ADDIS ABABAUNIVERSITY SCHOOL OF GRADUATE STUDIES FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

ISOLATION AND SELECTION OF ETHANOL TOLERANT YEASTS FOR THE PRODUCTION OF ETHANOL

BY

DECHASSA TOLESSA KUMSA

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Microbiology

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LISTS OF SYMBOLS AND ABRIVATIONS

^0C	Degree centigrade
CO_2	Carbon dioxide
D.T	Durham tube
g	Gram
GC	Gas chromatography
g/l	Gram per liter
hrs	Hours
KCl	Potassium chloride
KH_2PO_4	Potassium orthophosphate
L	Liter
ml	Milliliter
mg/ml	Milligram permilliliter
$MgSO_4.7H_2O$	Magnesium sulphate hydrated
NH_4Cl	Ammonium chloride
nm	Nanometer
$(NH_4)_2SO_4$	Ammonium sulphate
NH_3NO_3	*Ammonium nitrate*
$(NH4)_2PO4$	Ammonium phosphate
v/v	Volume by volume
TBY	Yeast isolates from Tela
TGY	Yeast isolates from Teji
HGY	Yeast isolates from Honey
DYP	Yeast isolates from dough
TBY1	Belaynesh *Tela* yeast isolate one
TGY2	Godere *Teji* yeast isolate two
YEPDA	Yeast extract peptone dextrose agar

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ABSTRACT

Twenty yeasts colonies were isolated from Tela, Teji, Honey and teff dough selection for ethanol tolerance on yeast extract peptone dextrose agar medium (YEPDA). All the isolates were first tested for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth using common fermentative carbohydrates. Ten isolates which were relatively high fermentative in Durham tube fermentation method were selected for testing of isolates for ethanol tolerance. Ethanol tolerance was tested using different concentration made from 96 %(v/v) of absolute ethanol in yeast extract peptone dextrose broth and their growth was determined by measuring optical density of the cells in broth using spectrophotometer at 615nm. Two strains of isolates showed measurable growth in medium containing above 14% (v/v) of ethanol were selected for further study. Strain TBY1 tolerates15.5% (v/v) ethanol and strain TGY2 tolerates 16% (v/v) of ethanol. Both isolates were classified under genus saccharomyces based morphological appearance of vegetative cell under microscope, ascospore production, ascospore character, colony character and physiological testing of carbohydrates using baker yeast as reference. They were found to be equally sugar tolerant having good growth in medium containing 24-28 % (w/v) sucrose. Fermentation of sucrose was optimized with respect to temperature, pH and sugar concentration. Results revealed that pH 5 and 28% sucrose concentration as optimum for fermentation for the three yeasts. The optimum temperature for selected strains and reference yeast was 30^0C. The maximum ethanol produced by TBY1and TGY2 from 28% of sucrose at pH 5 and temperature of 30 ^{0}C were 11.3 and 11.5% (v/v) of ethanol respectively, and 9.6%(v/v)ethanol by the standard baker's yeast. The amount of ethanol produced in the fermentation broth was measured using ebulliometer. The biomass was determined after the end of fermentation and the result showed it the biomass of more ethanol tolerant strain is greater than the others.

***Keywords/phrases**: Baker yeast, biomass, Durham tube, ethanol, ethanol tolerance, optical density Spectrophotometer, sugar tolerance*

1. INTRODUCTION

Ethanol is a liquid alcohol made of oxygen, hydrogen and carbon and is obtained from the fermentation of sugar or converted starch contained in grains and other agricultural or agri-forest feedstocks (Scheffers, 1987). It is color less liquid with acharacteristic,agreeable odor. In dilute aqueous solution, it has some what sweet flavor but in more concentrated solutions it has a burning taste (Stevenson, 1994). Ethanol ,CH_3CH_2OH,has been described asone of the most exonic synthetic oxygen containing organic chemicals (Weissermel and Arpe,2003). This is because of its unique combination of properties as a solvent, germicide, beverages, antifreeze, a fuel, a depressant, and specially because of its versatility as achemical intermediate for other organic chemicals (Beltize *et al*., 2004).

Ethanol is avolatile,flammable and amonohydric primary alcohol(Stevenson, 1994).it melts at -114.1^{0}C ,boils at 78.5^{0}C and has adensity of 0.789g/ml at 20^{0}C(Beltize *et al*., 2004). Its low freezing point made it useful as the liquid in thermometers for temperatures below -40^{0}C (steveson, 1994).

The production of pure ethanol apparently began in the 12-14th century along with improvements in the art of distillation permitting the condensation of vapors of lowers boiling liquids. During the middle ages, alcohol was not only mainly used for the production or as a constituent of medical drugs, but also for the manufacture of painting pigments and other chemical industries. It was only in the 19th century that this trade became an industry with enormous production, due to economic improvements of the distilling process (Roehr, 2001). Now, ethanol is an important industrial chemical with emerging potential as a biofuel to replace non-renewable fossil fuels (Alfenore *et al*., 2002).

The energy crisis necessitates studying and discovering new processes involved in the production of utilizable compounds as alternative energy sources among which fermentation to ethanol represents a significant strategy. Ethanol is being widely investigated as a renewable fuel source because in many respects it is superior to gasoline fuel (Jones and Ingledew, 1993). This situation has led many countries including Ethiopia are striving to develop and prepare preconditions for the use of ethanol as a fuel

especially from cane sources. Sugarcane resource can be used to produce a variety of commercial products that can be marketed domestically, regionally and internationally.

Ethiopia through its potential in developing large sugarcane production can play a pro-active role in mitigating the same. Molasses the non-crystallizable residue remaining after crystallizing sucrose, has additional advantage. It is relatively inexpensive raw material, readily available and already in use for industrial ethanol production using yeast strains. Despite the evolving trend of using bacteria for ethanol production, yeast is still the primary choice for fermentation (Chandra and Panchal, 2003). Yeasts are used in the fermentative production of ethanol, alcoholic beverages, baking products, and protein and vitamin supplements in human and animal diets as well as in the production of single cell proteins. However, efforts to characterize these yeasts were limited. In the assessment of yeasts ofthe yeast isolates for economic and efficient ethanologenicprocesses, certain specific physiological properties are important and required. These include good tolerance to high concentrations of ethanol, sugars and acids as well as high osmotic pressure (Ansanay-Galeote *et al*., 2001;Benitez *et al*., 1983; Ezeogu and Okolo, 1984a; Okolo *et al*., 1990; Okolo *et al*., 2004 and Stewart *et al*., 1984). Good flocculation/sedimentation ability depending on process requirements as well as good invertase activity and specific ethanol productivity are important characteristics of yeasts capable of converting sucrose to ethanol (Jameonoz and Benitez, 1986). The study therefore, was aimed at isolating and selecting of ethanol tolerant yeast strains from *Tella, Teji,* honey and dough with its potentials for industrial ethanol production.

1.2 Research objectives

General objective

To isolate and characterize ethanol resistant yeasts from indigenous feremented food and drinks (Tella,Teji, Honey and Teff dough) for the production of ethanol.

Specific objectives

1.2.1 To isolate and select ethanol tolerant yeast(s) for ethanol production from various sources

1.2 .2 To identify the selected ethanol tolerant yeasts

1.2.3 To study effects of pH, temperature, Substrate and ethanol concentration and nutrients on ethanol production by selected isolates

1.2.4 To apply the selected ethanol tolerant yeast for ethanol production.

2. LITERATURE REVIEW

2.1 Yeasts and Ethanol tolerant yeasts

2.1.1 Yeasts

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with about 1,500 species currently described (Kurtzman and Fell, 2006). They dominate fungal diversity in the oceans (Bass, *et al*., 2007). They are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). The yeast species are all characterized by a similar set of features, both morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy (Kreger-van Rij, 1984).

Identification of yeast genera can often be achieved by morphological tests supplemented with a few physiological tests. With regard to the latter, sole carbon and nitrogen source assimilation by yeasts may be determined by auxanography which nowadays can be conveniently carried out using commercially available kits: for example, Analytical Profile Index (API) strips (BioMérieux, France) or the automated/computerized BCCM/Allev 2.00 system (Louvain-la-Neuve, Belgium). Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, fructose, maltose, sucrose, lactose, raffinose, trehalose, dextrin, starch and xylose. With regard to fermentation of these sugars (Scheffers, 1987) has argued that the anaerobic liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast species. Ethanol production assays are deemed to be more appropriate determinants of sugar fermentation by yeasts (Walker, 1998).

Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products. Some of these products are produced commercially while others are potentially valuable in biotechnology (Kurtzman and Fell, 1997). Some yeast species have potential to be uses in food, beverage and fermentation industries (Jacobson and Jolly, 1989).

Table 1. Some presentand potential uses of yeasts in the food, beverage and fermentation industries.

Application	**Yeast species**
Ale fermentation	*Saccharomyces cerevisiae*
Bread and dough leavening	*S. cerevisiae, S. exiguus, S. rosei*
D-Arabitol (sweetener)	*Candida diddensiae*
Emulsifier	*C. lipolytica*
Ethanol fermentation	*S. cerevisiae*
Fish and poultry feeds (astaxanthin)	*Phaffia rhodozyma*
Fodder and single cell protein	*C. utilis*
Lactose and milk fermentation	*C. pseudotropicalis, Kluyveromyces fragilis, K. lactis*
Lager beer fermentation	*S. carlsbergensis*
Wine fermentation	*S. cerevisiae*
Xylitol (sweetener)	*T. candida*
D-xylose fermentation	*C. shehatae, Pichia stipitis,P. segobiensis*

Modifieified from **Jacobson and Jolly, (1989).**

Yeasts occur widely in nature and have been recovered from widely differing terrestrial as well as marine sources. Certain yeasts are more or less ubiquitous while others appear to be restricted to very specific habitats. Yeasts seldom occur in the absence of either molds or bacteria (Kreger-van Rij, 1984).

2.2 Classification and identification of yeasts

The chief characteristics used to classify yeasts are microscopically appearance of the cells, their mode of sexual reproduction certain physiological (especially nutritional) activities certain biochemical futures comparison of genomes in terms base sequences, by DNA hybridization or RNA/DNAsequence comparison (Barnett *et al*., 2000).

Table 2. Criteria used in yeast species classification and identification.

Morphological Characteristics	Physiological characteristics
- Giant colony morphology - cell morphology in liquid media - mode of vegetative and/or sexual reproduction - spore characteristics - presence /absence of hyphae or Pseudohyphae - pellicle formation at liquid surface -Flocculation in liquid media	- Fermentation of sole C source - Assimilation of sole carbon source - Assimilation of sole N sources - Pigment production - Acid production - Osmophilia

The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer and Nikaido, 1995).

Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. They are distinguished from most fungi by their usual existence as single ovoid cells about 8 µm long and 5 µm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990).

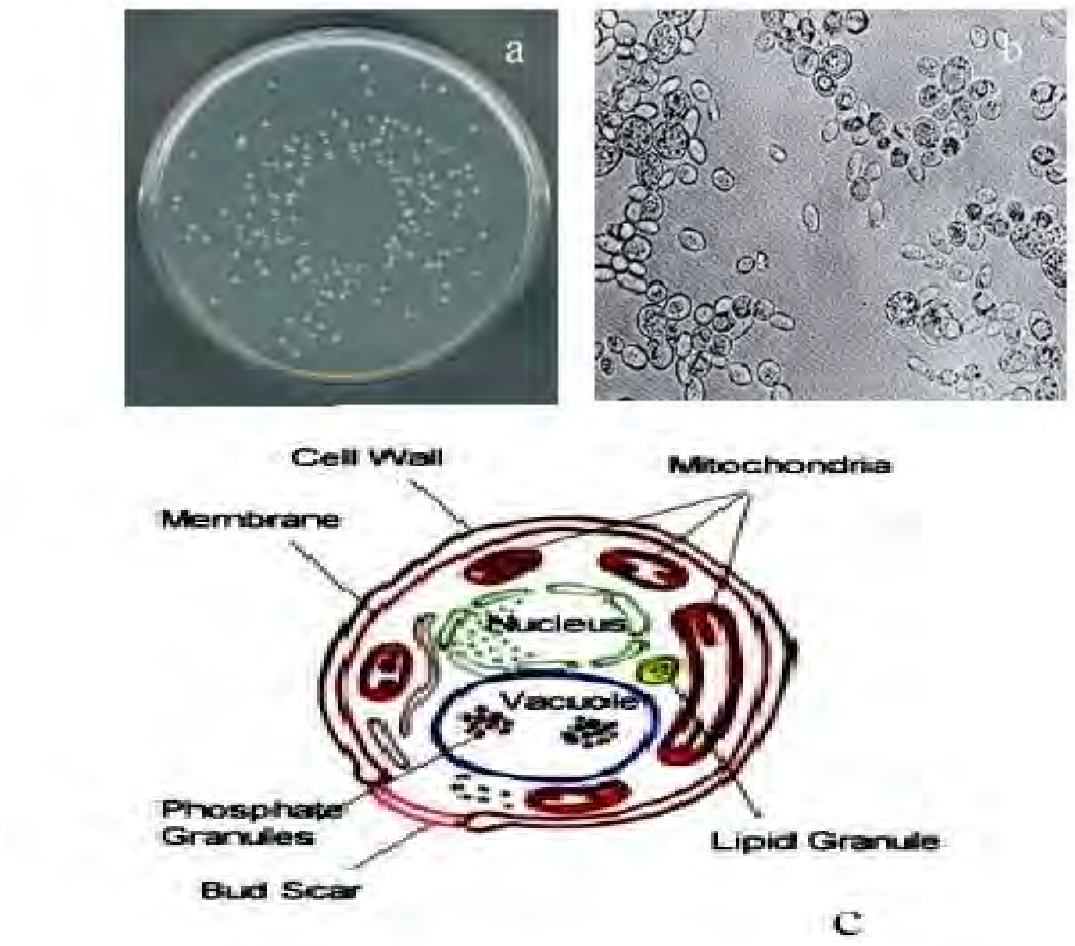

Figure 1. Yeast cell

a) colonies of *S. cerevisiae* on agar plate (www, 2005), b) *S. cerevisiae*

Under microscope (400x) (www, 2006) and c) Yeast cell composition (www, 2002)

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts (Figure2). Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962). Yeasts may reproduce asexually or sexually (Wayman and Parekh, 1990).

A. Asexual reproduction

Alexopoulos (1962) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. The budding yeasts reproduce by budding, in this process the protoplasm of the cell, covered by a thin membrane, pushes out of the cell wall in the form of a bud and forms daughter cells (Figure 3.). The bud enlarges until it is separated from the mother cell by a constriction at the base. Under some conditions, buds do not separate from the mother cell and a branched chain of cells called a pseudo mycelium forms (Figure 4).

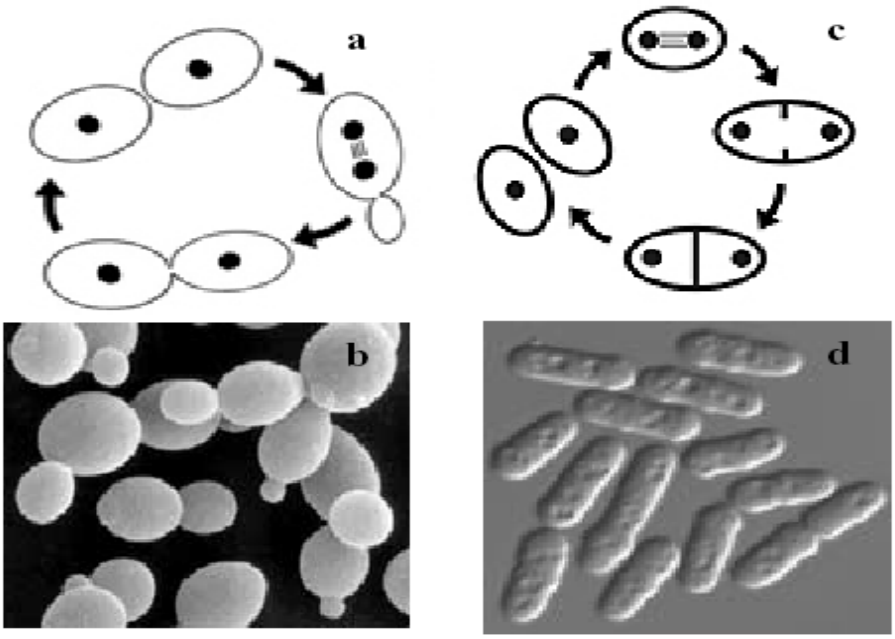

Figure 2**.** sexual reproduction in yeasts

a) budding yeast. Formation of new cell begins with blowing out of a new cell, at the pole of the cell. Mitosis follows with migration of one nucleus to the new cell. New wall material is then laid down in the passage between the two cells and separation of the cells will occur (www, 2005), b) shows *S. cerevisiae* cells reproducing asexually by budding (www, 2002) and c) fission yeast. Mitosis of the nucleus occurs and, follows by elongation of the cell and formation of a cell wall that divides the cell in half, and separates the two nuclei (www, 2005); d) shows *Schizosaccharomyces pombe* cells reproducing asexually by fission (www, 2002).

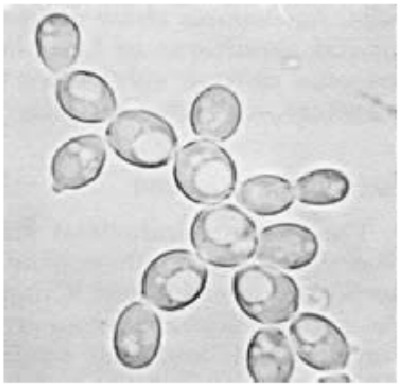

Figure 3. Chain of yeast cells (pseudo mycelium) produced by budding (www, 2005)

During the process of budding, the nucleus divides, one daughter nucleus passing into the bud, the other remaining in the mother cell. Most known yeasts reproduce by budding such as *S. cerevisiae.* The fission yeasts reproduce by transverse division. The parent cell elongates, the nucleus divides, and a transverse wall (septum) in laid down some where near the middle, separating the mother cell into two uninucleate daughter cells. This septum is formed by annular growth beginning at the wall and proceeding in ward. The new wall thickens before the daughter cells separation (Conti and Naylor, 1959).

B Sexual reproduction

Sexual union in the yeasts takes place either between two somatic cells or between two ascospores which assume the function of copulating gametangia, unite and form a zygote cell. Eventually, an ascus forms which contains ascospores, their number depending on the number of nuclear divisions which take place and on the subsequent development of the nuclei. Four or eight ascospores per ascus are the usual number, but other numbers may also be encountered.

Figure 4 shows the reproduction of yeast, proceeding by the formation of buds on the cell surface, but sexual reproduction can be induced under special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and á. Each type can develop by budding into order haploid cells. The mating of an a haploid cell

and an á haploid cell yields a normal á diploid cell. Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or á/ á) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer and Nikaido, 1995).

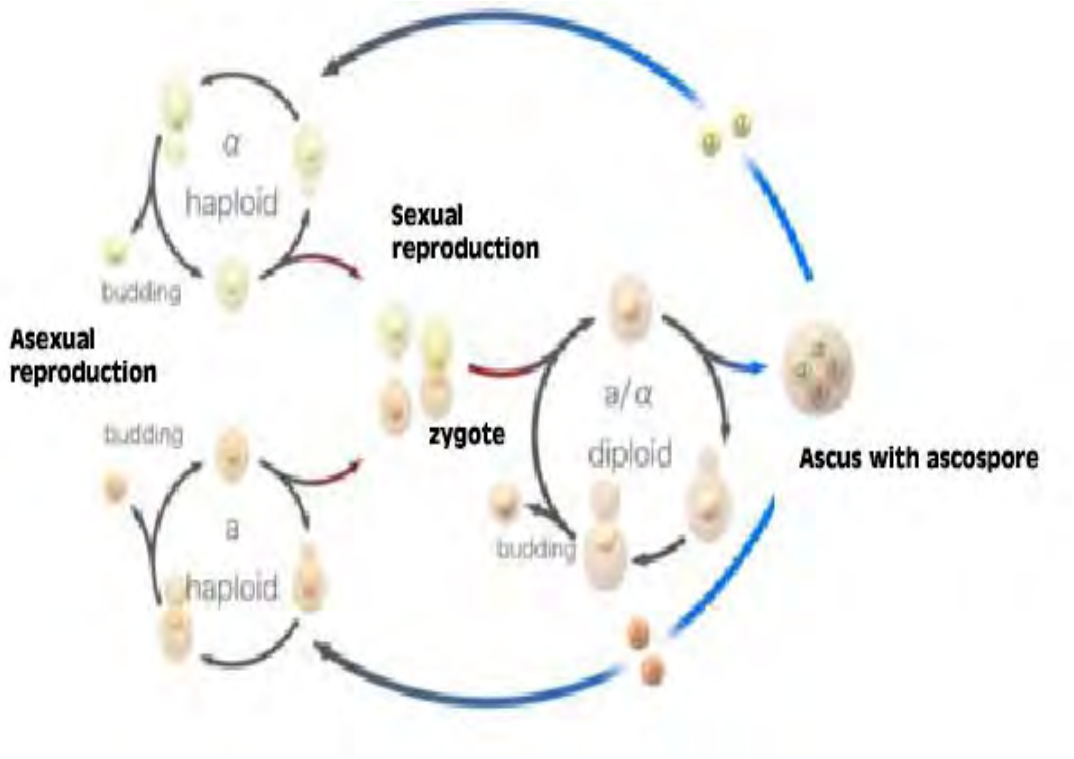

Figure 4. The reproduction of yeast by sexual and asexual (www,2005)

Ascospores formed by yeasts are often globose or ovoid, as in *Debaryomyces, Saccharomyces, Schizosaccharomyces*, and *Saccharomycodes* (Figure 5). Other yeasts form different types of ascospores. Thus, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped; in other species of *Hansenula* they may be hemispherical of shaped like the planet Saturn. Release of ascospores may occur when the ascus wall deliquesces; this is the usual method of release in species with hat- or Saturn-shaped spores. In other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopolos *et al*., 1996). Miller (1989) pointed out that yeast ascospores are much more durable than somatic cells and have the ability to withstand even snail gut enzyme, a distinct advantage in their natural environment.

