ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INSTITUTE OF BIOTECHNOLOGY

Characterization of Wild Yeasts Isolated from Selected Fruits for their Bread Leavening Capacity

BY

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This is to certify that the thesis prepared by Eshet Lakew, entitled with: **Characterization of Wild Yeasts Isolated from Selected Fruits for their Bread Leavening Capacity** and submitted in partial fulfilment of the requirement for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT: *Leavening agents are important in raising flour dough. Biological leavening agents are microorganisms that have the ability to produce carbon dioxide from the utilization of Sugar and thereby ferment and raise the dough. The present study was carried out to characterize yeast isolates isolated from selected fruits and to assess their leavening potential of wheat dough under laboratory scale. The collected fruit samples were processed to isolate yeasts using Potato Dextrose Agar (PDA) amended with 0.1 g/L chloramphenicol. Initially, 88 yeasts were isolated from the fruits and were first tested for their carbohydrate fermentation in yeast extract peptone dextrose (YEPD) broth medium. Six yeast isolates with their sugar fermentative abilities were selected and tested for H_2S production. Among them, AAUGr5, AAUOr7 and AAUPi3 found not produce undesirable H_2S for bread baking quality on both Kligler Iron Agar (KIA) and Bismuth Sulfite Agar (BSA) media. The three yeast isolates were identified as* Saccharomyces *using colonial, morphological parameters and biochemical tests. The optimum growth pH and temperature values for the three selected yeast isolates were recorded as 5 and 30 ^oC, respectively, in YEPD medium. In addition, 30% (w/v) D-glucose and 5 %(w/v) NaCl concentrations showed optimum growth of the three selected yeast isolates in yeast extract peptone broth medium. In all the cases, the maximum biomass was achieved at 96 hrs of incubation and there was a rapid decrease in biomass for all the yeast isolates after 96 hrs of incubation. In terms of CO_2 and biomass production as well as leavening potential, starter cultures which were formulated from the combination of the three yeast isolates (AAUGr5+AAUOr7+AAUPi3) showed better performance than starter cultures formulated from paired combination of the three isolates or each of the three isolates separately. However, isolate AAUGr5 was found to be satisfactorily potent for leavening action from the single isolates. The present study could therefore be important with respect to screening of wild yeast isolates that possess better bread leavening potential for extending the use of indigenous microbes as starter culture in bakery sector.*

Keywords/Phrases: biomass, carbon dioxide, fruits, hydrogen sulphide, laboratory scale leavening, yeast isolation

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List of Abbreviations

BSA	Bismuth sulfite agar
hrs	hours
KIA	Kligler Iron Agar
ml	Mili liter
OD	Optical Density
PDA	Potato Dextrose Agar
rpm	Revolution per minute
w/v	Weight per volume
YEPD	Yeast Extract Peptone Dextrose

1. Introduction

Bread is reported to be one of the most ancient human foods that was produced with the help of microorganisms by an ancient Egyptian bakery at the Giza Pyramid area in the year 4000 B.C (Willey *et al.,* 2008). *Saccharomyces cerevisiae* is the most commonly used species in the genus *Saccharomyces* for bread baking. It has been employed as baker's yeast in bread baking for at least 6,000 years (Bell *et al.,* 2001). It does not only induce and increase the volume of dough through gas incorporation but helps in creating the desired flavor and texture (Fleury *et al.,* 2002).

Today, baker's yeast is used for bread baking throughout the world at industrial scale. With the improvement of bread industry, the use of starter culture is increasing tremendously. Dough is usually leavened by baker's yeast, which ferments dough sugar and produces mainly carbon dioxide and alcohol (Hamelman, 2004; Edwards, 2007). However, other gas producing microorganisms e.g., wild yeasts, coliform bacteria, *saccharolytic* Clostridium species, heterofermentative lactic acid bacteria and various naturally occurring mixtures of these organisms have been used for leavening of dough instead of bread yeast alone (Bratovanova, 1996).

Baker's yeast is a mass of viable cells of *Saccharomyces cerevisiae*, which is a unicellular fungus that multiplies asexually through budding. *Saccharomyces cerevisiae* is a facultative anaerobe that can survive under both aerobic and anaerobic conditions. During bread baking, the yeast grows aerobically resulting in increased carbon dioxide production and minimum alcohol accumulation via fermentation of sugars (Willey *et al*., 2008).

Burrows (1970) listed four functions of yeast in bread baking: 1) to increase dough volume by evolution of CO_2 during fermentation of the available carbohydrates in the flour, 2) to develop structure and texture in the dough by the stretching due to expansion of gas bubbles, 3) to improve flavor and 4) to add some nutritive values of bread.

