples ranged from log 5.53 CFU/ml (sample TB) to log 6.45 CFU/ml (sample PC) (Table 9).

Table 9: Colony counts of the samples collected from different sites of Metehara Sugar Factory.

S.No.	Isolates	log (CFU/ml)	S.No.	Isolates	log (CFU/ml)
1	1MJ2	6.2	19	TA	6.14
2	TA1m	6.23	20	5M	6.16
3	TB	5.53	21	MP	6.15
4	1Ba	6.09	22	OP	6.05
5	1CJ	6.43	23	TA2	5.92
6	1MJ1	6.16	24	TA3	6.00
7	2TM1	6.17	25	4M	6.43
8	JTM2	6.22	26	1MP	6.43
9	2TM2	6.37	27	MJ	6.32
10	3MJ1	6.41	28	PC	6.45
11	MJ1	6.14	29	Ba	5.92
12	FB	6.08	30	CJ	5.94
13	JD	5.98	31	SPE	6.27
14	BS	5.94	32	LMT	6.11
15	MBBT	6.33	33	MMT	6.19
16	BMT	6.29	34	NSB	5.89
17	SST	5.81	35	DS	5.91
18	BMST	5.75			

Where 1MJ2=first mill juice2, TA1m=tandem A leakage first mill, TB=tandem B leakage, Ba= bagasse, CJ=clear juice, 1MJ1=first mill juice1, 2TM1=second tank mill1, JTM2= juice tanker mill2, 2TM2=second tank mill2, 3MJ1=third mill juice1, MJ1= mixed juice1, FB= fine bagasse, TA=tandem A, 5M= fifth mill juice tanker, MP=molasses pit before 2002G.C., OP=open pit before 2002G.C., TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, 4M=fourth mill juice tanker, 1MP=first mill pit, MJ=mixed juice and PC=prepared cane, JD=juice drainage, BS=bagasse storage, MBBT=mud from the base of big tanker, BMT=big molasses tanker, SPE=storage pit edge, LMT=left molasses tanker, MMT=medium molasses tanker, NSB= non stored bagasse, SST=soil sample near storage tanker, BMST=bottom of molasses storage tanker and DS= drainage site on the ground.

4.1.2. Morphological characteristics of yeast isolates

Of the total (305) yeast isolates, 33 yeast isolates were screened and found to be tolerant to 18% of ethanol and examined their cultural (colonies morphology, size, surface and color) and morphological (gram staining) characteristics of the purified isolates for further activities (Table 10 and Fig. 2). The macroscopic features of all these yeast isolates were shown to be raised, rough, smooth or flat and confirmed to be yeasts. The size of yeast colonies was small, medium and large (Table 10). Almost all of the screened purified yeast isolates from different samples were found to be oval in shape and gram positive under the microscope (Fig. 2).

Table 10: Morphological characteristics of yeast isolates.

		Macroscopic characteristics							
S. No.	Isolates	Colony color	surface	Size	S. No.	Isolates	Colony color	Size	Surface
1	TA2	White	Raised	M	18	SST	Creamy	M	Raised
2	TA3	White	Smooth	M	19	BS	Creamy	L	Smooth
3	TA4	White	Rough	M	20	MMT	Creamy	M	Raised
4	TA3$_{7}$	White	Smooth	M	21	NSB	Creamy	M	Rough
5	5M2	White	Smooth	L	22	MBBT1	White	L	Raised
6	5M3	White	Smooth	L	23	MBBT2	White	M	Smooth
7	5M9	White	Smooth	L	24	MBBT3	White	S	Smooth
8	OP6	Creamy	Rough	L	25	MBBT4	White	M	Smooth
9	4M8	White	Smooth	L	26	BMT	Creamy	S	Smooth
10	4M9	White	Smooth	M	27	SPE	White	L	Rough
11	4M10	White	Raised	M	28	LMT	White	L	Rough
12	MJ9	White	Smooth	M	29	BMMT	Creamy	L	Raised
13	MJ10	White	Smooth	M	30	DS	White	L	Smooth
14	Ba5	Creamy	Rough	L	31	5M1	White	L	Raised
15	Ba8	Creamy	Rough	L	32	OP1	Creamy	L	Raised
16	TB	Creamy	Raised	L	33	TA5	Creamy	M	Raised
17	2TM2	Creamy	Rough	L					

Where L= large colony, M= medium colony and S= small colony, 1MJ2=first mill juice2,TA1=tandem A leakage first mill, TB=tandem B leakage, Ba= bagasse, CJ=clear juice, 1MJ1=first mill juice1, 2TM1=second tank mill1, JTM2= juice tanker mill2, 2TM2=second tank mill2, 3MJ1=third mill juice1, MJ1= mixed juice1, FB= fine bagasse, TA=tandem A, 5M= fifth mill juice tanker, MP=molasses pit before 2002G.C., OP=open pit before 2002G.C., TA2=

tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, 4M=fourth mill juice tanker, 1MP=first mill pit, MJ=mixed juice and PC=prepared cane, JD=juice drainage, BS=bagasse storage, MBBT=mud from the base of big tanker, BMT=big molasses tanker, SPE=storage pit edge, LMT=left molasses tanker, MMT=medium molasses tanker, NSB= non stored bagasse, SST=soil sample near storage tanker, BMST=bottom of molasses storage tanker and DS= drainage site on the ground.

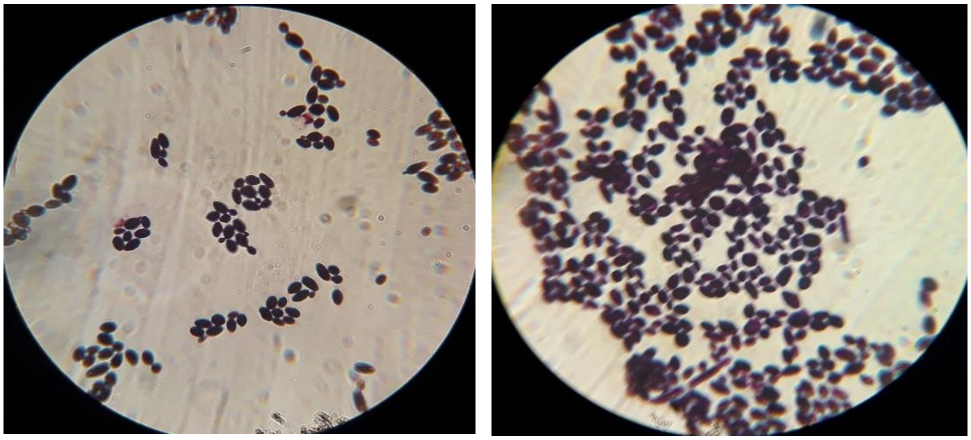


Figure 2: Microscopic morphology of the potent yeast isolates.

4.1.3. Screening ethanol tolerance of yeast isolates

The yeast isolates (n=305) tested for tolerance to ethanol (99.8%) at different concentrations (4 to 21% v/v) (Table 11 and Appendix 2). Accordingly, 100% (all the isolates), 85.90% (261 isolates), 69.51% (212 isolates), 33.44% (102 isolates), 26.23% (80 isolates), 10.82% (33 isolates) and 2.29% (7 isolates) were found to be tolerant to 4-8%, 10%, 12%, 14%, 16%, 18% and 20% of ethanol concentration (Appendix 2). None of the isolate was shown to tolerant to 21% of ethanol. Yeast isolates that tolerated 18% of ethanol and beyond were considered for further analysis as ethanol tolerant isolates. Of these 33 ethanol tolerant isolates, the survival of the 20 yeast isolates were found ranging from medium to high survival rate whereas the remaining 13 yeast isolates were characterized as low survival rate at 18% of ethanol. On the other hand, the survival rate of 7 yeast isolates were found as medium whereas the remaining 26 yeast isolates did not survive at 20 % of ethanol. The 7 yeast isolates those having medium survival rate at 20% ethanol were TA2, TA3, TA4, TA37, 4M8, 4M9 and 4M10 (Appendix 3).

Table 11: Ethanol tolerance of the yeast isolates.

Isolates	4%	6%	8%	10%	12%	14%	16%	18%	20%	21%
TA2	++++	++++	++++	++++	++++	+++	+++	+++	++	-
TA3	++++	++++	++++	++++	++++	+++	+++	+++	++	-
TA4	++++	++++	++++	++++	++++	+++	+++	+++	++	-
TA3$_7$	++++	++++	++++	++++	++++	+++	+++	+++	++	-
5M2	++++	++++	++++	++++	++++	+++	+++	+	-	-
5M3	++++	++++	++++	++++	++++	+++	+++	++	-	-
5M9	++++	++++	++++	++++	++++	+++	+++	++	-	-
OP6	++++	++++	++++	++++	++++	+++	+++	++	-	-
4M8	++++	++++	++++	++++	++++	+++	+++	++	++	-
4M9	++++	++++	++++	++++	++++	+++	+++	++	++	-
4M10	++++	++++	++++	++++	++++	+++	+++	+++	++	-
MJ9	++++	++++	++++	++++	++++	+++	+++	++	-	-
MJ10	++++	++++	++++	++++	++++	+++	+++	++	-	-
Ba5	++++	++++	++++	++++	++++	+++	+++	++	-	-
Ba8	++++	++++	++++	++++	++++	+++	+++	+	-	-
TB	++++	++++	++++	++++	++++	+++	+++	+	-	-
2TM2	++++	++++	++++	++++	++++	+++	+++	++	-	-
SST	++++	++++	++++	++++	++++	+++	+++	+	-	-
BS	++++	++++	++++	++++	++++	+++	+++	+	-	-
MMT	++++	++++	++++	++++	++++	+++	+++	+	-	-
NSB	++++	++++	++++	++++	++++	+++	+++	+	-	-
MBBT1	++++	++++	++++	++++	++++	+++	+++	++	-	-
MBBT2	++++	++++	++++	++++	++++	+++	+++	++	-	-
MBBT3	++++	++++	++++	++++	++++	+++	+++	++	-	-
MBBT4	++++	++++	++++	++++	++++	+++	+++	++	-	-
BMT	++++	++++	++++	++++	++++	+++	+++	++	-	-
SPE	++++	++++	++++	++++	++++	+++	+++	++	-	-
LMT	++++	++++	++++	++++	++++	+++	+++	+	-	-
BMMT	++++	++++	++++	++++	++++	+++	+++	+	-	-
DS	++++	++++	++++	++++	++++	+++	+++	+	-	-
5M1	++++	++++	++++	++++	++++	+++	+++	+	-	-
OP1	++++	++++	++++	++++	++++	+++	+++	+	-	-
TA5	++++	++++	++++	++++	++++	+++	+++	++	-	-