Figure 5. An ascus with four ascospores of *S. cerevisiae*

However, morphological, physiological and biochemical tests have commonly been used for phenotypic characterization of yeast species. These methods are often unreliable, due to strain variability and, therefore, do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular techniques provides more powerful means of strain identification and differentiation among strains (Recek *et al*., 2002).

For identification based on genomic analysis, ribosomes are an indispensable component of the protein synthesis apparatus, and their structures are strictly conserved, the DNA component of the small subunit ribosome has proved to be an important and useful molecular clock for quantifying evolutionary relationships between organisms. Generally, the rate of base substitutions, deletions or insertions in various regions of the rRNA gene is not uniform; some areas are highly conserved and unchanged through millions of years, some are highly variable and others are semi conserved (Nishimura and Mikata, 2000). Application of gene sequence analyses to yeast systematics has shown conflict between the placement of species on gene trees and their classification from phenotype. For example, the genus *Wingea* had been described because of the uniqueness of its lenticular ascospores, but phylogenetic analysis of rRNA sequences has placed it in the genus *Debaryomyces*, where species are generally characterized by roughened, spheroidal ascospores (Kurtzman and Robnett, 1994).

These is now a widespread pattern of disparity between phenotype and genotype as means for classifying yeasts, and these differences have been demonstrated from analyses of 18S ribosomal DNA (rDNA) (James *et al*., 1994; Cai *et al*., 1996; James *et al*., 1997), internal transcribed spacer (ITS) rDNA (James *et al*., 1996) and 26S rDNA (Kurtzman and Robnett, 1995 and 1998), leaving little doubt that phenotype is a poor predictor of genetic relationships among species (Kurtzman and Robnett, 2003).

2.3 Fermentative yeasts

The genera of yeasts that are most frequently involved in ethanol production are *Saccharomyces, Brettanomycespecies, Candida, Kluyveromyces, Debaryomyces, Trolopesis and clavispora.*

Table 3. Yeasts capable of producing ethanol from different carbohydrates

Glucose and sucrose	Lactose	Starch	insulin
S.cerevisiae *S. ellipsoideus* *S.carlsbegensis* *S. ovifofmis* *S .pome* **Xilose** *Kluyveromyces marxianus* *C. tropicalis* *C. shehatae* *k. cellobiovorus* *clavispora spp.* *P.tannophius* *P. stipitis* *B.anomalus* *B.clussenii*	*C.pseudotropicalis* *T.cremoris* *K.frangilis* *Brettanomycesspp.* *Torulopsis spp.* *Debaryomyces spp.* **Cellobios***e* *k. cellobiovorus* *B.anomalus* *C. lustanae* *C.molischiana* *C. versalis* *C.wickerhamii*	*S.diastaticus* *k. marxianus* *C. tropicalis* *C. shehatae* *S.occidentalis* *S. alluvius* *S. castelli* **Cellodextrin** *C.wickerhamii*	***C.pseudotropicalis*** ***K.frangilis*** ***k. marxianus*** ***Torulopsis spp.*** ***S.occidentalis*** ***S. castelli*** ***C. macedoniensis*** ***C. kefyr*** ***C.memebranaefaciens*** ***K.thermotolerans*** ***S. fermentati*** ***S. cheresiensis*** ***S. kluyveri*** ***S. moldevorans*** ***D.castellii*** ***T. delbreckii*** ***T. prectoriensis*** ***T.globosa***

Source :- Modified from **Spencer and Spencer(1997).**

Sacchromyces do not have the genes for amylases, cellulases or B-galactosidases or for degrading pentose (Ingledew, 1993). Several fermenting yeasts including recombinant strain have been evaluated in converting lignocellulosic hydrolysates to ethanol. *Candida shahatae* and *Pichia stipitis* were the most successful, where as *kluyveromyes marxianus* was also effective in cellulose to ethanol conversions.

2.4 Yeast isolates Selection for ethanol production

Criteria used for selection of yeast for ethanol production

Generally, to obtain high quality and yield of ethanol in ethanol industry, selection of fermentative yeast is very essential .The most important factors to select microbial culture are:

- ability to utilize wide range of carbohydrates
- absence of metabolites other than ethanol
- low pH optimum and high optimum temperature and resistance to several physicochemical stresses
- rapid growth and fermentation rate
- high osmo tolerance and ethanol tolerance (Spencer and Spencer,1997)

In selecting yeasts for the efficient production of ethanol fuel (as opposed to potable ethanol) ,microbiologist have set out certain requirements of yeasts ((Panchal *et al.,* 1981), the following are the most important one.

An "ideal" yeast for fuel ethanol production should be ethanol tolerant, osmo tolerant, acid tolerant, thermo tolerant, genetically stable, rapid and efficient fermentor, easy to propagate, able to utilize wide range of substrates, generate minimum heat during fermentation, possess flocculating or non flocculating characteristics depending upon the process requirements, Possess "killer"activity, derepressed for di- or polysaccharide uptake in the presence glucose, resistant to certain toxic wastes . It is safe to assume that there is no singe yeast strain used in the industry today posses all the above characteristic and hence the a research activity in this have continued in this area (Panchal *et al.*, 1981).

2.5 Ethanol Tolerant Yeasts

The conversion of carbohydrate to ethanol usually involve yeasts, and optimal conversion requires cells that are tolerant to high concentration of both substrates and products and are able to efficiently produce ethanol (Walker, 1998). Sacchromyces yeasts are the most ethanol tolerant of the eukaryotic organisms, and able to produce over 20% ethanol (Casey and Ingledew,1986). How ever, there are different ways of improving ethanol production: increasing the range of substrate used as feed stock, improving the efficiency of substrate

conversion to ethanol, raising fermentation temperature, or improving tolerance to ethanol and osmotic pressure. For these reason, attention has been given to yeasts other than saccharomyces capable of fermenting substrates not accessible to the former such as insulin, starch, lactose ,cellobiose, hemicelluloses, or xylose (Ingledew, 1993 and Walker, 1998).

Ethanol, the main end product of glycolysis in saccharomyes, inhibites sugar fermentation and cause other unfavorable effects in yeast cells. For examples, it is a non competitive inhibitor of growth rate and inhibits the transport of sugar and amino acids, and other processes associated with membrane lipids (Ingram and Buttke, 1984; Van Uden, 1989; and Walker, 1998). Lipid composition of plasma membrane is very important for ethanol tolerance, consistent with structural changes observed in the cell membrane of microorganisms tolerant to high concentration of ethanol (Ingram and Buttke, 1984). Ethanol-tolerance also depends on environmental and nutritional conditions (Ingledew, 1993). How ever under fixed conditions non-isogenic strains differs in their ability to tolerate ethanol, and tolerance is a reproducible characteristics implying that it is genetically controlled (Jime'nez and Beni'tez1987, 1988). Genetic analysis confirmed that the character s polygenic, and that the genes responsible for ethanol tolerance are different in different strains(Jime'nez and Beni'tez, 1986). For this reason hybridization has generated yeasts more tolerant to ethanol than their parental.

2.6 Ethanol fermentation

The fermentation of sugar to ethanol by yeast has an important among the different processes that are used in industry. The yeasts, which are of primary interest to industrial operations, are *S. cerevisiae*, *S. uvarum* (*carlsbergensis*), *Sch. pombe*, and *Kluyveromyces* species. Yeasts metabolize glucose to ethanol by the glycolysis pathway. The overall net reaction (Figure 6) involves the production of 2 moles each of ethanol, CO_2, and ATP per mole of glucose fermented. Therefore, on a weight basis, each gram of glucose can theoretically give rise to 51 % alcohol. The yield attained in practical fermentations, however, does not exceed 90-95% of the theoretical value. This is due to the requirement for some nutrients to be utilized in the synthesis of new biomass and other cell maintenance-related reactions. Side reactions also occur in the fermentation (usually to glycerol) which many consume up to 4-5% of the total substrate. If these reactions could be eliminated, an additional 2.7% yield of ethanol from substrate would result (Roehr, 2001).

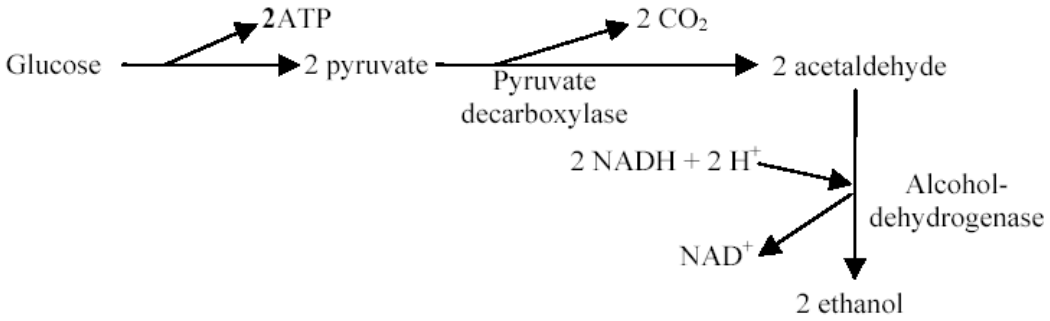

Figure 6. The ethanol fermentation pathway results in the formation of ethanol and carbon dioxide (Norr *et al.*, 2003).