Saccharomyces cerevisiae to bread baking accomplishes more than the mere catabolism of sugar into ethanol and carbon dioxide. A myriad of flavor compounds are also formed. While the desirability of some of these compounds are a function of their concentration, some others are contributors to off-flavors. One of such off-flavor compound is hydrogen sulfide. Hence, yeast to be used for bread baking should be free from producing this undesirable compound (Jiranek *et al.,* 1996).

The yeasts can be propagated using cheap raw materials and easily harvested due to their bigger cell sizes and flocculation abilities. The raw materials used as substrates for industrial yeast biomass production are usually agricultural, forestry and food wastes. These are materials like starch, molasses, distiller's wash, whey, fruit and vegetable wastes, wood, straw, etc.,(Jay, 1996).

Being a sugar loving microorganism, it is usually isolated from sugar rich materials. Fruits contain high sugar concentration so yeast species are naturally present on them and can be easily isolated. There is always a search for wild/nontoxic fermentative yeast species for their further industrial exploitation in baking industry. Discarded citrus fruits have also been proposed as growth media for the production of starter cultures like baker's yeast, for applications in bread baking (Plessas *et al.,* 2008). Moreover, baker's yeast produces not only high amount of CO_2 but also several proteins, vitamins, minerals and flavoring agents that aid in the overall taste, color and flavor of the bread.

The scientific knowledge and technology allow the isolation, construction and industrial production of yeast strains with specific properties to satisfy the demands of the baking and fermentation industry (baking, beer, wine) (Phaff,1990). Fermentative yeasts are utilized as starter cultures, for the production of specific types of fermented foods like bread, fermented meat and vegetable products, etc. The significance of yeasts in food technology as well as in human nutrition, as an alternative source of protein to cover the demands in a world of low agricultural production and rapidly increasing population makes the production of food grade yeasts extremely important (Bekatorou *et al.,* 2006*).*

Ethiopia is a developing country with high population pressure that posed increased demand for baker's yeasts. Most of the baking industries in Ethiopia use baking powder and the country imports most of its requirement of baking powder mainly from China (ESA, 2016). It is being imported in huge amount (385,107.88 kg) of baking powder every year for baking purpose (ESA, 2016).Very recently, there is a growing need in baking quality bread in the country that consequently increased the import size of starter culture for bakeries with considerable amount of local currencies 14,714,533.88 birr (ESA, 2016), which is highly detrimental for the Ethiopian economy. Therefore, the present study was initiated to assess the potential of indigenous yeasts as leavening agent for bread baking.

2. Objectives

2.1. General objective

The general objective of the current work was to:

- ♠ Characterize wild yeasts isolated from selected fruits and to determine their baking capacity

2.2. Specific objectives

The specific objectives of the present study were to:

- ♠ isolate, identify and characterize yeasts isolated from fermented fruits (avocado, banana, grape, mango, orange, papaya and pineapple) using cultural, morphological and biochemical tests
- ♠ screen the yeast isolates on the basis of sugars fermentation ability, production of H_2S, growth at different temperatures, different pH values, different NaCl concentrations and different D-glucose concentrations
- ♠ examine the effect of agitation and aeration on biomass of potent yeast isolates
- ♠ assess the wheat dough leavening potential of the screened yeast isolates separately and in combination

3. Literature review

3.1. General characteristics of yeast

3.1.1. Yeasts and classification

Ascomycete yeasts (phylum Ascomycota: subphylum Saccharomycotina: class Saccharomycetes: order Saccharomycetales) comprise a monophyletic lineage with a single order of about 1500 known species (Kurtzman *et al.,* 2011). The name "*Saccharomyces*" derived from Greek, and means "sugar mold". "*Cerevisiae*" comes from Latin, and means "of beer" (Balasubramanian and Glotzer, 2004). They are classified in the kingdom fungi, phylum Ascomycota and family *Saccharomycetaceae* (Mueller *et al.,* 2004). Yeasts (*Saccharomyces cerevisiae*) are living unicellular, eukaryotic and ubiquitous microorganisms commonly found on fruits, vegetables and other plant materials. Some yeasts are found in association with soil and insects (Slaviková and Vadkertiova, 2003). They are also called brewer's yeast, ale yeast, top-fermenting yeast or budding yeast.

3.1.2. Genus *Saccharomyces*

Genus *Saccharomyces* (previously called *Saccharomyces sensu stricto*) currently includes the species *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces bayanus* (Naumov, 1987), *Saccharomyces cariocanus, Saccharomyces mikatae*, *Saccharomyces kudriavzevii* (Naumov *et al.,* 2000), *Saccharomyces arboricolus* (Naumov *et al.,* 2010) and *Saccharomyces eubayanus* (Libkind *et al.,* 2011). *Saccharomyces bayanus* includes two varieties: uvarum and bayanus (Rainieri *et al.,* 2006).