Where 1MJ2=first mill juice2, TA1=tandem A leakage first mill, TB=tandem B leakage, Ba= bagasse, CJ=clear juice, 1MJ1=first mill juice1, 2TM1=second tank mill1, JTM2= juice tanker mill2, 2TM2=second tank mill2, 3MJ1=third mill juice1, MJ1= mixed juice1, FB= fine bagasse, TA=tandem A, 5M= fifth mill juice tanker, MP=molasses pit before 2002G.C., OP=open pit before 2002G.C., TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, 4M=fourth mill juice tanker, 1MP=first mill pit, MJ=mixed juice and PC=prepared cane, JD=juice drainage, BS=bagasse storage, MBBT=mud from the base of big tanker, BMT=big molasses tanker, SPE=storage pit edge, LMT=left molasses tanker, MMT=medium molasses tanker, NSB= non stored bagasse, SST=soil sample near storage tanker and DS= drainage site on the ground. The ++++= very high growth, +++= high growth, ++= medium growth, += low growth and - =no growth.

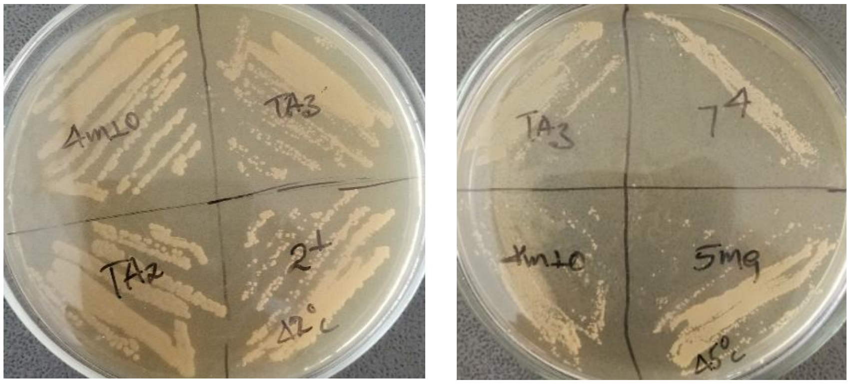
4.1.4. Temperature tolerance of yeast isolates

From 18% of ethanol tolerant yeast isolates, 20 yeast tolerated 25, 35, 37 and 40°C, 18 yeast isolates tolerated 42°C, 17 yeast isolates tolerated 45°C and 2 yeast isolates tolerated 46.5°C. None of the yeast isolate was found tolerant at 47.5°C (Table 12 and Fig. 3).

Table 12: Temperature tolerance of the yeast isolates.

Isolates	25°C	30°C	35°C	37°C	40°C	42°C	45°C	46.5°C	47.5°C
TA2	++	++++	++++	++++	+++	++	++	-	-
TA3	++	++++	++++	++++	+++	++	++	-	-
TA4	++	++++	++++	++++	+++	++	++	-	-
TA37	++	++++	++++	++++	+++	++	++	-	-
5M3	++	++++	++++	++++	+++	++	++	-	-
5M9	++	++++	++++	++++	+++	++	++	-	-
OP6	++	++++	++++	++++	+++	++	++	+	-
4M8	++	++++	++++	++++	+++	++	++	-	-
4M9	++	++++	++++	++++	++	-	-	-	-
4M10	++	++++	++++	++++	+++	++	++	-	-
MJ9	++	++++	++++	++++	+++	++	++	-	-
MJ10	++	++++	++++	++++	+++	++	++	-	-
Ba5	++	++++	++++	++++	+++	++	++	+	-
2TM2	++	++++	++++	++++	+++	++	++	-	-
MBBT1	++	++++	++++	++++	+++	++	++	-	-
MBBT2	++	++++	++++	++++	+++	++	++	-	-
MBBT3	++	++++	++++	++++	+++	++	-	-	-
MBBT4	++	++++	++++	++++	+++	++	+	-	-
BMT	++	++++	++++	++++	++	-	-	-	-
SPE	++	++++	++++	++++	+++	++	+	-	-

Where TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, TA4=tandem A, 5M= fifth mill juice tanker, OP=open pit before 2002G.C., 4M=fourth mill juice tanker, MJ=mixed juice, Ba= bagasse, 2TM2=second tank mill2, MBBT=mud from the base of big tanker, BMT=big molasses tanker and SPE=storage pit edge. The ++++= very high growth, +++= high growth, ++= medium growth, += low growth and - =no growth.



Where 7^4=MBBT4 and 2^1= SPE.

Figure 3: Thermotolerant yeast isolates on YMPD agar medium at 42°C and 45°C respectively.

4.1.5. Carbohydrate fermentation and gas production of yeast isolates

All of the 17 yeast isolates (those tolerant to 18% ethanol and 45°C) were considered for carbohydrate fermentation test. The ethanol-and-temperature tolerant yeast isolates were found utilize 10% sugars (glucose, fructose and sucrose) and produced gas (Fig. 4). From a total of 17 thermotolerant yeast isolates, 5 (29.41%) yeast isolates (TA2, TA3, TA3$_{7}$, 4M10 and MBBT1) were able to utilized sugars (glucose, fructose and sucrose) and produced gas at 24 hrs; the other 5 (29.41%) yeast isolates (5M3, 4M8, MJ10, 5M9 and TA4) utilized sugars and produced gas at 48 hrs and the remain 7 (41.18%) yeast isolates (MBBT4, OP6, Ba5, MBBT2, MJ9, SPE and 2TM2) utilized sugars and produced gas at 72 hrs (Fig. 4).

Figure 4: Sugar utilization and gas production test.

From 17 ethanol-and-temperature tolerant yeast isolates, all of them were found to utilize effectively 2% of galactose and maltose. The 6 yeast isolates (TA4, OP6, 2TM2, MBBT4, SPE and MBBT1) were shown to utilize all the given 2% of carbohydrates (Table 13). It was observed that all the 17 yeast isolates were observed utilize trehalose except Ba5. It was observed that isolate Ba5 did not ferment 2% of the 5 carbohydrates (starch, dulcitol, lactose, trehalose and xylose) (Appendix 9). Isolate TA3 was also found unable to ferment lactose and xylose. Isolates 5M9 and MJ10 did not ferment starch, dulcitol, lactose and xylose (Table 13).

Table 13: Carbohydrate fermentation test for thermotolerant yeast isolates.

Isolate	Gal.	Star.	Dul.	Lac.	Mal.	Tre.	Xyl.	Isolate	Gal.	Star.	Dul.	Lac.	Mal.	Tre.	Xyl.
TA4	++	+	+	+	++	+	+	MBBT4	++	+	+	+	++	++	+
4M10	++	-	-	-	++	++	+	MJ10	++	-	-	-	++	+	-
OP6	++	+	+	+	++	++	+	MBBT2	++	-	-	+	++	+	+
5M3	++	+	-	-	++	+	-	SPE	++	+	+	+	++	++	+
4M8	++	+	-	-	++	++	-	MBBT1	++	+	+	+	++	+	+
5M9	++	-	-	-	++	+	-	MJ9	++	-	-	-	++	+	-
TA2	++	-	-	-	++	++	+	Ba5	++	-	-	-	++	-	-
TA3	++	+	-	-	++	+	-	2TM2	++	+	+	+	++	+	+
TA3$_7$	++	+	+	-	++	+	-								

Where TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, TA4=tandem A, 5M= fifth mill juice tanker, OP=open pit before 2002G.C., 4M=fourth mill juice tanker, MJ=mixed juice, Ba= bagasse, 2TM2=second tank mill2, MBBT=mud from the base of big tanker and SPE=storage pit edge and gal.= galactose, star.= starch, dul.=dulcitol, lac.= lactose, mal.= maltose, tre.= trehalose and xyl.= xylose. The signs ++= yellow color and gas production, + = indicates color change to yellow only and - = no color change and gas production that incubated till 72 hrs.

4. 1.6. Sugar tolerance of yeast isolates

Almost all of the ethanol-and-temperature tolerant yeast isolates were found effectively utilized and tolerant 10 to 25% of sugars (glucose, fructose and sucrose), whereas isolates MBBT2 and MJ10 were unable to utilized 25% of sucrose and fructose, respectively. Isolates MJ10 and Ba5 failed to utilize 30% sugars and above. The yeast isolates 4M10, TA2, TA3, TA3$_7$ and MBBT1 were exhibited tolerance to 35% of all the 3 sugars without gas production (Table 14). There was no isolate tolerated 40% of sugars. Of the total of 17 ethanol-and temperature tolerant yeast isolates, the 12 yeast isolates that tolerated 35% of one or more sugars were selected for further analysis (Table 14).