The reducing power of NADH, produced by glycolysis, must be transferred to an electron acceptor to regenerate NAD+. In ethanol fermentation, it is not pyruvate but rather acetaldehyde, its decarboxylation product, which serves as the terminal electron acceptor. With respect to glycolysis, ethanol fermentation contains two additional enzymatic reactions, the first of which (catalyzed by pyruvate decarboxylase), decarboxylates pyruvic acid which have thiamine pyrophosphate (TPP) as cofactor (Ribéreau-Gayon *et al.*, 2000).

2.4 FACTORS AFFECTING FERMENTATION

Ethanol fermentation is influenced by different environmental, biological and chemical conditions. the influential parameters are temperature, sugar concentration, available nutrients and vitamins,pH,the amount of oxygen and ethanol concentration.

2.4.1 Effect of ethanol on yeast fermentation

A limitation of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because ethanol inhibits alcoholic fermentation, which limits the concentration of ethanol which can be produced by a given strain of yeast. The maximum concentration of ethanol which can be produced by yeast varies with species up to 20% by volume. The degree of inhibition is also related to other environmental factors, in particular high sugar concentration and high temperature which cause the inhibition of ethanol fermentation. Ethanol, which is produced during fermentation, is more inhibitory to cell growth than that from an exogenous source (Wayman and Rarekh, 1990).

Navarro (1980) studied the high intracellular ethanol concentrations were a consequence of resistance to diffusion through the membrane to the outside. At elevated temperature, the rate of ethanol production increased faster than the rate of excretion. Navarro and Durand (1978) also concluded that the effects of temperature on ethanol accumulation in *S. uvarum*. They found growth was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures.

The toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of certain yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman and Rarekh, 1990).

2.4.2 Effect of Nutrients

Yeasts grow in simple media which contain fermentable carbohydrates to supply energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, mineral salts and one or more growth factors. Sources of carbon included monosaccharides, disaccharides and trisaccharides (Priest and Campbell, 1996).

The metabolic activities of yeasts are greatly affected by the temperature at which they grow. Temperatures above the optimum lower the growth rate, oxygen solubility and also change the cellular composition of yeasts. It is known that under oxygen-limited conditions, yeasts require nutritional supplements for growth (Slapack *et al*., 1987 and Thomas *et al*., 2002). An increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels (Slapack *et al*., 1987).

Helena da Cruz *et al*. (2003) concluded that nitrogen and carbon are the main nutrients in fermentation medium and this implies that the mutual interaction of these nutrients may play an important role in the metabolism of yeasts The supplementation of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production. In *S. diastaticus*, Amore *et al*. (2002) reported by doubling the nutrient components in the medium, resulting in the production of 9.1% (w/v) ethanol. Increasing the fermentation temperature from 40 to 45 °C Most yeast grows well on a variety of amino acids, purines, and pyrimidines as the sole source of nitrogen. They require trace amounts of biotin, thiamine, pyridoxine, calcium

pantothenate and inositol for the maximum growth and fermentation rate (Wayman and Parekh, 1990). Amore *et al*. (2002) have also shown that role of magnesium in relieving the detrimental effect of high temperature may to some extent be related to the requirement of some of the glycolytic enzymes for this cation. In addition, increasing the cell density also resulted in an increase in ethanol production at the higher temperature resulted in a decrease in the rate and extent of glucose utilization and ethanol production.

2.4.3 pH

Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates and by-product formation. The best ethanol yields are generally obtained at pH 4.5-4.7. At higher pH, more glycerol and organic acids are formed at the expense of ethanol (Wayman and Parekh, 1990).

Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0. The gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast. A greater proportion of glucose is converted to ethanol if the pH is adjusted to 4.5. This increased conversion is independent of the presence of nutrient supplements in the medium (Thomas *et al*., 2002). If the pH is adjusted to 7 or above, acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Wang *et al*., 2001).

2.4.4 Inhibition of growth and fermentation by substrate

The production of high concentration of ethanol is frequently limited by the inhibitory effect on productivity of the fermenting microorganism exerted by the substrate, the concentration of which affects osmotic pressure (Van uden, 1989). Musts with lower sugar concentrations start to ferment sooner, and the sugar is completely fermented. High sugar concentration inhibits fermentation by their high osmotic pressure, which draws water from the yeast cells (Rehm and Reed, 1995).

Direct substrate inhibition of fermentative ability becomes significant some where between 15-25% sugar concentrations (Van Uden, 1989). Values of specific ethanol production rate and specific uptake decrease almost linearly with the increase sugar concentration.

When the substrates are introduced in several batches ethanol yields are higher (Casey and Ingledew, 1986; D,Amore and Stewart, 1987) and cell viability is close to 95% compared to 40% for single batch run(Casey and Ingledew, 1986).

2.4.5. Effect of temperature

Temperature has an important influence on growth rate of organisms and rate of ethanol production. Yeasts capable of utilizing a variety of substrate effectively to yield ethanol at temperature range of 28-35^{0}c (Kosaric and Vardar-Sukan, 2001). Though the intial rate of ethanol production is higher at increased temperatures the over all productivities of fermentation is decreased due to ethanol inhibition (Jones *et al*., 1983).

Many investivators are attemting to isolate termophilic bacteria culture which can grow and ferment at elevated temperature (Atkinson *et al*., 1975; Sree *et al*.,2000 and Limtong *et al*., 2007). Thus shifts in temperature may vary the amount of pyruvate going to ethanol, organic acid and other alcohols.

The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. As a result, it becomes necessary to cool the large fermenters in the distilleries. This necessity often becomes a major operation and a cost factor in the production of ethanol. Temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. Fermentation in industries is usually carried out at ambient temperature of 25-35ºC but temperature exceeds 40ºC during fermentation which decreases the cell viability and productivity. Maintenance of high cell viability is a major characteristic of fermentation to get high ethanol yield. Fermentation at 35-40 ºC or above has advantages such as ethanol recovery and significant savings on operational costs of refrigeration for alcohol production. Therefore many studies have been carried out or development of yeast to ferment at high temperature of up to 40-45 ºC.

2.4.6 Effect of Oxygen

The microorganisms involved in ethanol fermentation are facultative microbes since they are able to grow with or without the utilization of oxygen. Thus, two of different path ways of pyruvate metabolism are available (Abbott, 2005). In the presence of oxygen, more cell biomass is produced from initial substrate and the growth rate is increased (Alfenore, 2005). How ever for ethanol production, oxygen must be restricted from entering the fermenter. But, small concentration of oxygen must be proved to the fermenting yeast as it is a necessary component of in the biosynthesis of polyunsaturated fats and lipids (Cysewaski and Wilke, 1977 and Sa'nchez and Cardona, 2008). According to Ksaric and Vardar-sukan(2001), typical amount of oxygen maintained in the broth is 0.05-0.10 mmHg. Any value higher than this will promote cell growth at the expense of ethanol productivity. The oxygen concentration which triggers aerobic or anaerobic growth processes, is how ever, varies from culture to culture depending on substrate concentration and cell density (Munnecke, 1981).

2.5 Current status of ethanol production in Ethiopia

With inevitable depletion of world's energy supply and with the gradual increase in the price of fossil fuel, there has been an increasing world-wide interest in alternative sources of energy. It is now understood that it is important to use biomass energy as means of providing modern energy to billions who lack it. It is the renewable energy mix of the future (Gallagher *et al*., 2006).

Ethanol has emerged as an alternative for petroleum based liquid fuels. Now days, its use in automobiles as an alternative fuel has attracted many countries including Ethiopia. Currently in Ethiopia, there is only one sugar mill producing ethanol and few distilleries participating in down stream chemicals from ethanol. Among molasses driven products, ethanol takes the largest part, but its utilization must attract the attention of government policy makers in order to utilize as a bioethanol. With the present trend sugar sector expansion and modernization in the country, implementation of different domestic measures for bioethanol production utilization has to take place. At present about 5.6 million liter of ethanol is annually produced, but there are projects towards increasing the products to cover 142000 cubic meter (Ethiopian sugar develop agency (ESDA, 2005).

An efficient ethanol production requires four components: fermentable carbohydrate, an efficient yeast strain, a few nutrients and simple culture conditions. Among the widely used substrates for ethanol production molasses of sugar cane and molasses of sugar beet are the most important. This is because they are ready for conversion with limited pre-treatments as compared with starchy or cellulosic materials (Yadav *et al*., 1997)

Ethiopia through its potential in developing large sugar cane production can play a productive role in mitigating sugar, ethanol and electricity demands in the country. Molasses non-crystallizable residue remaining after crystallizing sucrose has additional advantage. For it is relatively inexpensive, readily available and readily in, use for industrial ethanol production. Immobilization offers advantages of modern technique of continuous fermentation along with low cost design and optimization of available expertise. So studies on isolation of new potent strain and improvement of the available strain to increase higher productivity are necessary for the existing and newly emerging ethanol industries of the country.

The three sugar factories that are the main supply line in the domestic market are Fincha, Wonji-Shoa and Metehara. The combination of their annual production capacity reached 2.8 million quintals of sugar. While Fincha produces eight million liters of ethanol, Wonji-Shoa and Metehara together yield 64,000 tones of molasses per year.

3. MATERIALS AND METHODS

3.1 sampling site and sample collection

The samples of Dough, Honey, *Teji* and *Tela* were collected from Godere, Gambella region and from Addis Ababa using sterile plastic bottles and transported to Mycology Laboratory of Biology Department Addis Ababa University and were kept in refrigerator until use.

3.2 Microorganism isolation

The yeast isolates used in these studies were isolated from Dough, Honey, *Teji* and *Tela* samples after dilution followed by plating aliquots of appropriate dilution of samples on yeast extract peptone dextrose agar (YEPDA). One ml of each of the sample was transferred to nine ml of sterile distilled water to be successively diluted to 10^{-1}up to $10^{-6.}$ Aliquots of 0.1 ml from final dilutions were spread plated on YEPDA (Pons *et al.,* 1986).