The ecology of *Saccharomyces* species is diverse. Several species of this genus have been only found in natural environments, this is the case of *Saccharomyces mikatae* (partially decayed leafs), *Saccharomyces kudriavzevii* (decayed leafs, soils and oaks), *Saccharomyces arboricolus* (oak trees), *Saccharomyces cariocanus* (Drosophila sp.) and *Saccharomyces eubayanus* (bark); whereas *Saccharomyces cerevisiae*, *Saccharomyces paradoxus* and *Saccharomyces bayanus* have been found associated to both natural and biotechnological environments (Phaff, 1990).

3.1.3. Reproduction and cell cycle

Yeast typically grow asexually through vegetative multiplication but can also reproduce sexually by forming ascospores (Fig.1). The cell cycle in budding or vegetative multiplication consists of four distinct phases (G1, S, G2 and M). The sexual reproduction involves the formation of four haploid spores (two MATa and two MATα) and is induced during nutrient starvation (Taxis *et al.,* 2005). During conjugation, two cells of opposite mating type (MATa and MATα) fuse to form a diploid zygote (Jackson and Hartwell, 1990). Strains that can be maintained stably for many generations as haploid are termed heterothallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homothallic. The large majority of *Saccharomyces cerevisiae* industrial strains are homothallic (Jackson and Hartwell, 1990).

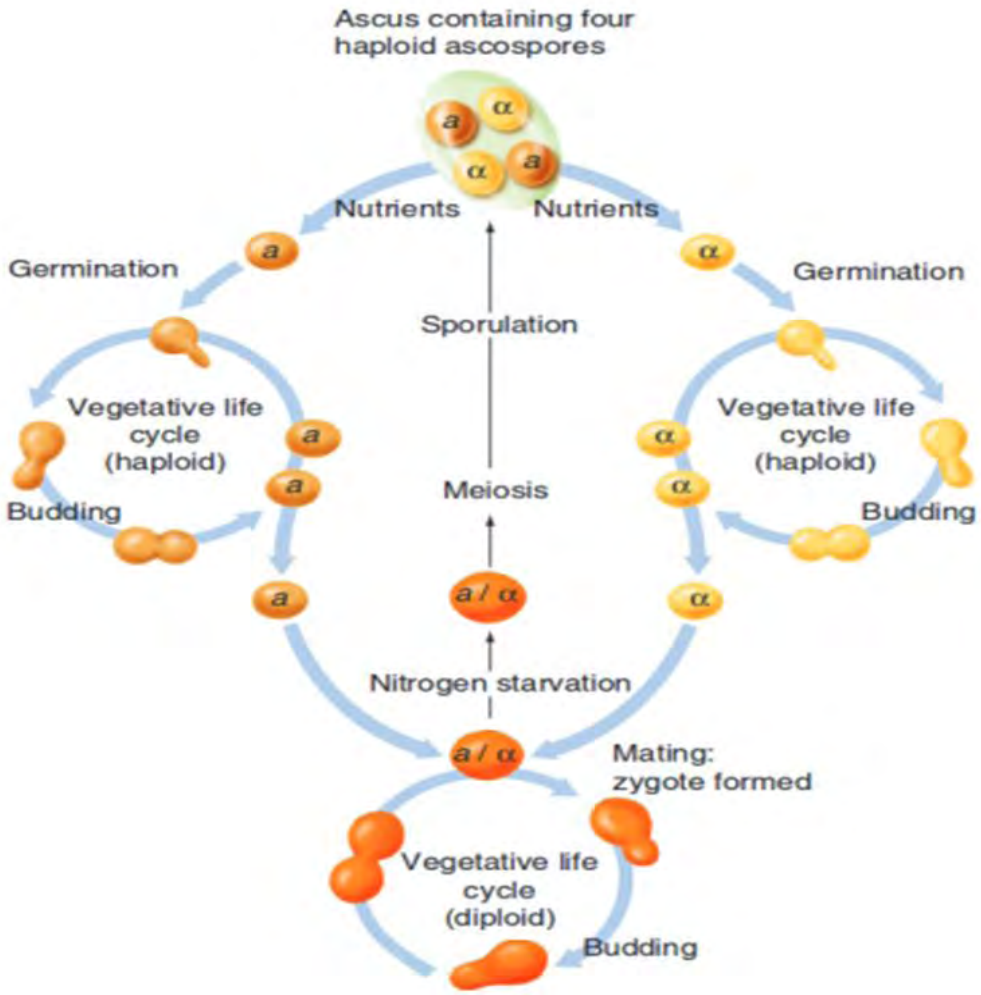


Fig.1 Life cycle of yeasts, (Adapted from Clara, 2015).