Table 14: Sugar tolerance test at different concentration.

	sugars utilization and tolerance test																				
	10%			15%			20%			25%			30%			35%			40%		
Isolates	Glu	Fru.	Suc	Glu	Fru.	Suc	Glu	Fru.	Suc	Glu	Fru.	Suc	Glu	Fru.	Suc	Glu	Fru.	Suc	Glu	Fru	Suc
TA4	++	++	++	++	++	++	++	++	++	++	+	+	++	-	-	+	-	-	-	-	-
4M10	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-
OP6	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-	-	-	-	-	-
5M3	++	++	++	++	++	++	++	++	++	++	+	+	++	+	-	+	-	-	-	-	-
4M8	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	-	-	-	-	-
5M9	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	-	-	-	-	-
TA2	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-
TA3	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-
TA3₇	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-
2TM2	++	++	++	++	++	++	++	++	++	++	++	+	+	-	-	-	-	-	-	-	-
MBBT1	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	-
MJ9	++	++	++	++	++	++	+++	++	++	++	+	++	+	-	-	+	-	-	-	-	-
MBBT2	++	++	++	++	++	++	++	++	++	++	+	-	+	-	-	-	-	-	-	-	-
SPE	++	++	++	++	++	++	++	++	++	++	++	+	+	-	+	+	-	-	-	-	-
MBBT4	++	++	++	++	++	++	++	++	++	++	+	+	+	-	-	+	-	-	-	-	-
MJ10	++	++	++	++	++	++	++	+	++	++	-	+	+	-	-	-	-	-	-	-	-
Ba5	++	++	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

Where TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, TA4=tandem A, 5M= fifth mill juice tanker, OP=open pit before 2002G.C., 4M=fourth mill juice tanker, MJ=mixed juice, 2TM2=second tank mill2, MBBT=mud from the base of big tanker, SPE=storage pit edges and Ba= bagasse. The representation shows, glu= glucose, fru= fructose and suc= sucrose. The description indicates gas production and the color change from pink/phenolic to yellow due to acid production during utilization of sugars in different concentration. The ++ = gas produce and yellow color, += the color change only (yellow) and - = indicates that no gas production in Durham tube and no color change in 72 hrs of incubation time.

4.1.7. Acid tolerance of yeast isolates

From a total of 12 yeast isolates that tolerated 35% of glucose, the 7 of them tolerated pH 2 (Table 15). However, the 5 yeast isolates TA4, 4M8, MJ9, MBBT4 and SPE failed to tolerate pH 2. Isolate MJ9 was also unable to tolerate pH 2.5 (Table 15). The yeast isolates that tolerated at pH 2 were 4M10, 5M3, 5M9, TA2, TA3, TA3₇ and MBBT4 and considered for further investigations.

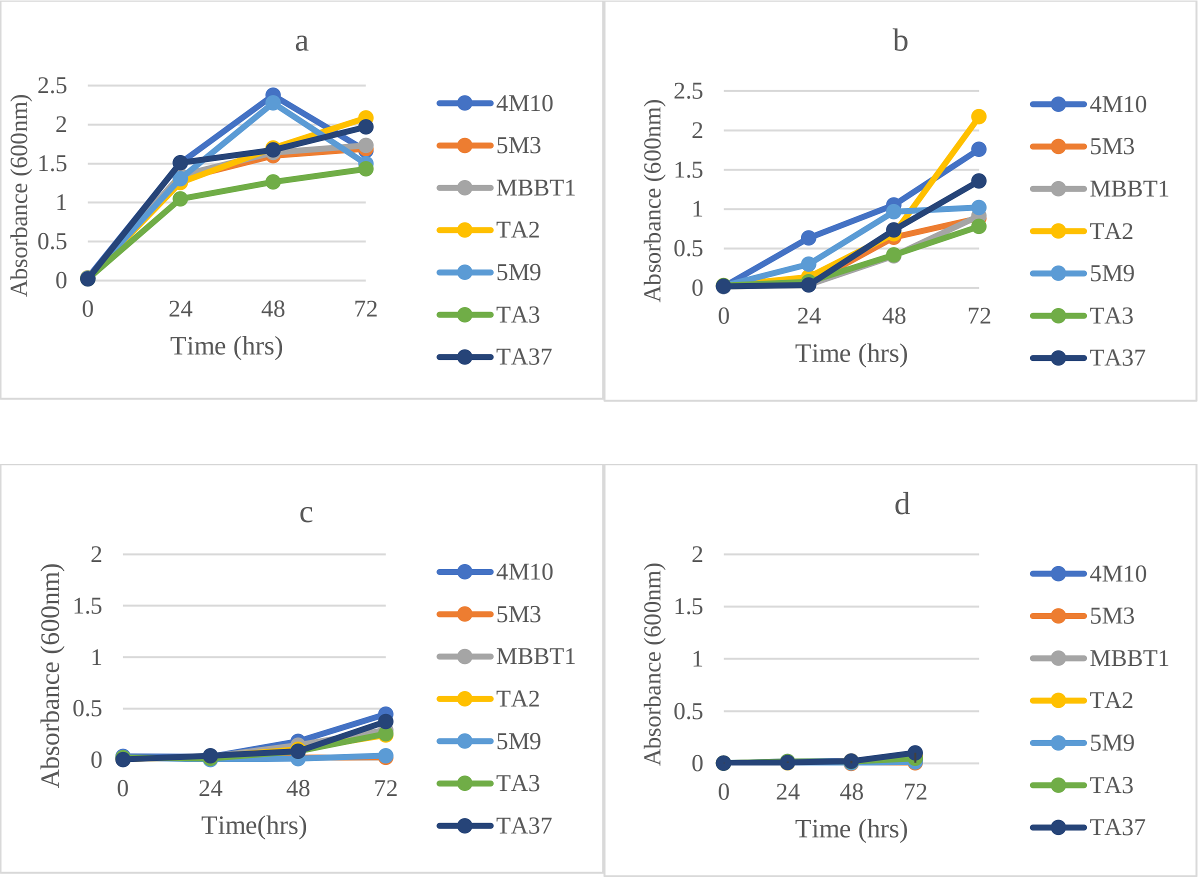
Table 15: Acid tolerance test for yeast isolates.

Isolates	pH1.5	pH2	pH2.5	pH3	pH3.5	Isolates	pH1.5	pH2	pH2.5	pH3	pH3.5
TA4	-	-	+	+	+	TA3	-	+	+	+	+
4M10	-	+	+	+	+	TA3$_7$	-	+	+	+	+
5M3	-	+	+	+	+	MJ9	-	-	-	+	+
4M8	-	-	+	+	+	MBBT4	-	-	+	+	+
5M9	-	+	+	+	+	SPE	-	-	+	+	+
TA2	-	+	+	+	+	MBBT1	-	+	+	+	+

Where TA2= tandem A 2nd mill juice tanker, TA3=tandem A 3rd mill juice tanker, TA4=tandem A, 5M= fifth mill juice tanker, 4M=fourth mill juice tanker, MJ=mixed juice, MBBT=mud from the base of big tanker and SPE=storage pit edges.

4.1.8. Salt tolerance of yeast isolates

Almost all of the 7 yeast isolates at 0% NaCl, the growth was high growth and had higher optical density (OD) value. At 4% of NaCl, the yeast isolates were shown linear increasing. At 7% of NaCl, the yeast isolates took 24 hrs to adapt the environment and start slight increasing its survival up to 72 hrs. Five yeast isolates (4M10, TA2, TA3, TA3$_7$ and MBBT1) had better proliferation to 7% of NaCl than isolates of 5M3 and 5M9 when the time was extended to 72 hrs and consider for further analysis. At 10% of NaCl, the yeast isolates had shown no growth (Fig. 5).



where a= 0 %, b=4%, c=7% and d=10% of NaCl.

Figure 5: The effect of NaCl on the growth of yeast isolates.

4.1.9. Identification of selected yeast isolates

The potent yeast isolates 4M10, TA2, TA3, TA3$_7$ and MBBT1 were identified as *Kluyveromyces lodderae*, *Pichia guilliemodii* B, *Saccharomyces boulardii*, *Zygosaccharomyces rouxii* and *Torulaspora globosa*, respectively as indicated in Table 16. The similarity must be 0.5/1 and above to confirm the probability of the species.

Table 16: Biochemical identification of yeast species for bioethanol production.

S.No.	Isolate’s code	Yeast species	Probability	Similarity
1	4M10	*Kluyveromyces lodderae*	0.896	0.787
2	TA2	*Pichia guilliemodii* B	0.713	0.563
3	TA3	*Saccharomyces boulardii*	0.730	0.573

4	TA3$_7$	*Zygosaccharomyces rouxii*	0.611	0.511
5	MBBT1	*Torulaspora globosa*	0.634	0.561

4.1.10. Yeast propagation for bioethanol production

Yeast species such as *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa* were found potent yeasts (ethanol-temperature-sugar-pH-and salt tolerant isolates) and selected for propagation and production of bioethanol via fermentation. During propagation, the brix of fermented molasses was 5.1 at 24 hrs; 8.6 and 8.5 at 48 hrs by *K. lodderae* and *P. guilliemodii* B, respectively. It was also 11.5, 13.1 and 7.6 at 48 hrs by *S. boulardii*, *Z. rouxii* and *T. globosa*, respectively (Table 17). The maximum and minimum cell density of active culture inoculated for propagation were 3.4 and 2.5 x10^8 cells/ml for *Z. rouxii* and *T. globosa*, respectively. However, the highest cell counts after propagation was observed by *K. lodderae* (1.83x10^8 cells/ml) (Table 17). The cells density was decreased due to the new environment and adapting to maximize its size.