The YEPDA medium contains g/l: yeast extract 10, peptone 20, dextrose (glucose) 20, and agar 20. Supplemented with 0.1 mg/ml streptomycin sulphate as previously described (Osho, 2005) to inhibit the bacterial growth. The plates were incubated at 30^{o}C for 24 to 48 hrs. Morphologically distinguished colonies were then selected under a dissection microscope. The yeasts were purified by subsequent streaking on YEPD medium. Pure culture of each strain was kept on YEPD agar slants and stored at 4^{o}C until needed.

3.3 Testing of Isolates for Carbohydrate Fermentation using Durham tube method

Yeast fermentation broth base with Durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast fermentation broth media were used for identification yeasts based on fermentation of specific carbohydrates of fermentation pattern. The carbohydrate used were; glucose (dextrose), galactose, maltose, sucrose, lactose trehalose raffinose and xylose. Yeast fermentation broth was modification of media developed by Wickerham for the determination carbohydrate fermentation by yeasts for fermented carbohydrates by yeasts, the color of the medium changed from blue to yellow due to the formation of acids and gas produced (Warren and shadomy, 1991).

Composition of yeast fermentation media (broth) per liter deionized filtered water

1) Yeast fermentation broth with carbohydrate and Durham tube(D.T)

   Yeast extract ---------- 4.5g

   Peptone ---------- 7.5g

   Lactose ----------80g

   Raffinose ----------120g

   Other carbohydrates----------60g

   Bromcresolbule ----------17g final pH 7.1+or – 2 at 25^0c

2) Yeast fermentation broth control with Durham tube is the Same as (1) above except it does not contain a carbohydrate

Preparation. 4.5 g of Yeast, extract and 7.5 g of peptone were transferred to 1L of deionized distilled water and thoroughly mixed gently heat and brought to boiling and bromcresol blue was added to yeast extract peptone broth after heating. 10 ml of the broth was distributed in to the larger tube with about 150 mm by 15mm and inserted with the Durham tube of about 50mm by 6mm. Each carbohydrate was transferred in to separate flasks with specified (g/l) of deionized distilled water gently heated, filtered and sterile separately. That is all were autoclave for 15min at 15psi pressure -121^0c and cooled to room temperature, and 1ml of teste carbohydrate was transferred to test tube that contains 10ml of yeast extract peptone broth. Finally one drop of 72 hour old culture yeast suspension was added to each test tube and was incubated at 25^0c for one week. Each day the test tubes were shaken, to help sediment the yeast, and examined for bubbles of gas (CO_2) in the inserted tubes and color change of the media (Barnett *et al.,* 2000).

3.4 Screening of Yeast for ethanol tolerance

The method of Novak *et al.* (1981) was used for the screening of the yeast for ethanol tolerance. YEPD liquid medium was used for detecting yeasts for ethanol tolerance. 40 ml aliquot of the media were distributed into 100 ml conical flask. The medium was sterilized at 121^oC for 15 minute in an autoclave and cooled. Absolute ethanol was then added to different flask of the same medium to constitute varying percentages of ethanol from 8% to 18 % v/v) differing by 1% (v/v) from one flask to the other. 40 ml portion of the media were distributed into 100 ml conical flask. The media were inoculated with one ml of 48 hrs old culture yeast isolates separately. The

initial optical density of each flask was read off on a Pye-Unicam SP6 spectrophotometer at 615 nm against the medium as blank. The inoculated flasks were transferred in a gyratory shaker incubator set at 120 ppm at room temperature for72hrs. The increase in optical density in a flask was recorded as evidence of growth.

3.5 Identification of the selected ethanol tolerant yeast

In this study selected yeast isolates were characterized based on their morphological, cultural, sexual and physiological characteristics (Lodder, 1971).

3.5.1 Morphological characterization

According to the method of Kreger-van Rij(1984) and Kurtzman and Fell (1997), the morphology of the vegetative cells was grown in liquid and on solid media. Yeast cells were observed microscopically to examine their size and shape, how they reproduce vegetatively (by multilateral, bipolar or unipolar budding, by fission, by forming filaments), and the form, structure and mode of formation of ascospores.

3.5.1.1 Growth on solid medium

The morphology of cells of ethanol tolerant yeasts and their appearance on solid medium, on YEPD agar was examined, after incubating at 30°C for 3 days. The following cultural features of the isolates were recorded; texture, color and surface of colonies. Their ascospore and pseudo mycelium formation were determined.

3.5.1.2 Ascospore formation

The selected isolates examined for ascospore formation according to Kurtzman *et al*. (2005). Accordingly, two types of media were prepared, i.e. presporulation and sporulation media. The presporulation media, composed . 20g of glucose, 2g of ammonium sulfate ($(NH_4)_2SO_4$), 2g of potassium dihydrogen phosphate (KH_2PO_4), 5g of yeast extract and 1000ml of distilled water, were kept in sterile state for 7 days. The media were inoculated with a loopful young culture of 48hrs old and incubated at 25^{o}C, on shaker for 3 days.

The sporulation media, i.e. 1g of glucose, 8.2g of potassium acetate, 2.5g of yeast extract, 1.86g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 1000ml of distilled water, were inoculated with a drop of yeasts from the presporulation media. In contrast to the presporulation medium, this medium was used immediately after sterilization. The inoculated media were further incubated at 25^oC to induce ascospore formation. The culture was examined microscopically for ascospores production at weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation (Kreger-van Rij, 1984) carbol-fuchsin after have been steamed gently 5 min. Slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue; the mature ascospores stain red and vegetative cells blue.

3.5.1.3 Pseudo mycelium formation

Following to Kreger-van Rij (1984), the formation of pseudo mycelium was investigated by slide culture technique. A Petri dish was containing a U-shaped glass rod supporting glass slide, was sterilized by dry heat at 180 °C for 2 hrs. YPD agar was melted and poured into a second Petri dish. The glass slide was quickly removed from the glass rod with a flame-sterilized, and was dipped into the agar after which it was replaced on the glass rod support.

After solidification of the agar on the slide, the isolates were inoculated very lightly in two lines along slide and a sterile cover slip was placed over part of the lines. Sterile water was poured into the Petri dish to prevent the agar from drying out. The culture was incubated at 25 °C for 5 days. For observation, the slide was taken out of Petri dish and the agar was wiped off the back of the slide. The edges of the streak under and around the cover slip were examined microscopically.

3.5.1.4 Growth in liquid medium

The morphology of cells was cultured in YPD liquid medium. Cells from a young actively growing culture were inoculated into test tube containing 7 ml of medium, incubated at 30 °C for 3 days. The culture was examined the growth of ethanol tolerant yeast visually on the surface of YEPD liquid medium and the shape of cells by compound microscope (Alphaphot-2, Nikon, Japan).

3.5.2. Physiological characterization

3.5.2.1 **Fermentation of carbohydrates**

Using the same method under testing of isolates for carbohydrate fermentation ,the ability to ferment twelve different carbohydrates an aerobically, was assessed for ethanol tolerant isolates by looking for the formation of gas (CO_2) in Durham tube.

3.5.2.2 Assimilation of carbohydrates

In addition to Durham tube methods, assimilation of carbohydrates was also determined according to the method of Barnett *et al.* (2000) by the auxanographic technique on nitrogen base agar medium prepared from 5g peptone, 3g yeast extract, 15g agar, 1000ml distilled water. A volume of 15ml aliquots dispensed in test tubes were sterilized, cooled to 45^{o}C and inoculated with 500µl young cultures. These were thoroughly mixed and poured into sterile Petri dishes to solidify. Twelve carbohydrates were used for the assimilation test. About 5mg of the carbohydrates were placed on the dried agar surface at the edge of the Petri dishes opposite to one another. Three test carbohydrates were tested on each Petri dish. The Petri dishes were incubated at 28 ^{o}C for up to about 10 days and examined for growth.

3.6 Laboratory scale of ethanol production using selected ethanol tolerant yeast isolates.

3.6.1 Inoculum preparation

The yeast inoculum was prepared in YEPD broth. A loop full of selected 48 hour old culture was inoculated in to 250ml flask containing 100 ml of the medium and incubated at room temperature under shaking conditions at 25ºC on a rotary shaker (121rpm) for 72 hours. The inoculums was used at 20 percent or as specified to inoculate sterilized fermentation broth media.

3.6.2 Ethanol fermentation Media composition (g/l)

Yeast extract	5
KH_2PO_4	5
NH_4Cl	1.5
$MgSO_4.7H_2O$	0.5
KCl	1.7 and different concentration of sucrose
PH	5 (miller,1959)

3.6.3 Optimization of fermentation process

Fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature, N-source and inoculums size. Fermentation was carried out in batch mode under static condition at 30^0c. The sterile ethanol production media at various initial sucrose inoculated with selected ethanol tolerant yeast isolates and *S. cerevisiae* in to different flasks with 20 % of inoculums size.

Figure 7. Experimental setup for batch fermentation

3.6.3.1 Effect of sugar concentration

The effect of sugar concentration on growth and ethanol production by Ethanol tolerant yeast isolates and the baker yeast *S.cerevisiae* was tested by incorporating 24, 28, and 32 percent in sucrose in ethanol production media the production media.

Fermentation was carried out in 500 ml conical flasks. 72 hour old inoculums of yeast were added at the rate of 20 percent to the medium. Samples were withdrawn after every 12-hour

interval for determination of ethanol content in the media (Caputi *et al.*, 1968). Ebulliometer was employed for estimating the percentage of ethanol produced. The instrument was obtained from from Balezaf Alcohol and Liquors Factory. The initial sugar concentration that was efficiently utilized by the isolates was selected and maintained in fermentation media analyzing the other parameters.

3.6.3.2 Effect of temperature on fermentation of sucrose

In order to evaluate the effect of temperature on fermentation process the media was supplemented with 24% sucrose nitrogen source, NH_4Cl 1.5 g and phosphorous source, KH_2PO_4 5g were inoculated and incubated at 25^0C,30^0C, and 35^0C for 72hrs and the samples were analyzed.

3.6.3.3 The effect of pH

The of pH on ethanol production was tested on the same media by adjusting the pH to 3.5 pH,5 and pH 7and incubated at the temperature of 30^0C for 72 hours.

3.6.3.4 The effect of supplement on ethanol production

The effect of nutrient supplement for ethanol production was tested as follows. A 24% sucrose at pH5 was prepared and supplemented with different additives such as ammonium nitrate (1g/l), ammonium phosphate(1g/l),yeast extract(1g/l) and urea (5g/l) (Munnecke,1981). The effect of these supplements on ethanol yield was evaluated after measuring the ethanol content of the fermented broth.