Yeast population growth is the result of cell division and the progression through the cell cycle. Under optimal growth conditions, yeast growth kinetic follows the typical microbial growth curve, comprising four phases: lag phase, exponential phase, stationary phase and death phase (Fig. 2). The lag phase reflects the time required for yeast cells to adapt to their new environment by synthesizing ribosomes and enzymes needed to establish growth at a higher rate. The duration of this phase depends firstly on the initial population size and secondly on environmental conditions. Once the cell starts actively metabolizing, they begin DNA replication and shortly after the cells divide. This begins the second phase of growth called the exponential phase of growth. This is the period in which the cells reproduce at maximum specific growth rate (μmax). The time it takes the population to double is called generation time (Clara, 2015).

Yeast strain, growth medium, and temperature are important factors in determining the generation time. Industrial fermentations aim to extend this phase for maximizing the output of biomass and metabolites production (López *et al.,* 2004). The third phase in yeast growth is stationary phase; a period of no growth when metabolism slows and cell division is stopped. The factors that cause cells to enter stationary phase are related to change in the environment, such as nutrient deprivation, toxic metabolites and high temperatures. After prolonged periods in stationary phase, cells may die and autolysate which constitutes the last phase of yeast growth called death phase (López *et al.,* 2004).

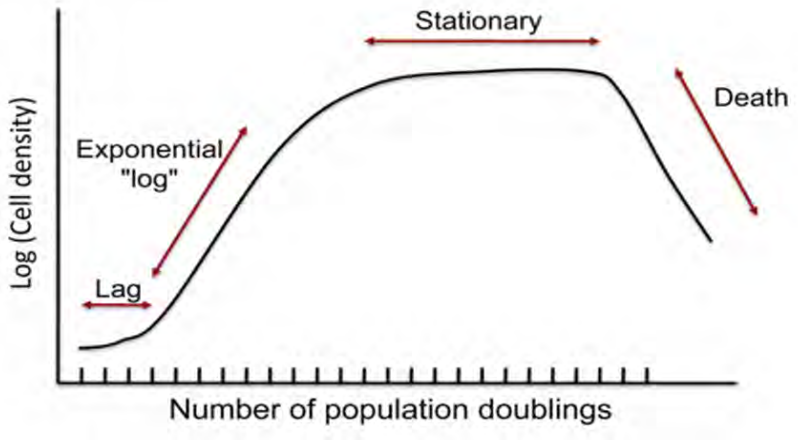


Fig.2 Standard yeast growth curve, (Adapted from Werner, 1996).

3.1.4. Yeast Identification

Classical culture-based diagnostic methods use morphological characteristics of yeasts (size, colour and shape of the colony), as well as biochemical (fermentation of selected carbohydrates, assimilation of carbon or nitrogen from selected organic compounds, acid production, etc.). These methods, however, require long time waiting for the final score, some lasting up to 1-2 weeks. Therefore, laboratories are increasingly choosing rapid diagnostic tests, such as API® Candida, API® 20C AUX, and ID32C® that shorten identification time to 24-48 hrs.

Commercial tests are based on evaluation of selected biochemical properties with assigned values, which in turn are given a numerical code designating the species (Katarzyna, 2011). Yeast identification using molecular taxonomy with improved speed and accuracy in identification due to their established and comprehensive databases for comparisons of strains have been reported (Kock *et al.,* 1985; Botha and Kock, 1993). These techniques have also found application in production environments such as in monitoring the succession of active yeast species during bread baking (Esteve-Zarzoso *et al.,* 1999), in analysis of restriction fragment length polymorphism of the ITS region, allowing for detection and quantification, of different yeast species (Vasdinyei and Deak, 2003).

The complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved), are useful in measuring close fungus genealogical relationships (Fig.3). This is due to their ability to exhibit far greater interspecific differences than the 18S and 25S rRNA genes (Cai *et al.,* 1996). Ribosomal regions evolve in a concerted fashion and hence show a low intraspecific polymorphism and a high interspecific variability. This has proved very useful in the classification of *Saccharomyces* species (Wyder and Puhan, 1997), *Kluyveromyces* species (Belloch *et al.,* 1998) and for the identification of a small collection of baker's yeast species (Guillamón *et al*., 1998). The use of two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA and subsequent sequencing of this domain allows for rapid and accurate species identification (Daniel and Meyer, 2003). According to Frutos *et al.,* (2004), the use of D1/D2 domain is generally accepted as the main tool for yeast taxonomy allowing for identification of new ascomycetous yeasts previously not recognized as novel through use of conventional identification techniques (Kurtzman, 2000).