Table 17: Propagation of yeasts species.

	Brix (°Bx)		Final pH		Before propagation	After propagation	Viable cells after fermentation
Yeast species	24 hrs (10)	48 hrs (15)	24 hrs	48 hrs	(cells/ml x 10^8)	(cells/ml x 10^8)	(cells/ml x10^8)
K. lodderae	5.1	8.6	4.2	4.4	2.96	1.83	3.08
P. guilliemodii B	5.1	8.5	4.2	4.5	2.93	0.59	1.81
S. boulardii	7.6	11.5	4.3	5.1	3.20	0.54	1.36
Z. rouxii	8.2	13.1	4.4	5.1	3.40	0.48	1.69
T. globosa	7.3	7.6	4.7	5.3	2.50	0.45	1.56

4.1.11. Bioethanol production

The brix and pH of the raw molasses was found as 79°Bx (39.5*2) and 6.2 pH. During fermentation, the percent of bioethanol (% v/v) for *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z.*

rouxii and *T. globosa* yeast species was 12.62, 11.61, 10.58, 10.82 and 10.44, respectively at 48 hrs and 12.56, 12.52, 11.82, 11.11 and 11.48, respectively at 72hrs (Table 18). The highest bioethanol production in this study was 12.62% at 48 hrs and 12.56% at 72 hrs by *K. lodderae* followed by *P. guilliemodii* B (12.52%) at 72 hrs. The lowest bioethanol production was 11.11% by *Z. rouxii* species at 72 hrs. During fermentation of sugarcane molasses using local indigenous wild yeast species, the percent of bioethanol was significant both within and between yeasts species ($p<0.05$) (Appendix 5). The fermentation efficiency (%) for yeasts of *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *T. globosa* and *Z. rouxii* was 98.0, 97.8, 93.1, 90.8 and 88.1, respectively. The *K. lodderae* had been the highest fermentation efficiency (98.0%) followed by *P. guilliemodii* B (97.8%) whereas *Z. rouxii* had been the lowest fermentation efficiency (88.1%) (Table 18).

Table 18: Bioethanol yield (% v/v) using local wild yeast species.

	Bioethanol (% v/v) (mean ± sd)				
	Fermentation time				
Yeast species	24 hrs	48 hrs	72 hrs	TY (%)	FE (%)
K. lodderae	11.04±0.05a	12.62±0.08a	12.56±0.05a	12.81	98.0
P. guilliemodii B	10.82±0.05a	11.61±0.11b	12.52±0.17a	12.79	97.8
S. boulardii	4.97±0.67c	10.58±0.25c	11.82±0.74bc	12.69	93.1
Z. rouxii	8.39±0.05b	10.82±0.23c	11.11±1.03c	12.61	88.1
T. globosa	5.03±0.75c	10.44±0.91c	11.48±1.34c	12.65	90.8

Where sd= standard deviation, TY=theoretical yield and FE= fermentation efficiency. Mean values (of duplicates) followed by the same letter (s) with in column (between species) indicates no significant difference ($p>0.05$) at 95% of confidence interval using Duncan multiple range test.

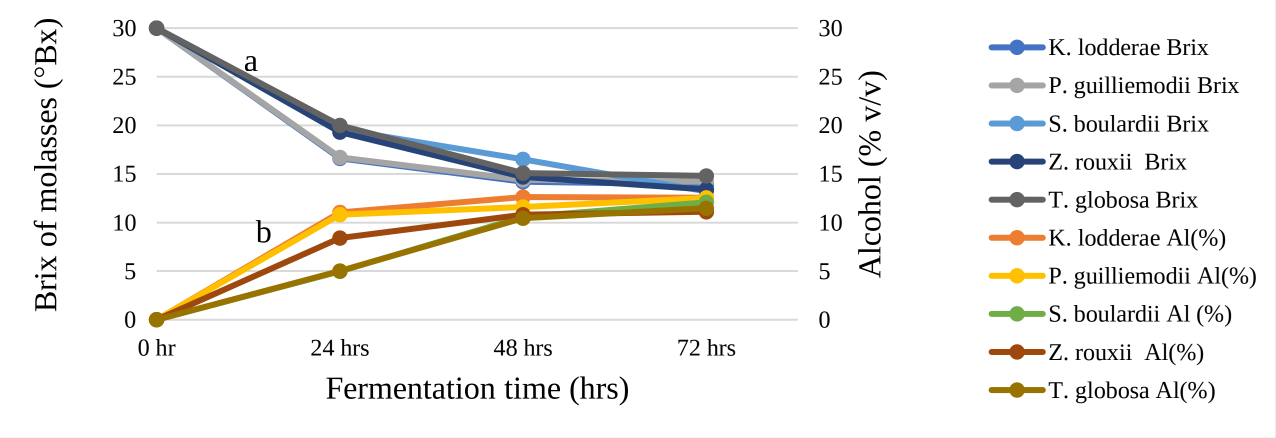
The total sugar in working volume (1300ml of molasses) was 21.6%. The total fermentable sugar was also 19.6%. During fermentation, the residual sugar (%) was 1.71, 1.73, 1.89, 2.02 and 1.95 by *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa* yeast species at 72 hrs, respectively (Table 19). The pH was increased from 4.7 to 5.3 by *K. lodderae* and *P. guilliemodii* B at 72 hrs. The pH of fermented molasses by *S. boulardii*, *Z. rouxii* and *T. globosa* ranged between 4.7 to 4.9 (Table 19 and Appendix 4). During fermentation of sugarcane molasses using local indigenous wild yeasts, the pH had been significant difference within the species ($p<0.05$) (Appendix 5).

Table 19: Residual sugars and pH of yeast species during fermentation.

	Residual sugars (%)			pH		
Yeast species	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
K. lodderae	2.41	1.78	1.71	5.2±0.07a	5.3±0.07a	5.3±0.00a
P. guilliemodii B	2.06	1.81	1.73	5.2±0.07a	5.3±0.07a	5.3±0.07a
S. boulardii	7.89	2.78	1.89	4.7±0.07b	4.8±0.00b	4.9±0.07b
Z. rouxii	5.34	2.25	2.02	4.8±0.00b	4.8±0.00b	4.8±0.00b
T. globosa	8.02	2.80	1.95	4.7±0.00b	4.8±0.00b	4.8±0.00b

Mean values (of duplicates) followed by the same letter (s) with in column (between species) indicates no significant difference ($p>0.05$) at 95% of confidence interval using Duncan multiple range test.

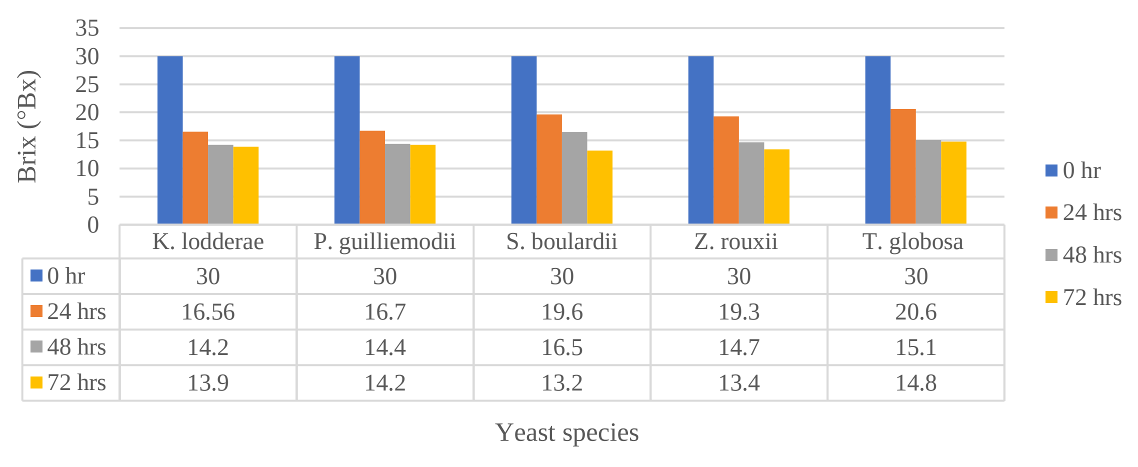
The *K. lodderae* and *P. guilliemodii* B yeast species rapidly utilized sugars in molasses and produced equivalent amount of bioethanol within the first 24 hrs, slightly increased up to 48 hrs and level of up to 72 hrs. The brix became decreased when fermentation was continued from its initial (30°Bx). Similar trend but with slightly lower rate of utilization of molasses was observed during the first 24 hrs and the production of bioethanol was increased when the time extended to 48 hrs by *Z. rouxii*. However, *S. boulardii* and *T. globosa* yeasts poorly utilized fermentable sugars found in molasses and produced low percent of bioethanol within the first 24 hrs and utilized more sugars in molasses and produced more bioethanol at 48 hrs (Fig. 6).



where a=decreasing the value of brix of molasses, b= an increase in the alcohol content with increase in fermentation time, ° Bx = Brix of molasses

Figure 6: Relationship between percent of alcohol and brix of molasses across fermentation time.

During fermentation, the brix of molasses was decreasing from 30°Bx to 13.9, 14.2, 13.2, 13.4 and 14.8°Bx by *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa* at 72 hrs, respectively. The brix of molasses was 16.6 and 16.7°Bx by *K. lodderae* and *P. guilliemodii* B within the first 24 hrs, respectively. It was also 19.6, 19.3 and 20.6°Bx within the first 24 hrs by *S. boulardii*, *Z. rouxii* and *T. globosa*, respectively (Fig. 7 and Appendix 4). There was high significant difference ($p<0.05$) between the species of yeasts regarding to reduction of brix of cane molasses and increasing percent of bioethanol during fermentation (Appendix 5).