3.6.4 Cell biomass Determination

Following the methods used by Campelo and Belo (2004) after completion of fermentation, the fermentation broth in each was filtered the suspension in each flasks were centrifuged at 5000Xg for 10 minutes several times with intermittent washing with cold distilled water. Each yeast biomass (pellet) was dried in an oven at 60 ºC to constant weight. dry weight of yeast was measured using Methler balance (Scaltec).

3.6.5. Detrmination of ethanol content in the fermentation broth

For this specific study Ebulliometer obtained from Balezaf alcohol and liquors factory for measuring the concentration of ethanol. Ebulliometer is an equipment designed to evaluate the boiling point of different types of liquids. Its use in the wine industry is based on the fact that alcohol boils at (78.4°C) a lower temperature than water, so the boiling point of alcohol-water mixtures changes as a function of their concentration. A precision thermometer is involved, to determine the boiling temperature of the ethanol.

4. RESULTS

4.1 Isolation of yeasts for ethanol tolerance

A total of 20 of yeast-like colonies were isolated from twelve samples of *Tela, Teji*, Honey and teff dough. Six isolates from *Tela*, six isolates from Teji, six isolates from Honey and two isolates from dough. It was possible to get pure colonies that to be used fermentation experiment .most colonies whitish convex and dom shaped except one isolate from honey was pink and convex (appendix 1). All the samples isolates were reproduce vegetatively budding . None of the isolates form fission. The isolates from *Tela* were designated by TBY (TBY1, TBY2, TBY3, TBY4, TBY5, and TBY6).The yeast isolates from *Teji* were designated by TGY(TGY1,TGY2,TGY3, TGY4, TGY5 and TGY6).The isolates from Honey were designated by HGY (TGY1, TGY2, TGY3,TGY4,TGY5,TGY6) and for the Teff dough the designation was DYP (DYP1 and DYP2).

4.2.1 Durham tube fermentation tests for selection of isolates

In this study isolates showed variation of utilization of nine different sugars (Table4). The fermentation set up was indicated by (appendix 2). Almost all isolates utilized Glucose,Galactose,sucrose maltose and Rafinose,and many were capable of metabolizing Trehalose. all isolates were failed to grow on lactose exceptTGY2, HGY2and HGY4and xylose, but isolate TGY2 and TGY6 . The most vigorous isolates were TBY1, TGY1, TGY2,TGY3, HGY2, HGY4 and HGY5 that were fermentative on several of the test carbohydrate(Table 4),but the rate of fermentation variesin all isolates . The most fastidious once were TBY4, TBY5, TBY6 TGY4 and TGY6.

Table 4. Comparison and selection of isolates by Durham tube carbohydrates fermentation method.

Isolates	Fermentation									
	Glucose	Galactose	Fructose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Xylose	Total
TBY1	++	++	+++	++	+	-	++	+	-	7
TBY2	++	++	++	++	+	-	+	+	-	7
TBY3	+	+	+	+	+	-	+	+	-	7
TBY4	+	+	+	+	-	-	+	+	-	7
TBY5	+	+	+	+	+	-	+	-	-	6
TBY6	+	+	+	+	+	-	+	-	-	6
TGY1	++	++	+++	+++	++	-	+	+-	-	7
TGY2	+++	+++	+++	++	++	+	+	+	+	9
TGY3	++	++	++	++	+	-	+	-	-	6
TGY4	+	+	+	+-	+	-	+	-	-	6
TGY5	++	++	++	++	+-	-	+	+	-	7
TGY6	+	+	+	+	+	-	+	+	+	8
HGY1	+	+	+	++	++	-	+	+	-	7
HGY2	+	+	++	+	+	+	+	+	-	8
HGY3	++	+	+	+	+	-	+	+	-	7
HGY4	++	++	++	++	++	++	+	+	-	**8**
HGY5+	++	++	++	++	++	-	+	+	-	7
TGY6	+	+	+	+	+	-	+	-	-	6
DYP1	++	+	++	+	+	-	++	+	-	7
DYP2	+	+	+	+	+	-	+	+	-	7
Total	20	20	20	20	20	2	20	15	1	

Key: +=fermentative,++= high fermentative,+++=very high fermentative(Durham tube empty)

4.3 Screening of Yeast for ethanol tolerance

Ten isolates which were rapid fermentative were selected for screening of yeasts ethanol tolerant (table 5).The most ethanol tolerant was TGY2 with ethanol toleranceto 16%(v/v) followed by isolateTBY1which was resistant to 15.5% ethanol in the media .the least ethanol tolerant was HGY4 to ethanol concentration of 9 % (v/v) followed by HGY3 which was resistant to10%(v/v) ethanol in the media. Accordingly TBY1 and TGY2 were relatively rapid fermentative and the highest ethanol tolerant. HGY2 was resistant to high ethanol concentration in the media but slow fermentative when compared to TBY1 and TGY2. Depending on the data obtained, TBY1and TGY2 were selected for further analysis.

Table 5. Ethanol tolerance levels of selected yeasts isolates.

Isolate	Mean ethanol tolerance level (% v/v)	Sugar utilization (9 sugars)
TBY1	15.5	7
TBY2	12	7
TBY3	11	7
TGY1	13	7
TGY2	16	9
TGY3	12	6
HGY1	13	7
HGY2	14	8
HGY3	10	7
HGY4	9	8

4.3 Identification of the yeast isolates TBY1and TGY2

4.3.1 Morphological and physiological characteristics

Appendix 3 shows the features of the appearance of cultures when cells grown in YEPD broth and on YEPD agar. After 3 days of incubation at 30^0C, heavy, dry climbing pellicles were formed on the surface of YPED broth medium. The growth was butyrous ,and white cream color on YPD agar. The cell morphology of the ethanol tolerantTBY1and TGY2 under compound microscope (example TGY2 appendix 4), are ovoidal to elongate have single, pairs, or triple budding cells were present and pseudo mycelia were also developed.

Following to the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997), ascospores formation was detected for indication of the ascomycetous yeasts. As shown in appendix 5 ascospore formed in ascospore forming media after incubating for 3 weeks at 25 °C. Morphological characteristic of the isolates were summarized in table 6.

Table 6. Morphological characteristics of yeast isolates

character	TBY1	TGY2	Baker yeast
Surface	Smooth	Smooth	smooth
Margin	crispulate	crispulate	slightlycrispulate
Colour	Cream,white	Cream,white	Cream,white
Elevation	Convex	slightlyConvex	convex
Cells	spheroidal,ellipsoidal Multilaterialbudding	spheroidal, ellipsoidal Multilaterialbudding	spheroidal,ellipsoidal Multilaterialbudding
Ascospores	+	+	+
Pseudomycelium	+	+	+

In addition to morphological test, ethanol tolerant yeast isolates TBY2 and TGY2 were identified based on fermentation and assimilation carbohydrates using twelve different carbohydrates(appendix 6 and 8). The result which was compared the similarity percentages of sugar acidification and or enzymatic reactions patterns with reference strain of the *Saccharomyces cevisiae* was demonstrated in table 7. According to the data obtained TGY2 was highly fermentative and assimilated most of the sugar, but TBY1 showed almost similar fermentation and assimilation compared with *Saccharomyces cerevisiae*

Table7. Physiological properties of the selected yeast isolates using Durham tube fermentation and auxanographic technique (V = variable, + = positive, - = negative)

Isolates	Fermentation												Assimilation											
	Glucose	Galactose	Fructose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Starch	Dextrin	Xylose	Cellulose	Glucose	Galactose	Fructose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Starch	Dextrin	Xylose	Cellulose
TBY1	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	v	-	-	-	-
TGY2	+	+	+	+	v	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-
S.cer	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	v	-	-	-	-

4. 4 Growth studies and effect of sugar concentration

The growth of TBY1 and TGY2 in gradually increasing concentrations of sugar showed an increase in optical density up to 32% sugar concentration in YEPD broth medium as shown in Table 8. Samples were taken every 12 hours for the study of growth kinetics. The growth measured at 600nm using spectrophotometer. However at the sugar concentration of 32 % the growth was decreased in the first 12 hrs. As the concentration sugar increase from 24% to 32% the optical density of both the isolates and baker yeast were decreased gradually. Their optical density were highest at24% and lowest at 32% of sugar concentration.

Table 8. Effect of increasing sugar concentration on growth of TBY2, TGY2 and baker yeast

Time	Sugar concentration in % (g/l)								
	TBY1			TGY2			Baker yeast		
	24%	28%	32%	24%	28%	32%	24%	28%	32%
0	0.44	0.53	0.75	0.45	0.54	0.78	0.45	0.54	0.74
12	0.59	0.58	0.48	0.62	0.60	0.69	0.60	0.59	0.46
24	1.38	1.37	1.30	1.40	1.38	1.31	1.49	1.58	1.31
36	1.56	1.55	1.44	1.57	1.56	1.44	1.57	1.55	1.43
48	1.78	1.77	1.45	1.79	1.78	1.46	1.81	1.76	1.45
72	1.99	1.96	1.78	2.00	1.98	1.79	2.01	1.97	1.78

4.5 Effect of temperature on ethanol yield

Temperature is one of the major constraints that determine the ethanol production. To know the optimum temperature for ethanol fermentation, the solutions were kept at 25, 30, 35 and with 24% initial sugar concentration. The sucrose solution of 24% and fermentation was carried out in 500ml flasks. Samples were withdrawn every 12 hours and the fermentation was carried out for 72 hours. A low ethanol yield, 5.5 %(v/v) was observed at 25°C in 72 hours by the baker yeast . As shown in Table 9 at 30°C. Ethanol yield was maximum and turned out to be 11 %(v/v) by the isolate TGY2. However increasing the temperature beyond 30°C the growth as well as concentration of alcohol decreased. This decrease was pronounced at 35 so 30°Cwas selected as optimum temperature for ethanol production.

Table 9. Effect of temperature on ethanol production

Time in hrs	Temperature in (^{0}C)								
	TBY1			TGY2			Baker yeast		
	25^{0}C	30^{0}c	35^{0}c	25^{0}C	30^{0}c	35^{0}c	25^{0}C	30^{0}c	35^{0}c
12	1.2	3.2	2.1	1.5	4.5	3.2	1.12	4.0	2.8
24	3.2	5.2	4.1	3.2	5.4	4.3	2.3	4.0	3
36	4.3	7.4	5.2	4.8	8.0	5.6	3.80	5.2	4.01
48	5.6	9.3	7.6	6.0	9.3	7.5	4.2	6.2	5.3
72	6.7	10.2	8.6	7.5	11	8.55	5.5	7.8	6.5

4.6 Effect of pH on ethanol yield

Initial sugar concentration of 24% and optimum temperature of 30°C was selected for further studies and subjected to pH treatments 3.5, 5 and 7 .The results were shown in table 10.At pH 3.5 fermentation took place but it gave low ethanol content. Best results were obtained at pH5 where maximum ethanol production was noticed for the isolates and baker yeast. How ever yeast isolate TGY_2 was not much affected by pH in range 5 -7 as indicated in table10. Decrease in pH from 5 to 3.5 was found to hinder the fermentation of sucrose.