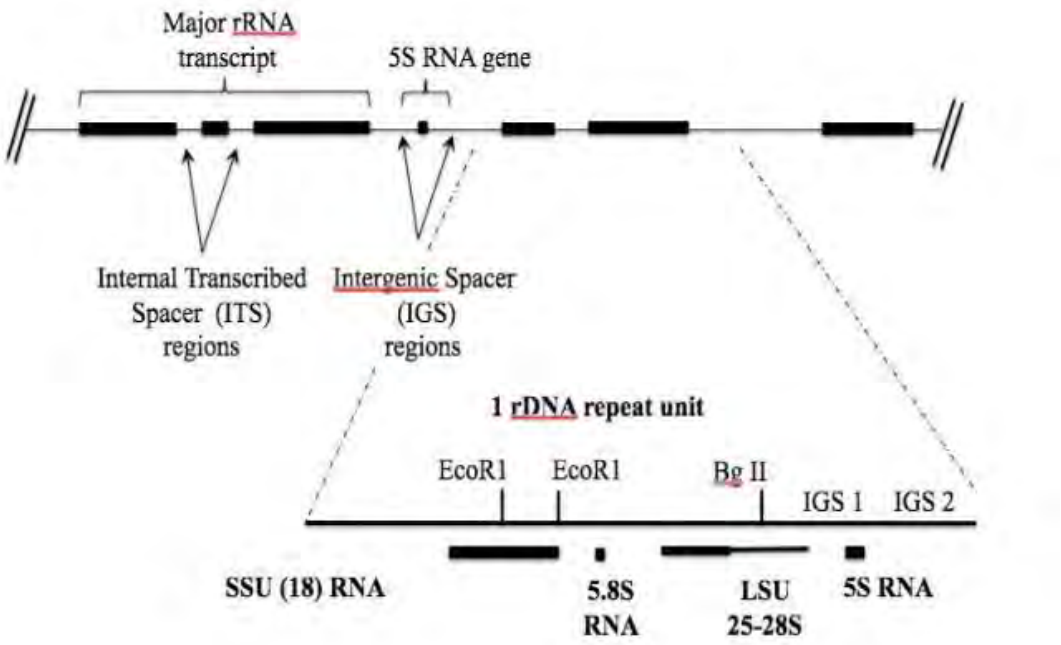


Fig.3 Schematic representation of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) (Adapted from Gargas and De Priest, 1996).

Databases of the D1/D2 sequences are available for all currently recognized ascomycetous and basidiomycetous yeasts. This extensive database makes species identification much easier and could serve as reliable and practical criteria for identification of most known yeasts (Hesham *et al*., 2006).

3.1.5. Importance of yeast in food fermentation

Genus *Saccharomyces* possesses a series of unique characters that are not found in other genera (Vaughan-Martini and Martini, 1998). One of these unique characteristics is their high capability to ferment sugars vigorously, either in the presence or in the absence of oxygen, to produce ethanol. This ability allows them to colonize sugar rich substrates (plant saps and fruits) and compete with other yeasts, which are not so tolerant to alcohol (Martini, 1993).

The apparition of angiosperm plants with sugar rich saps and fruits introduced a new ecological niche with a different selection regime that likely imposed altered physiological demands to the ancestors of *Saccharomyces* yeasts (Wolfe and Shields, 1997). Under such circumstances, adaptive evolution took place in this new ecological context favoring the acquisition of such high fermentative capability. This capability has unconsciously been used by humans to produce fermented foods and beverages, which introduced new selective pressures on these yeasts. Neolithic human populations likely observed that fruit juice spontaneously ferment producing an alcoholic beverage (Mortimer *et al.,* 1994). Since then, the yeast *Saccharomyces cerevisiae* and related species become an essential component of many important human activities including baking, brewing, distilling and wine making. In general, these industrial *Saccharomyces* strains are highly specialized organisms, which have evolved to utilize the different environments or ecological niches that have been provided by human activity. This process can be described as "domestication" and is responsible of the peculiar genetic characteristics of the industrial yeasts. During the last years, intensive researches have been focused on elucidating the molecular mechanisms involved in yeast adaptation to the industrial process, and the reshaping of genomic characteristics of the industrial yeast which have been unconsciously selected over billions of generations (Querol *et al.,* 2003). Among them, the most useful and widely exploited species are those from the *Saccharomyces* genus, especially *Saccharomyces cerevisiae*. The ability of this genus to degrade carbohydrates has been unconsciously used by humans for thousands of years to ferment a broad type of beverages; cider, beer, wines, etc.(Querol and Fleet, 2006).