	K. lodderae	P. guilliemodii	S. boulardii	Z. rouxii	T. globosa
🟦 0 hr	30	30	30	30	30
🟧 24 hrs	16.56	16.7	19.6	19.3	20.6
⬜ 48 hrs	14.2	14.4	16.5	14.7	15.1
🟨 72 hrs	13.9	14.2	13.2	13.4	14.8

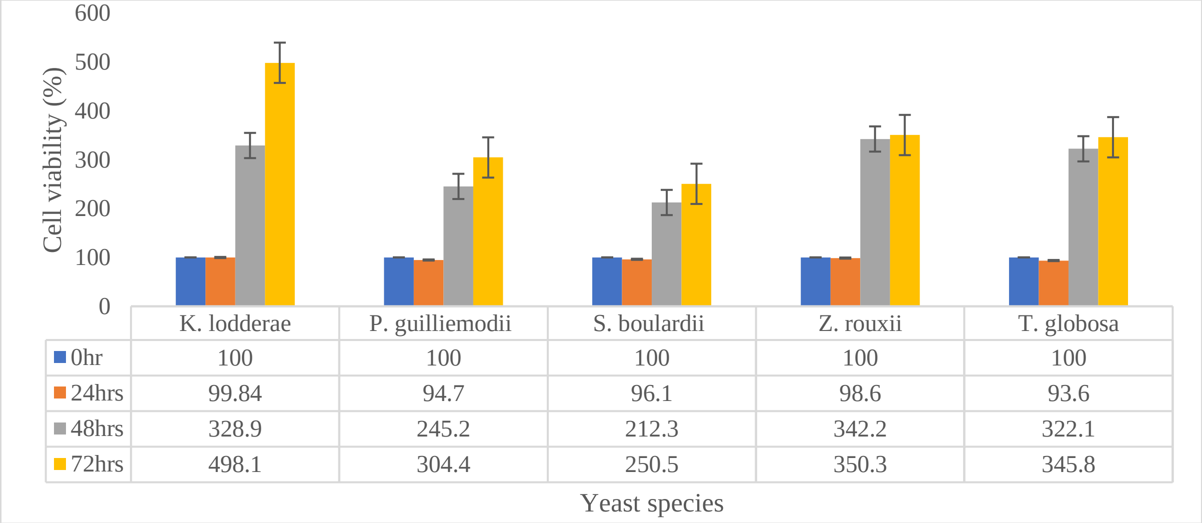
Figure 7: Brix of fermented molasses during fermentation for bioethanol production.

During fermentation, the viability of cells decreased due to its harsh environment (high sugar content of brix) with in the first 24 hrs and its viablity yeast were increased from its inoculum size (1.83 to 3.08, 0.59 to 1.81, 0.54 to 1.36, 0.48 to 1.69 and 0.45 to 1.56) x10^8 cells/ml at 72 hrs by *K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*, respectively (Table 20).

Table 20: Cell viability during fermentation.

Yeast species	Cells/ml x10^8			
	0 hr	24 hrs	48 hrs	72 hrs
K. lodderae	1.83	1.83	2.03	3.08
P. guilliemodii B	0.59	0.56	1.23	1.82
S. boulardii	0.54	0.52	1.15	1.36
Z. rouxii	0.48	0.47	1.65	1.69
T. globosa	0.45	0.42	1.45	1.56

The maximum cell density was found 498.1% by *K. lodderae* (that increased by 398.1% from its initial inoculum size) and followed by *P. guilliemodii* B (350.1%) (that increased by 250.1% from its initial inoculum size) whereas the minimum cell density was observed 250.5% by *S. boulardii* (that only increased by150.5% from its initial inoculum size) at 72 hrs (Fig. 8).



	K. lodderae	P. guilliemodii	S. boulardii	Z. rouxii	T. globosa
🟦 0hr	100	100	100	100	100
🟧 24hrs	99.84	94.7	96.1	98.6	93.6
⬜ 48hrs	328.9	245.2	212.3	342.2	322.1
🟨 72hrs	498.1	304.4	250.5	350.3	345.8

Figure 8: Increment of cell density (%) during fermentation for bioethanol production.

The correlation (r) values for *K. lodderae* were shown as between: time and cell density r= 0.85, time and bioethanol r= 0.86, pH and bioethanol r= 0.99, brix and bioethanol r= -0.99, brix and time r= -0.84 (Table 21). Similar values for other 4 yeast species (*P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) are presented in Appendix 6.

5. DISCUSSION

In this study, yeast isolates were oval shaped and some with buds. The colonies of yeast isolates on solid YMPDA plates showed that the isolates were white and creamy in color, rough, raised, irregular and smooth in their textures. Similarly, Lodder (1970) and Nasir *et al*. (2017), the cell morphologies of ethanol tolerant yeast isolates were observed ovoidal to elongate in shape and having single, pairs or triple budding cells. Some of them had flat, raised and rough texture on YPDA plates. Other investigators also reported that isolates were white to cream, ovoid, rough, mucoid, irregular, raised, round and ellipsoidal to cylindrical (Talukder *et al*., 2016).

The yeast isolates those tested for ethanol tolerance especially *K. lodderae*, *P. guilliemodii* B, *S. boulardii* and *Z. rouxii* were found tolerant to 20% (v/v) while *T. globosa* was tolerant to 18% (v/v) of ethanol. Most of the yeast isolates were fully tolerant to 4-8% of ethanol while their percentage of tolerance sharply decreased as ethanol concentration increased to 20% (Table 11). Similarly, Kechkar *et al.* (2019) reported that yeast isolates highly tolerant up to 20% (v/v) ethanol. According to Taye Negera (2016), the yeast isolates were tolerated ethanol higher than 15% (v/v) of ethanol concentration. It was the most ethanol tolerant (tolerated at 16.5%) but their survival was declined when percentage of ethanol was increased to 20% (v/v). Other investigators also reported that yeast isolates were shown to tolerant to ethanol concentration of 7, 10 and 13% (v/v) of ethanol concentration after 48 hrs of incubation (Techaparin *et al.*, 2017). Therefore, these top ethanol tolerant yeasts are important as potent yeasts and used for bioethanol production because of that might not be inhibited by the product during fermentation.

The yeast isolates that tolerated 20% of absolute ethanol in YMPD broth medium (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) were grown highly at 45°C. However, a few isolates (OP6 and Ba5) were tolerated at 46.5°C but were not able to grow at 20% of absolute ethanol (Table 12). Similarly, Talukder *et al*. (2016) have reported that thermotolerant isolates that were grown at temperatures ranging from 30 to 50°C in YPD agar plates and most isolates grew between 37 and 42°C. Some of the isolates also grew at higher temperature (45-50°C). A few isolates (especially those isolated from cane molasses) grew even at 50°C. Those isolates that grew at high temperatures (45, 48 and 50°C) had rough, irregular, elevated and pink or deep brown color while between (37-45°C) temperature had smooth, creamy or white. Another study conducted by Techaparin *et al.* (2017) have verified that thermotolerant yeast isolates grew well with better performance at 37, 40 and 45°C after 24 hrs of incubation in YMPD agar plates. These thermotolerant yeasts can be used for bioethanol production because of the advantages of tolerance of higher temperatures for the fermentation processes that leads to increase the rate of hydrolysis of substrate that resulted easily accessible to yeasts and minimize the time required to complete the fermentation process.

In this study, the thermotolerant yeasts (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) were able to utilized diverse carbohydrate sources (glucose, fructose, sucrose, galactose, trehalose and maltose) effectively. Some yeast isolates in this study utilize xylose (Table 13). Galactose and maltose were utilized by all isolates whereas trehalose also consumed by all isolates except Ba5 with acid and gas production. Some of the yeast isolates also utilized lactose (Table 13). Similarly, Sundarrajan *et al.* (2016) have reported that yeasts isolated from sugarcane bagasse and fruit juices were tested for assimilation of different carbohydrates (2%) and observed that all the isolates were able to assimilate glucose, fructose, sucrose with acid and gas production after 48 and/or 72 hrs. The thermotolerant yeasts such as *S. cerevisiae* HG1.1 and HG1.2, *T. globosa*, *Pichia manshurica* and *Pichia kudriavzevii* could be assimilated several types of carbon sources including glucose, sucrose, galactose, trehalose, maltose, raffinose and melibiose (Phong *et al.*, 2019). According to Taye Negera (2017), the thermotolerant yeast isolates fermented some carbohydrates such as glucose, galactose, maltose, sucrose, fructose and trehalose but not xylose. Therefore, these yeasts are important for bioethanol production because of that capacity to use different substrate and produce gas and acid which indicates that have a good character to produce bioethanol.

In this study, yeast species (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) utilized glucose, fructose and sucrose which are mostly abundant in molasses and tolerated 30% of the test sugars and produced gas but their effectiveness was reduced at 35% of sugars concentration (Table 14). Similarly, Taye Negera (2016) have reported that yeast isolates effectively tolerated sugar with high concentration up to 30% whereas the ability to utilize sugar was decreased, took long time and did not produce gas when the concentration of sugars increased (up to 45%). These yeasts are important for bioethanol production may be at industrial level because of that they are consumed the sugars and not inhibited by the sugars at higher concentration.