Table 10. The effect of pH on ethanol production.

pH	Ethanol produced by isolates in %(v/v)		
	TBY1	TGY2	*S.cervisiae*
3.5	5.2	6.6	5.1
5	10.2	11.0	8.4
7	8.2	8.5	7.8

4.7. The effect of supplements on ethanol yield

The effect of additives in fermenting media was evaluated. Compared to control (sucrose solution with out any additive) ethanol yield with supplements effect was shown on (Figure 8). The additives were urea, yeast extract, ammonium phosphate and ammonium nitrate. The supplemented sucrose did show a slight increase in yield in all supplementation case when

supplemented with $(NH4)_2PO4$ showed the best ethanol yield increase than the other supplemented media compared to control. The least ethanol yield was observed in a media supplemented with yeast extract when compared to the other supplemented medi

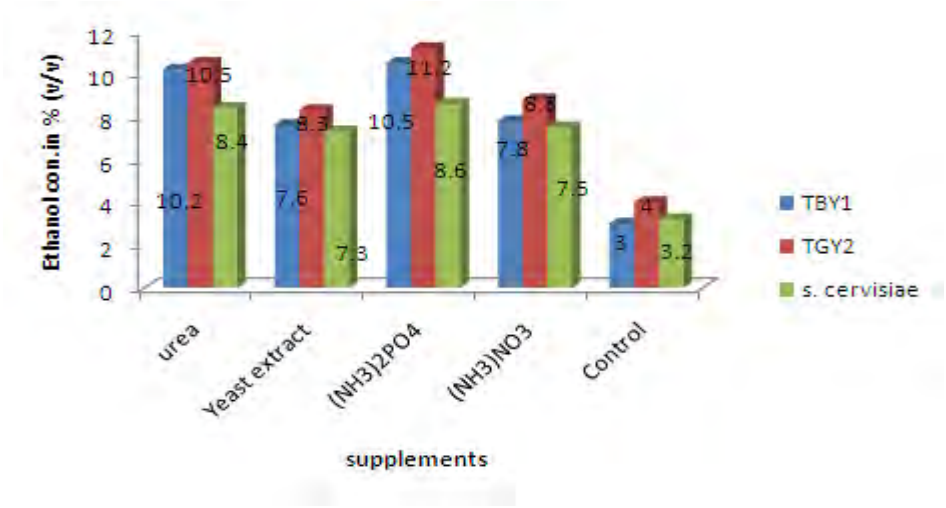

Figure 8. Comparison of the effect of supplements on ethanol yield

4.8 Evaluation ethanol production in an increasing concentration of sucrose

After optimizing the various parameters like pH, temperature, and intial sugar concentration ethanol production was evaluated in increasing concentration sucrose in 24%, 28% and 32% at PH 5 and temperature of 30^0C. And the result was described in appendix 9 and table 11).

Table 11. The amount ethanol produced by the selected yeast strains in different sucrose solution

Substrate	Ethanol concentration by the yeast strains in (v/v)		
	TBY1	TGY2	*S. cerevisiae* (Baker yeast)
24% sucrose	10.2	10.5	8.4
2 8% sucrose	11.3	11.5	9.6
32% sucrose	10.5	10.5	9.6

4.9 Biomass yield at the end of fermentation

The yeast biomass was determined after the end of fermentation i.e after 72 hours. The maximum cell density was recorded for *TGY2* in Batch fermentation was determined with initial sugar concentration of 24% g/l as it was indicated in table 12.

Table 12. Biomass of isolates after fermentation

Isolates	Net weight in (g/l)
TBY1	1.46
TGY2	2.14
Baker yeast	1.39

5. DISCUSSIONS

In this study, twenty yeasts were isolated from *Tella, Teji*, Honey and dough. Based on the their colony characteristics (creamy and white texture), they have apex ovoid microscopic shape. All the isolates were tested for fermentation of carbohydrates isolates were capable utilizing 6-9 sugars out of the totally nine tested sugar indicating that they were diverse in sugar utilization(table). Among the isolates ten of them which were relatively rapid fermentative were screened for ethanol tolerance and showed variation from resistance to 9% -16% ethanol content in the growth media. All the ten isolates were diverse in sugar utilization (table 5).

The most ethanol tolerant was TGY2 with ethanol tolerance to16 %(v/v) followed by isolateTBY1which was resistant to 15.5% ethanol in the media. The *Teji* isolate yeast (TGY2) and the *Tella* isolate yeast(TBY1) were compared with the commercial baker's yeasts. Based on their colony characteristics (white and creamy texture), ovoid microscope shape, the presence of four ascospore in ascus, and budding pattern (multipolar), all isolates were found to belongs to *sacharomyces* type unicellular ascomycete according to Lodder (1971) and (Boekhout and Kurtzman, 1996). These results were consistent with the previous findings that yeast from *teff* dough and *tella* are *saccharomyces* type (BerhanuAbegazGashe *et al.*,1982 ;Samuel Sahle and Birhanu Abegaz Gashe,1991 and Tamene Milkessa,2009).

Like the palm wine yeast, TBY_1 and TGY_2 isolates exhibited remarkably high ethanol tolerance comparable to industrial yeasts such as sake and distiller's yeasts associated with high level of ethanol tolerance (Casey and Ingledew ,1986 and Hayashida *et al.*,1974). The level of ethanol tolerance of 16 %(v/v) by the yeast strain TGY_2 is in agreement with Nwachukwu *et al*. (2006) on *saccharomyces cevisiae* isolated from raffia palm wine. It is interesting to note that isolates TBY_1andTGY_2showed resistance to 15.5% and16 % (v/v) ethanol concentration, Almost Similar tolerance of at16.5 % (v/v) ethanol have been observed for *saccharomyces cerevisiae* (Teramoto *et al.*, 2005).

The yeast isolates, TBY1 ,TGY2 and reference yeast were able to grow in the concentration from 24% to 32% of the media containing sucrose .Their optical density were decreases as the sucrose concentration increases and their optical density at the concentration of 32% of sucrose deceases for all the isolates in the first twelve hrs and less than the initial optical density of the

inoculums and increases gradually after twelve hrs of incubation at 30^0C. The isolates and the reference strain were tolerance to osmotic pressure up to 32% (w/v) sucrose (table 8). Some workers (D'amore *et al*., 1988) have reported change in growth dynamics of yeasts up on exposure to various osmotic stress conditions, and the decrease in logarismic growth rate constants of the test yeasts in relation to increasing osmotic pressure is there for consistent with the views expressed by these workers (Osho, 2005)

Bekatorou *et al*. (2006) and Verstrepen *et al*. (2004) showed that high substrate concentration would lead to catabolic repression by glucose and sucrose, which is known as crab tree effect, may leads to several problems, such as incomplete fermentation, development of off flavors and undesirable by products as well as decreased biomass and yeast vitality.

The ability of the two ethanol tolerant yeast isolates to withstand osmotic stress has been amply demonstrated by the fact the yeasts were able to grow in media containing relatively high degree of sugar concentrations. Ekunsanmi and Odunfa, (1990) asserted that the combination of sugar tolerance and alcohol tolerance is an advantage when a yeast is being considered for industrial use especially when ethanol is being produced.

When one compares the over all pattern of growth of the three isolates along substrate concentration and incubation periods they showed difference in optical density in substrate concencentrations of 24% - 32 % suggesting that optimization is very important to obtain the maximum yield.

Optimization of growth and ethanol yield was evaluated on the bases of different pH (pH3.5,pH5 andpH7) at 24%substrate concentration and 30^0C. the isolates showed different pattern of ethanol production of 5.2%-10.2% (v/v) for isolate TBY1, 6.6%-11.0 % (v/v) for isolate TGY2 and and 5.1% - 8.4% (v/v)for the baker 's yeast. This shows that TGY2 isolate was found to be produce 11% (v/v) ethanol and more tolerant to pH than TBY1 and bakers yeast .All the isolates were more effective at pH5,and were produce less ethanol at the 3.5 pH (table 10). Similar study Yeasts specifically *Saccharomyces* are an acidophilic organism and, as such, grows better under acidic conditions. The optimal pH range for yeast growth can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. This likely is due to the optimum pH value for

the activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath and Power, 2005).

The effect of temperature on growth and ethanol production was showed that the highest ethanol production was displayed at 30^0C by the isolates TGY2 11% (v/v) and TBY1 10.2% (v/v) after incubation for 72hrs.There was lowest ethanol yield in all isolates at 25^0C (table 9).The fermentation activities of yeasts will decrease the temperature of 25^0C and below due to catabolic activates of the enzymes decreases as temperature lower temperature Du Preez,*et al* .(1987) and at35^0C the ethanol production was also reduced for all isolates. The evolution of heat at higher temperature during fermentation inhibited ethanol yield (Morimura *et al.,* 1997).

After having identified the optimization of substrate concentration, pH and temperature additional nutrient supplements were also tested further increase the ethanol yield. Consequently, supplementation of additional nitrogen sources (urea, yeast extract, ammonium phosphate and ammonium nitrate) and with no supplementation was analyzed. All the isolates were produced the high ethanol with supplementation of ammonium phosphate, TGY2,11.2% (v/v),TBY1 10.5% (v/v) and baker'syeast8.6% (v/v) ethanol (figure 8).

Walker (1998) reported that ammonium phosphate is a commonly used as nitrogen source for yeast growth media since it also provides a source of assimilable phosphate. Some yeast can also grow on nitrate as a source of nitrogen and can utilize low, sub-toxic concentration of nitrate. Ammonium salts of organic acid are better utilized than salts of inorganic acids, since their decomposition gives rise to weak acids that can serve as and additional carbon source. Utilization of inorganic salts gives rise to strong inorganic acid that change the pH and have an inhibitory effect on cells (Kocková-Kratochvílová, 1990).