3.2. Nutrition and their growth

Saccharomyces cerevisiae requires the following essential nutrients for the multiplication of its cells, in the presence of atmospheric oxygen; 1. A source of fermentable organic carbon and energy, 2. Assimilable nitrogen composition, and 3. The essential minerals $PO4^{-3}$, K^{+}, $SO4^{-2}$, Mg^{2+} and trace element ions. A complete understanding of the nutrition of *Saccharomyces cerevisiae* is required in order to optimize its growth and metabolic activities (O'dell *et al.,* 1997).

3.2.1. Carbon Sources

Saccharomyces cerevisiae is able to use various organic compounds as sources of carbon and energy. Carbon can be used aerobically or anaerobically as it is shown in Fig.4.

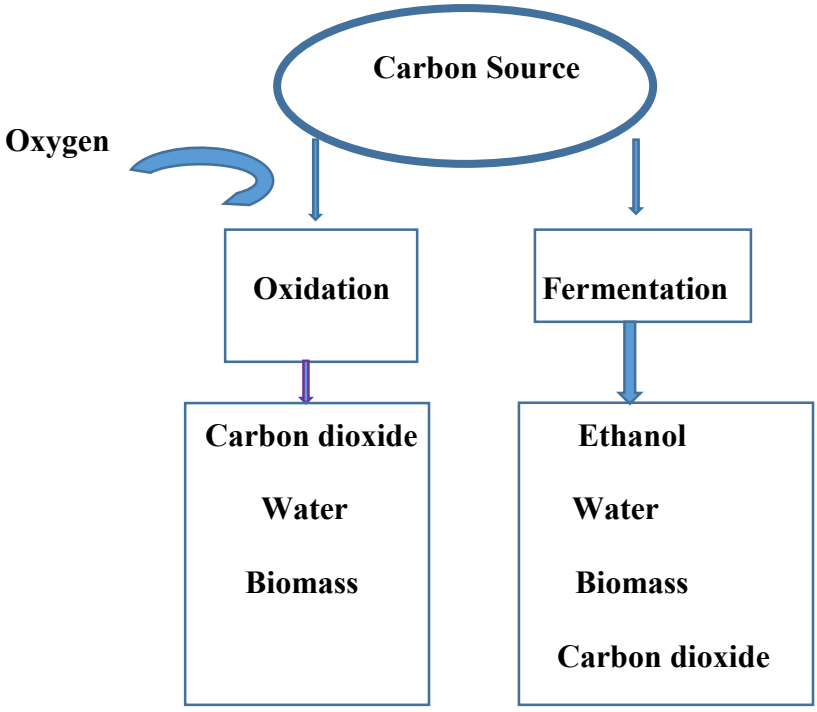


Fig.4 Utilization of carbon by yeast, (Modified from Berna, 2002).

However the speed of cell multiplication, cell yield and catabolism then differ, in particular some sources of carbon are oxidizably assimilable by the yeast, but not fermentable. Only a small portion of sugars is fermentable. Combined sources of carbon and energy useful to *Saccharomyces cerevisiae* as shown in Table 1;

Table 1 Useful carbon and energy sources for *Saccharomyces cerevisiae*

Source of carbon	**Oxidatively Assimilable**	**Fermentable**	**Source of carbon**	**Oxidatively Assimilable**	**Fermentable**
Starch	-	-	D-erythritol	-	-
Dextrine	-	-	Mannitol	+-	-
Maltotriose	+-	+-	Glycerin	+	-
Maltose	+	+	Ethanol	+	-
Cellobiose	-	-	Methanol	-	-
Sucrose	+	+	Gluconic a.	-	-
Lactose	-	-	5-keto	-	-
Glucose	+	+	Lactic acid	-	-
Fructose	+	+	Citric acid	-	-
Galactose	+	+-	Succinic acid	-	-
Arabinose	-	-	Acetic acid	-	-
Xylose	-	-	Paraffins	-	-
Sorbite	+-	-			

(Adapted from Berna, 2002)

+ = The yeast isolates ferments and assimilates the carbon source, - = The yeast isolates do not ferment and assimilate the carbon source and +- = The yeast isolates may or may not ferment and assimilate the carbon source

The carbon source for baker's yeast propagation usually consists of assimilable sugars, such as glucose, fructose, mannose, galactose, sucrose, maltose and hydrolyzed lactose. Ethanol has also been used, at least partially, as a substrate for yeast production. In order to be assimilated, these compounds must be transported into the yeast cells. Lactose can be used after being hydrolyzed (Bronn, 1985).