In this study, yeasts (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) observed that tolerate extremely low pH (acidic YMPD broth medium at pH 2) even if with light turbidity and slight growth when streaked on solid YMPDA plate medium. When the pH values increased to 3.0 and above, their turbidity was higher as compared to the lower pH values (2.0 and 2.5). There is high turbidity above pH 3. Similarly, Taye Negera (2017) have reported that the growth of yeast isolates did not show significant difference for pH values from 3.0 to 6.5 with higher turbidity achieved at pH 5 but beyond these values the growth of yeast isolates was decreased at both ends (extreme acidic and basic medium). According to Sundarrajan *et al.* (2016), no yeast isolate tolerated below pH of 3.5 and beyond 7.5. Therefore, these yeasts those isolated and screened in this study are important for bioethanol production because of that did not worry the formation of acid during fermentation that may lower the pH significantly.

In this study, the growth of yeasts (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) without NaCl (YMPD broth medium free from NaCl) found highly increased during the first 48 hrs but declined when incubation time was extended to 72 hrs that might be due to the nutrient insufficient and that leads to death of cells. The growth of yeast isolates linearly increased at 4% (w/v) of NaCl. When the concentration of NaCl was increased to 7% (w/v), there was no proliferation for the first 24 hrs that might be due to adapting the harsh environment and then had slight grown when incubation time was extended to 72 hrs. Similarly, Logothetis *et al.* (2010), who determine the effect of NaCl on yeast that the concentration of NaCl increased from 0-10% (w/v), the growth was decreased gradually and that the growth was arrested at 10%. When *S. cerevisiae* also was exposed to NaCl (8.19% w/v), it had been reduced its viability as compared to lower concentration of NaCl (4% w/v). These yeasts are important for bioethanol production because of that cannot be inhibited by the salt (NaCl) found in molasses (about 4%) and that might not be precipitated during pre-treatment like Calcium.

In this study, the thermotolerant yeasts produced high bioethanol yield (% v/v) and that significantly increased within the isolate (11.01 and 12.62 by *K. lodderae* and 10.82 and 11.61 by *P. guilliemodii* B at 24 and 48 hrs, respectively) during fermentation ($p<0.05$). During fermentation of sugarcane molasses using locally isolated indigenous *S. boulardii* yeast, the percent of alcohol (% v/v) was found significantly increasing from 4.97, 10.58 and 11.82 as the fermentation time was extended from 24 to 48 and further to 72 hrs ($p<0.05$). During fermentation of sugarcane molasses using local indigenous wild yeasts, the percent of bioethanol produced by the yeasts was found increased significantly as time progress from 24 to 48 and further to 72 hrs ($p<0.05$) and had high fermentation efficiency. Generally, yeast species (*K. lodderae*, *P. guilliemodii* B, *S. boulardii*, *Z. rouxii* and *T. globosa*) had been significant difference for the production of bioethanol both within and between species ($p<0.05$). Generally, when fermentation time was increased, the total reducing sugars (21.6%) in molasses were depleted whereas the percent of alcohol produced by those yeasts was found increased (Fig. 6). The *K. lodderae* and *P. guilliemodii* B were also consumed the sugars in molasses and the cells were highly proliferated (that increasing from 1.8 to 3.08 $x10^8$ and 0.59 to $1.82x10^8$ cells/ml, respectively) during fermentation. Similarly, yeasts such as *P. kudriavzevii* and *T. globosa* were efficient thermotolerant ethanol producing yeasts at 40°C after 48 hrs (Jutakanoke *et al.*, 2014). Fadel *et al.* (2013) have reported that the 9.6 (%v/v) of bioethanol was produced with 3.60 $x10^8$ cells/ml of propagated yeast and 86.3% of fermentation efficiency while 9.7 (%v/v) of bioethanol was also produced with 3.75 $x10^8$ cells/ml of propagated yeast and 87.2% of fermentation efficiency at 24 hrs. Another previous study also showed that the maximum bioethanol (%v/v) produced was 8.5 with initial inoculum size of 4.0×10^8 cells/ml at 32°C, 4.6 pH by *S. cerevisiae* at 40 hrs. The brix of fermented molasses highly reduced (from 27 to 10.61°Bx) and the final viable cell count also decreased (from 4.0 to 3.05×10^8 cells/ml that was decreased by 23.75% from its initial inoculum) (Mukhtar *et al.*, 2010). Other investigators also reported that throughout fermentation of sugar cane molasses, reducing sugars were well utilized by yeasts that indicates with increasing the number of cells and produced bioethanol. There was a sharp drop of reducing sugars (consumed more than 50%) during the first 36 hrs of incubation and further slowly reduced until 72 hrs (Jayus *et al.*, 2016). Therefore, these yeasts have been high potential of producing bioethanol with high fermentation efficiency and important for bioethanol production at industrial scale after optimization of some important parameters.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The potent five yeasts were isolated from mill juice, tandem juice and mud around the molasses tanker and morphologically characterized as oval shape, white colonies and their texture were smooth [*S. boulardii* (TA3) and *Z. rouxii* (TA3$_7$)] and raised [*K. lodderae* (4M10), *P. guilliemodii* B (TA2) and *T. globosa* (MBBT1)]. The four yeast *K. lodderae*, *P. guilliemodii* B, *S. boulardii* and *Z. rouxii* tolerant to 20% of ethanol while *T. globosa* tolerant to 18% of ethanol. Physiologically, they tolerated high temperature at 45°C, extreme acidic condition (pH 2), salinity (7 % of NaCl) with 30% of osmotic stress tolerant. These yeasts could utilize different carbohydrates and produce excess gas. These potent yeast species (*K. lodderae*, *P. guilliemodii* B, *S. boulardii* and *Z. rouxii* and *T. globosa*) could produce bioethanol (% v/v) 12.62, 11.61, 10.58, 10.82 and 10.44, respectively at 48 hrs and 12.56, 12.52, 11.82, 11.11 and 11.48, respectively at 72 hrs. Among the five yeasts, *K. lodderae* and *P. guilliemodii* B relatively high promising potent yeasts for bioethanol production at industrial scale because of that they consumed the given substrate and provide high yield with high fermentation efficiency.

6.2. Recommendations

Based on the findings of this study, it is possible to provide the following points as recommendation:

- Molecular characterization and identification of the potent yeasts should be done at strain level.
- Fermentation kinetics should be done for each potent yeast species in order to identify at which time it can successfully consuming the substrate and produce the product.
- The potent species should be utilized for bioethanol production at large scale after optimizing fermentation parameters.