In addition to optimizing the different parameters, ethanol production was optimized using 24%,28% and 32% of sucrose concentration at pH5 and temperature 30^0Cfor 72hrs.the maximum ethanol yield was observed at 28%sucrose for the three isolates, TGY2 11.5% (v/v),11.3% TBY1 and baker's yeast 9.6% (v/v), when the concentration of sugar increases from 24%to 28% in the media ethanol yield also increases. But the yield was not increased when the concentration of sugar increased from 28% to 32% the maximum yield was obtained at the sugar concentration of 28% so, 28% was used for further ethanol production (table 11). The ability of

the two ethanol tolerant yeast isolates to withstand osmotic stress during fermentation has been amply demonstrated by the fact the yeast strains were able to grow in media containing relatively high degree of sugar concentrations. This observation is in agreement with the suggestion of Gray (1945) who stated that ethanol tolerant yeasts tend to be sugar tolerant. Ekunsanmi and Odunfa, (1990) asserted that the combination of sugar tolerance and alcohol tolerance is an advantage when a yeast is being considered for industrial use especially when ethanol is being produced.

The biomass of the isolates were determined the end of fermentation accordingly, they showed differences in biomass accumulation. The biomass of the isolates TGY2 2.14g/l, TBY1 1.46 and Baker's yeast 1.39 g/l (table12). This shows that biomass accumulation was directly proportional to the ethanol yield. In the conversion of carbohydrates to ethanol were yeasts are involved, optimal conversion requires cells that are tolerant to high concentration of both substrate and product and are able to efficiently produce ethanol (Walker, 1998). The effect of ethanol on the cell of yeast depends on permeability of cell membrane which is depends on the accumulation of unsaturated fatty acid in the membrane, and the extent of ethanol tolerance of certain yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman and Rarekh, 1990).

6. CONCLUSION

The highest ethanol tolerant yeast were isolated from *Tela* and *Teji* ,TBY1and TGY2 where able to tolerate ethanol concentration of 15.5 and 16 % (v/v) respectively. The isolates where able to produce 10 to 11.5% (v/v) by using 28% of sucrose as the substrate. The isolates were grouped under the genus saccharomyces depending up on their morphological and physiological characteristics and were closely related to *S. cerevisiae* .The optimum temperature required for the isolates was 30^0c at optimum pH5 .Generally both isolates were able to produce relatively high amount of ethanol from concentrated sugar solution.

The result obtained from this study reveal a strong indication of yeast's great potential in the production of ethanol using locally available substrates specially molasses.

7. RECOMMENDATIONS

The potential of ethanol tolerant yeasts from different alcoholic beverages and sugar sources were not exploited. Therefore, efforts should have to be directed to ward exploitation of ethanol tolerant yeasts specially from honey, honey wine, different types of *Tella* and other sugar sources by collecting samples from different regions of the country for novel strain discovery and application of it for industrial ethanol production.

Further studies should be conducted on characterization and utilization of the selected isolates.

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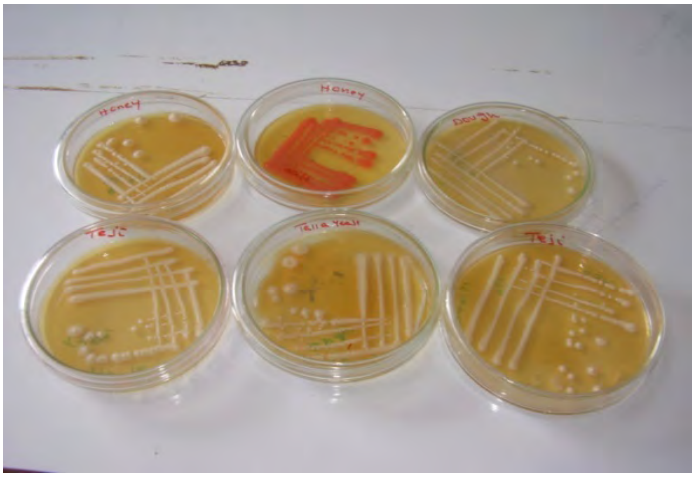
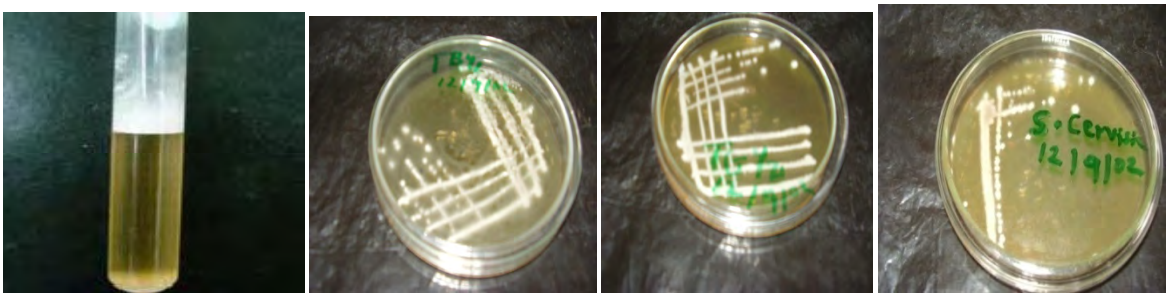
8. APPENDICES

Appendix 1. Pure isolates of the four samples



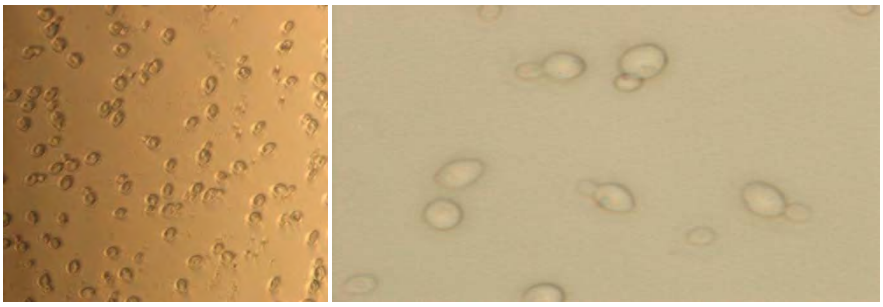
Appendix 2. Durham tube carbohydrate fermentation set up

Appendix 3. The growth of yeast cells inYEPD broth and on YEPD agar medium



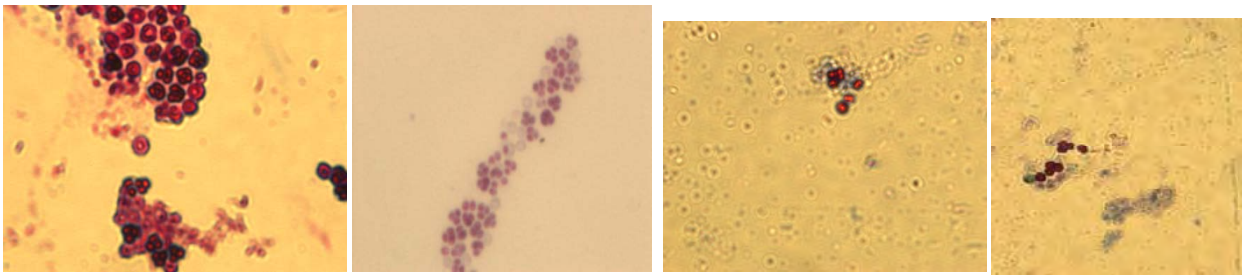
A.growth in YEPD broth B. TBY1 C.TGY2 D. *S.cervisiae*

Appendix 4. Morphological characteristics of the ethanol tolerant yeast strain TGY2 under compound microscope.



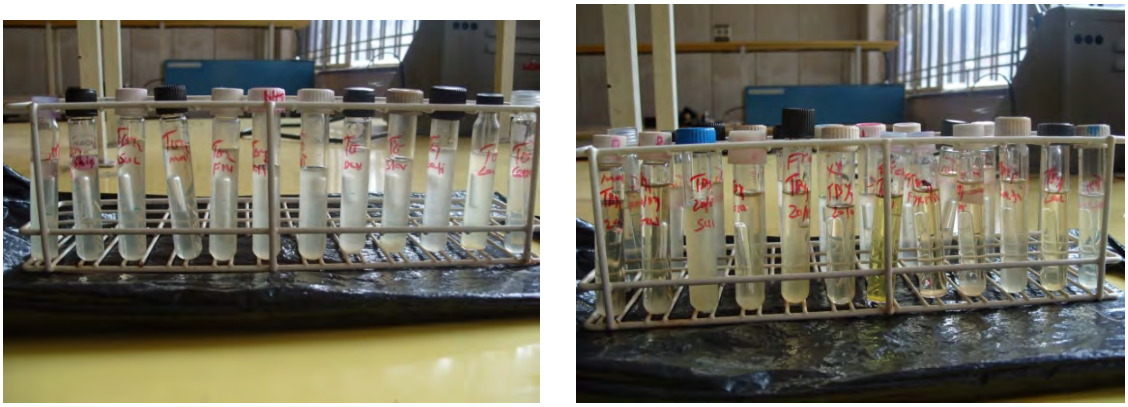
TGY2 Vegetative cell of TGY2

Appendix 5. Ascospores of the ethanol tolerant yeast strains and baker yeast



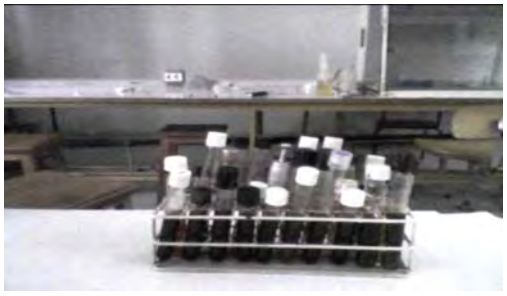
TBY1 **Baker yeast** TGY2

Appendix 6. Carbohydrate fermentation test for characterizing of isolates.



TGY2 TBY2

Appendix 7. Media for carbohydrate assimilation test



Appendix 8. Carbohydrate fermentation and assimilation for identification of ethanol tolerant yeast strains of TBY1 and TGY2 ,with referance to baker yeast

TBY1 TGY2 *S. cerevisiae* (Baker yeast)

Appendix 9. Laboratory scale ethanol production by batch fermentation system

DECLARATION

I the undersigned person declaredthat this thesis is my original work and that all sources of material used for the thesis have been genuinely acknowledged.

Name : Dechassa Tolessa

Signature: _____

Date:_____

This thesis has been submitted for the examination with my approval as

Advisor's Name : Dawit Abate (Ph.D)

Signature :_____

Date : _____