3.2.2. Nitrogen Sources

Nitrogen can be assimilated by *Saccharomyces cerevisiae* as shown in Table 2;

Table 2 Assimilation of N_2 derivatives by *Saccharomyces cerevisiae*

Source of N_2	**Assimilation of N_2**
$NH4^+$	+
$NO3^-$	-
Protein hydrolysate	+
Simple amino acids	
Aspartic acid	+
Glutamic acid	+
Amides	
Asparagine	+
Glutamine	+
Urea	+

(Adapted from Berna, 2002)

+ = The yeast isolates assimilates the N_2 source and - = The yeast isolates do not assimilate the N_2

The type of nitrogen assimilation with the least problem is with ammonium ions in the form of ammonium hydroxide solution or ammonium salts and when an organic source of nitrogen is replaced by amino acids, due to the amino acid's carbon content, another source of carbon is added which will influence the yeast yield. Amino acids have a regulatory effect on the yeast metabolism they accelerate or slow down the process (Bronn, 1985).

3.2.3. Minerals

An essential but often neglected part of the nutritional requirements of yeast is the ionic constituents of the medium. The chelation of metal ions by organic components of the fermentation substrate, e.g. molasses or corn step liquor and other physicochemical attributes that can affect ionic availability such as pH or ionic strength. Limitation of trace elements may therefore be more common than is realized. In other cases, the removal of potentially toxic metal ions from solution by binding or complexation may even enable yeast growth and fermentation to proceed in the presence of total metal concentrations that would otherwise be toxic (Bronn,1985).The essential mineral phosphorus is assimilated by yeast only in the form of the anion phosphate ($PO4^{3-}$). If the quantity of phosphate is insufficient, not all available nitrogen is absorbed, despite a possible surplus of nitrogen. Conversely, if there is a lack of nitrogen, not all available phosphate is assimilated, despite a possible surplus of phosphate.

3.3. Yeast Metabolism

Yeasts are facultative anaerobes and can grow with or without oxygen. In the presence of oxygen, they convert sugars to CO_2, energy and biomass. In anaerobic conditions, as in alcoholic fermentation, yeasts do not grow efficiently, and sugars are converted to intermediate by-products such as ethanol, glycerol and CO_2 (Balls *et al.,* 2007). Therefore, in yeast propagation, the supply of air is necessary for optimum biomass production. The main carbon and energy source for most yeast is glucose supplied from molasses, which is converted to the glycolytic pathway to pyruvate and by the Krebs cycle to anabolites and energy in the form of ATP. Yeasts are classified according to their modes of further energy production from pyruvate to respiration and fermentation. These processes are regulated by environmental factors, mainly glucose and oxygen concentrations. In respiration, pyruvate is decarboxylated in the mitochondrion to acetyl-CoA, which is completely oxidized in the citric acid cycle to CO_2, energy and intermediates to promote yeast growth. In anaerobic conditions, glucose is slowly utilized to produce the energy required just to keep the yeast cell alive. This process is called fermentation, in which the sugars are not completely oxidized to CO_2 and ethanol (Bekatorou *et al.,* 2006; Scragg, 1991).Yeasts can metabolize various carbon substrates but mainly utilize sugars such as glucose, sucrose and maltose. Sucrose is metabolized after hydrolysis into glucose and fructose by the extra cellular enzyme invertase.

Maltose is transferred in the cell by maltose permease, and metabolized after hydrolysis into two molecules of glucose by maltase. Some yeast can utilize a number of unconventional carbon sources, such as biopolymers, pentoses, alcohols, hydrocarbons, fatty acids and organic acids (Bailey *et al.,* 1977). Elements like; N, P, S, Fe, Cu and Zn are essential to all yeasts and are usually added to the growth media. Most yeast are capable of assimilating directly ammonium ions and urea, while very few species have the ability to utilize nitrates as nitrogen source. Phosphorus and sulphur are usually assimilated in the form of inorganic phosphates and sulphate, respectively.

3.4. Food Grade Yeasts

Various microorganisms are used for human consumption worldwide as single cell protein or as components of traditional food starters, including algae (Spirulina, Chlorella, Laminaria, Rhodymenia, etc.), fungi (Aspergillus, Penicillium, etc.) and yeasts (*Saccharomyces*, *Candida*, *Kluyveromyces*, *Pichia* and *Torulopsis*) (Bekatorou *et al.,* 2006). Among the yeast species, *Saccharomyces cerevisiae* and *Candida utilis* are fully accepted for human consumption, but very few species of yeast are commercially available. The most common food grade yeast is *Saccharomyces cerevisiae*, also known as baker's yeast, which is used worldwide for the production of bread and baking products (Ravindra, 2000). *Saccharomyces cerevisiae* is the most widely used yeast species, whose selected strains are used in breweries, wineries and distilleries for the production of beer, wine, distillates and ethanol (Suh *et al.,* 2006). Baker's yeast is produced utilizing molasses from sugar industry by products as a raw material. Commercial *Saccharomyces cerevisiae*, *Saccharomyces carlsbergenesis* and other yeast products available to cover the needs of the baking and single cell protein fermentation industries or for use as nutritional supplements for humans (Haider *et al.,* 2003).