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8. APPENDICES

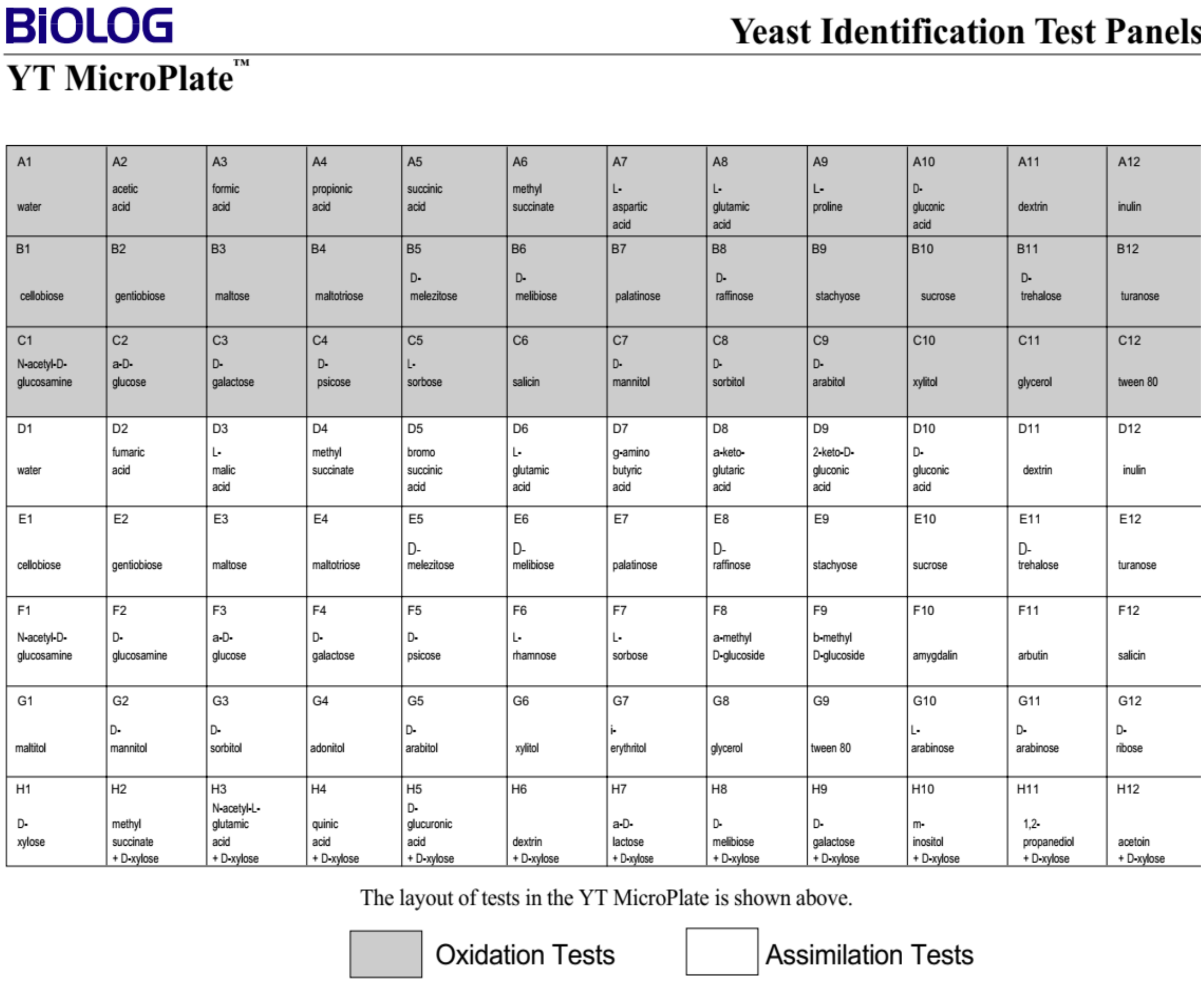
Long term preservation method

The 20 potent yeast isolates (ethanol tolerant) were preserved using 40% of glycerol. The glycerol (40%) was prepared as follows; C1V1=C2V2 where C1=99.8%, C2=80%, V2=100ml, and V1=? C1V1=C2V2, 99.8*V1= 80%*100ml, V1= 8000/99.8=80.16ml. V2=V1+H_2O or saline solution, 100ml-80.16ml=19.14ml. Then, the 99.8% of 80.16ml of glycerol was taken and 19.14ml of 0.93% of saline solution was added to prepare 80% of glycerol. The saline solution was prepared as weighed 0.93g of NaCl and put in clean measuring flask and added distilled water till 100ml and shake well for complete dissolve. The yeast isolates were prepared by Eppendorf tubes (overnight active culture in 1ml of YPD broth), then centrifuge and discarded the supernatant and taken the pellet (to remove old media and minimize osmotic shock) and washed by 0.85% of saline water without vertexing (to avoid the lysis of cells). After getting the pure pellet, aliquot with sterilized distilled water and added in 1ml of new YPD broth in cryopreserve tubes and then 1ml of 80% of glycerol was added at the top of each suspension of isolate. The tubes were put in -20 and -80°C in duplicate.

Procedure for determination of residual sugar in fermented mash

Standard method of Titration

The 50g of the filtrate was weighed, transfer quantitatively to 200ml volumetric flasks and make up to mark with distilled water and mix (dilution =1:4 m/v). The burette rinse with a portion of the sample and fill to 50ml mark. The 5 ml of Fehlingis solution "B" pipette into 300ml Erlenmeyer flask. The 5 ml of Fehling's solution “A” pipette into the 300ml flask with solution “B’’ and mix with swirling motion. The preliminary titration only 0.5-1ml was required later to complete the titration. The few fragments of anti-bumping granules or pumice powder was added to prevent bumping during boiling. The content of the flask was mixed by swirling then heat to boiling over a suitable heater, switch on the stopwatch as soon as boiling started. The liquid was boiled at moderate for exactly 2 minutes. The methylene blue indicator (3 to 5 drops) was added. Complete the titration during the next 1st one minute by adding 2 to 3 drops of the sugar solution at a time until the color of the methylene. Blue indicator is completely discharged and the boiling reaction liquid regains the bright orange appearance, caused by Cuprous oxide, which it had before the indicator was added. Duplicate titration should agree within 0.1ml. The titration should be completed within three minutes from the commencement of ebullition and during this time the mixture must be kept boiling continuously to expel the air. Record the number of ml of sample required for titration from the burette. From the % polarization of the original sample, calculate % pol of diluted sample. From the obtained titer corrected to the concentration of Fehligis solution and %polarization of diluted sample, read mg of reducing sugars per 100ml. From the obtained residual sugars mg per 100ml of diluted juice calculate the reducing sugars per 100 ml of original undiluted juice (Handbook for Ethiopian Sugar Factory Laboratories, 2010).



BIOLOG

Yeast Identification Test Panels

YT MicroPlate™

A1 water	A2 acetic acid	A3 formic acid	A4 propionic acid	A5 succinic acid	A6 methyl succinate	A7 L-aspartic acid	A8 L-glutamic acid	A9 L-proline	A10 D-gluconic acid	A11 dextrin	A12 inulin
B1 cellobiose	B2 gentiobiose	B3 maltose	B4 maltotriose	B5 D-melezitose	B6 D-melibiose	B7 palatinose	B8 D-raffinose	B9 stachyose	B10 sucrose	B11 D-trehalose	B12 turanose
C1 N-acetyl-D-glucosamine	C2 a-D-glucose	C3 D-galactose	C4 D-psicose	C5 L-sorbose	C6 salicin	C7 D-mannitol	C8 D-sorbitol	C9 D-arabitol	C10 xylitol	C11 glycerol	C12 tween 80
D1 water	D2 fumaric acid	D3 L-malic acid	D4 methyl succinate	D5 bromo succinic acid	D6 L-glutamic acid	D7 g-amino butyric acid	D8 a-keto-glutaric acid	D9 2-keto-D-gluconic acid	D10 D-gluconic acid	D11 dextrin	D12 inulin
E1 cellobiose	E2 gentiobiose	E3 maltose	E4 maltotriose	E5 D-melezitose	E6 D-melibiose	E7 palatinose	E8 D-raffinose	E9 stachyose	E10 sucrose	E11 D-trehalose	E12 turanose
F1 N-acetyl-D-glucosamine	F2 D-glucosamine	F3 a-D-glucose	F4 D-galactose	F5 D-psicose	F6 L-rhamnose	F7 L-sorbose	F8 a-methyl D-glucoside	F9 b-methyl D-glucoside	F10 amygdalin	F11 arbutin	F12 salicin
G1 maltitol	G2 D-mannitol	G3 D-sorbitol	G4 adonitol	G5 D-arabitol	G6 xylitol	G7 i-erythritol	G8 glycerol	G9 tween 80	G10 L-arabinose	G11 D-arabinose	G12 D-ribose
H1 D-xylose	H2 methyl succinate + D-xylose	H3 N-acetyl-L-glutamic acid + D-xylose	H4 quinic acid + D-xylose	H5 D-glucuronic acid + D-xylose	H6 dextrin + D-xylose	H7 a-D-lactose + D-xylose	H8 D-melibiose + D-xylose	H9 D-galactose + D-xylose	H10 m-inositol + D-xylose	H11 1,2-propanediol + D-xylose	H12 acetoin + D-xylose

The layout of tests in the YT MicroPlate is shown above.

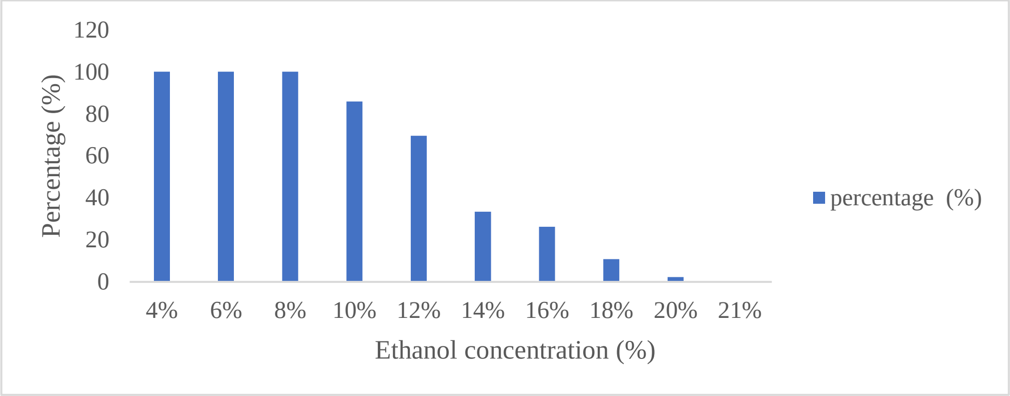
▨ Oxidation Tests ☐ Assimilation Tests

Appendix 1: Yeasts isolated from different sugary substrates.

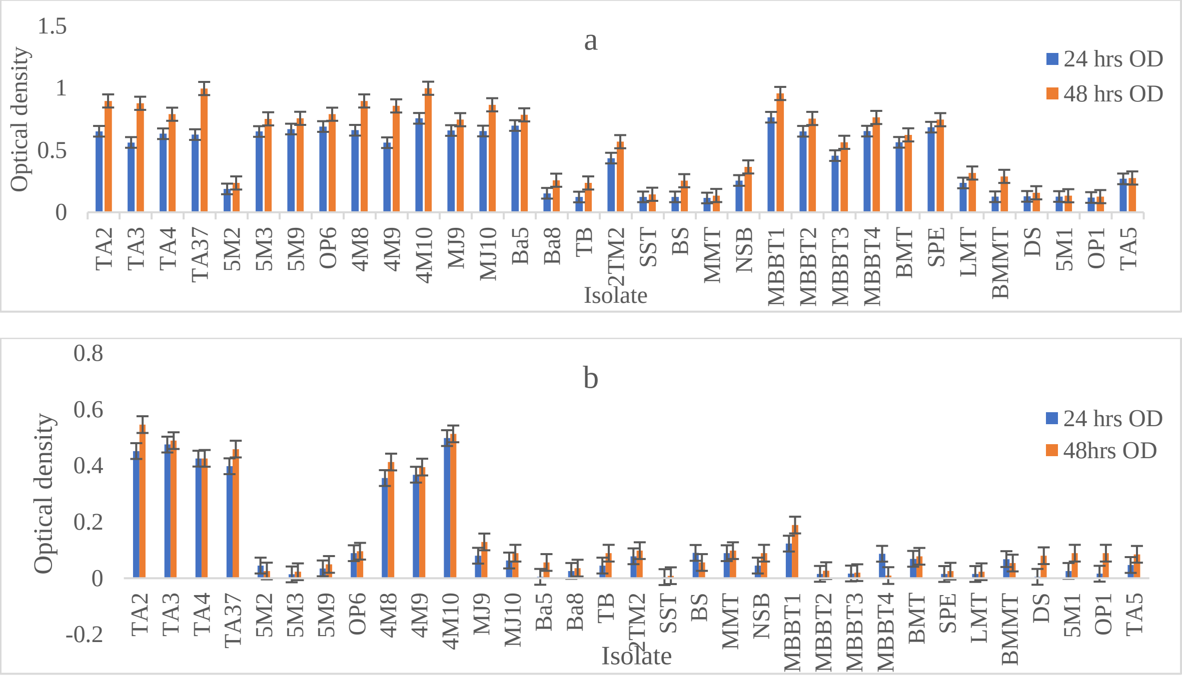
S.No.	Code	S.No.	Code	S.No.	Code	S.No.	Code	S.No.	Code
1	TA1	62	4m2	123	F32	184	2n	245	8h
2	TA2	63	4m3	124	F33	185	3a	246	8i
3	TA3	64	4m4	125	F34	186	3b	247	8j
4	TA4	65	4m5	126	F41	187	3c	248	9a
5	TA5	66	4m6	127	F42	188	3d	249	9b
6	TA6	67	4m7	128	F43	189	3e	250	9c
7	TA7	68	4m8	129	F6	190	3f	251	9d
8	TA8	69	4m9	130	F7	191	3g	252	9e
9	TA9	70	4m10	131	F71	192	3h	253	9f
10	TA10	71	1Mp1	132	F72	193	3i	254	9g
11	TA21	72	1Mp2	133	F73	194	3j	255	9h
12	TA22	73	1Mp3	134	F74	195	4a	256	9i
13	TA23	74	1Mp4	135	F81	196	4b	257	9j
14	TA24	75	1Mp5	136	F82	197	4c	258	9k
15	TA25	76	1Mp6	137	F91	198	4d	259	9l
16	TA26	77	MJ1	138	F92	199	4e	260	9m
17	TA27	78	MJ2	139	f93	200	4f	261	9n
18	TA28	79	MJ3	140	F1	201	4g	262	10a
19	TA29	80	MJ4	141	F2	202	4h	263	10b
20	TA210	81	MJ5	142	F3	203	5a	264	10c
21	TA31	82	MJ6	143	F11	204	5b	265	10d
22	TA32	83	MJ7	144	F12	205	5c	266	10e
23	TA33	84	MJ8	145	F13	206	5d	267	10f
24	TA34	85	MJ9	146	F14	207	5e	268	10g
25	TA35	86	MJ10	147	F21	208	5f	269	10h
26	TA36	87	PC1	148	F22	209	5g	270	10i
27	TA37	88	PC2	149	F23	210	5h	271	10j
28	TA38	89	PC3	150	F31	211	5i	272	10k
29	TA39	90	PC4	151	F32	212	5j	273	10l
30	TA310	91	PC5	152	F41	213	5k	274	10m
31	5m1	92	PC6	153	F42	214	5l	275	10n
32	5m2	93	PC7	154	F43	215	6a	276	10O
33	5m3	94	PC8	155	F51	216	6b	277	10P
34	5m4	95	CJ1	156	F52	217	6c	278	11a
35	5m5	96	CJ2	157	F53	218	6d	279	11b
36	5m6	97	CJ3	158	F54	219	6e	280	11c
37	5m7	98	CJ4	159	F61	220	6f	281	11d
38	5m8	99	CJ5	160	F62	221	6g	282	11e
39	5m9	100	CJ6	161	F63	222	6h	283	11f
40	5m10	101	CJ7	162	1a	223	6i	284	11g

41	MP1	102	CJ8	163	1b	224	6j	285	11h
42	MP2	103	CJ9	164	1c	225	6k	286	11i
43	MP3	104	CJ10	165	1d	226	7a	287	11j
44	MP4	105	Ba1	166	1e	227	7b	288	11k
45	MP5	106	Ba2	167	1f	228	7c	289	11l
46	MP6	107	Ba3	168	1g	229	7d	290	12a
47	MP7	108	Ba4	169	1h	230	7e	291	12b
48	MP8	109	Ba5	170	1j	231	7f	292	12c
49	MP9	110	Ba6	171	2a	232	7g	293	12d
50	MP10	111	Ba7	172	2b	233	7h	294	12e
51	OP1	112	Ba8	173	2c	234	7i	295	12f
52	OP2	113	3e	174	2d	235	7j	296	12g
53	OP3	114	9k	175	2e	236	7k	297	12h
54	OP4	115	F11	176	2f	237	7l	298	12i
55	OP5	116	F12	177	2g	238	8a	299	12j
56	OP6	117	F13	178	2h	239	8b	300	12k
57	OP7	118	F14	179	2i	240	8c	301	12l
58	OP8	119	F15	180	2j	241	8d	302	12m
59	OP9	120	F21	181	2k	242	8e	303	F10
60	OP10	121	F22	182	2l	243	8f	304	F29
61	4m1	122	F31	183	2m	244	8g	305	F26

Appendix 2: Screening yeast isolates using different ethanol concentration.



Appendix 3: Optical density values for viability of yeast isolates at 18 and 20% of ethanol.



Where a=18% and b=20% of ethanol.

Appendix 4: The mean difference for brix of molasses during fermentation.

	Brix (°Bx)		
Yeast species	24 hrs	48 hrs	72 hrs
K. lodderae	16.6±0.42a	14.2±0.49a	13.9±0.42a
P. guilliemodii B	16.7±0.71a	14.4±0.14a	14.2±0.28b
S. boulardii	19.6±0.21b	16.5±0.35b	13.2±0.42a
Z. rouxii	19.3±1.41b	14.7±0.35a	13.4±0.63a
T. globosa	20.0±0.85b	15.1±0.56b	14.8±0.21b

Where mean values (of duplicates) followed by the same letter (s) with in column (between species) indicates non-significant difference (p>0.05) at 95% of confidence interval using Duncan multiple range test.

Appendix 5: F-tables and p-values.

ANOVA for pH during fermentation

Source of Variation	SS	DF	MS	F	P-value
Within species	0.924	4	0.231	77***	2.0123E-06
Between species	0.016	2	0.008	2.667ns	0.1296
Error	0.024	8	0.003		

ANOVA for brix of molasses

Source of Variation	SS	DF	MS	F	P-value
Within species	9.79817333	4	2.44954333	1.98775 ns	0.18942551
Between species	50.5894533	2	25.2947267	20.5261**	0.0007075
Error	9.85854667	8	1.23231833		

ANOVA for bioethanol yield (%v/v)

Source of Variation	SS	DF	MS	F	P-value
Within species	16.86876	4	4.21719	4.40461*	0.03566763
Between species	23.46988	2	11.73494	12.2565**	0.00366542
Error	7.65952	8	0.95744		

Where *= stands for significant, **=high significant, ***= very high significant and ns = non-significant at @=0.05.

Appendix 6: Correlation of parameters during fermentation process.

K. lodderae	Time	Cell density	pH	Brix	Bioethanol
Time	1				
Cell density	0.851948	1			
pH	0.9135	0.613075	1		
Brix	-0.84535	-0.48333	-0.98793	1	
Bioethanol	0.863222	0.504108	0.990681	-0.99899	1

P. guilliemodii B	Time	Cell density	pH	Brix	Bioethanol
Time	1				
Cell density	0.967105	1			
pH	0.853986	0.943507	1		
Brix	-0.84619	-0.94084	-0.99973	1	
Bioethanol	0.860541	0.956569	0.995096	-0.99627	1

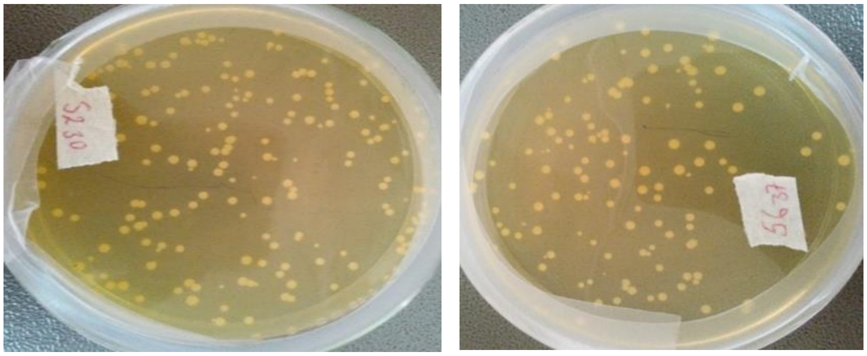
S. boulardii	Time	Cell density	pH	Brix	Bioethanol
Time	1				
Cell density	0.966082	1			

pH	0.774597	0.635322	1		
Brix	-0.94806	-0.85434	-0.93485	1	
Bioethanol	0.97321	0.95378	0.83304	-0.95727	1

Z. rouxii	Time	Cell density	pH	Brix	Bioethanol
Time	1				
Cell density	0.956968	1			
pH	0.894427	0.941294	1		
Brix	-0.92329	-0.95346	-0.79587	1	
Bioethanol	0.923504	0.955488	0.799885	-0.99996	1

T. globosa	Time	Cell density	pH	Brix	Bioethanol
Time	1				
Cell density	0.959329	1			
pH	0.774597	0.714757	1		
Brix	-0.91752	-0.91576	-0.93361	1	
Bioethanol	0.967826	0.970479	0.861419	-0.98327	1

Appendix 7: Colonies of yeast isolates on solid YMPD agar medium.



Appendix 8: Carbohydrates (dulcitol, xylose) fermentation, Ebulliometer reading of bioethanol by *K. lodderae* at 48 hrs and biolog identification.