3.5. Characteristics of baker's yeast fermentation

A selected strain of baker's yeast, *Saccharomyces cerevisiae*, is used for industrial-scale production. These strains are selected for stable physiological characteristics, vigorous sugar fermentation in dough, rapid growth and high cell yields, and easy maintenance during storage.

The fermentation of baker's yeast has to produce a product with minimum variation in yeast performance, maximum yield on raw material, and minimum production of undesirable side products (Haider *et al.,* 2003). Under aerobic conditions, *Saccharomyces cerevisiae* uses sugars such as glucose to grow cell mass rather than produce alcohol. If the rate of sugar uptake is higher than the transport rate of respiratory intermediates into the mitochondrion, the metabolism favored ethanol production and limited the specific oxygen uptake rate. If the respiratory intermediates transport rate into the mitochondrion was equal, the transport of sugar into the cell, carbon dioxide (CO_2) was the major metabolite with little or no ethanol produced and a much higher specific oxygen uptake rate occurred (Gelinas *et al.,* 1998). In aerobic batch fermentation, *Saccharomyces cerevisiae* can only produce a limited amount of respiration enzymes. If the glucose concentration is more than 5% in the medium, the respiratory intermediate enzymes are suppressed and the ethanol mechanisms dominate (Darlington, 1964).

3.6. Formulation methods of bakery yeasts

Baker's yeast as a commercial product has several formulations that can be grouped into two main types: compressed yeast, called fresh yeast, has moisture content 70-75 % (Cook, 1958) and the dried yeast (Daramola and Zampraka, 2007). Compressed yeast is the traditional formulation of baker's yeast, and is ready for immediate use. Dried (dehydrated) yeast is available in two forms: Active dry yeast (ADY) and instant dry yeast (IDY). Active dry yeast (ADY) is normally sold in air tight packages, vacuum seal or filled with an inert gas such as nitrogen. It is not a problem to maintain quality, but it should be rehydrated before use. Unlike ADY, instant dry yeast (IDY) does not have the cell damage during rehydration (Daramola and Zampraka, 2007).

4. Materials and Methods

4.1. Sampling site and sample collection

A total of fifty six fruit samples eight each from avocado (*Persea americana*), banana (*Musa acuminate),* grape *(Vitis vinifera),* mango *(Mangifera indica),* orange *(Citrus sinensis L.),* papaya *(Carica papaya)* and pineapple *(Ananas comosus)* were collected from local markets (Atikilit Tera and Merkato) of Addis Ababa City.

4.1.1. Sample preparation

Fruit samples preparation was done following the method of Thais *et al.,* (2006). The collected samples were placed aseptically in sterile plastic bags and brought to the laboratory. The fruit samples were stored at 4 ^{o}C until use. Before any pretreatment, each fruit sample was washed in sterile distilled water to make it free of any extraneous matter. The fruit samples were separately cut, crushed with mortar in sterile plastic bags. The homogenates of avocado (300g), banana (400g), grape (250g), mango (450g), orange (250g), papaya (600g) and pineapple (800g) were prepared and were separately transferred into the sterile beakers along with 50 ml of sterile distilled water. The beakers with homogenates were covered with aluminum foil. Thereafter the mixtures were kept at normal room temperature for three days to allow fermentation to take place.

4.1.2. Isolation of yeasts

One ml of each of the samples were transferred to 9 ml of sterile distilled water and mixed thoroughly. A tenfold serial dilutions (10^{-1} -10^{-6}) were done. From appropriate serial dilutions, aliquots of 0.1 ml were spread plated on potato dextrose agar (PDA, India) plates containing (dextrose, 20 g/L; potato infusion, 200 g/L; agar, 15 g/L; pH, 5.6 and 1L sterile distilled water). Chloramphenicol (0.1 g/L) was added to inhibit bacterial growth. The samples were incubated at 30 ^{o}C for 48 hrs. After incubation, different colonies were picked (each colony represented one isolate) on the basis of their colony shape and color (Barnett *et al*., 2000). The colonies were purified by repeated sub culturing using streak plate method on freshly prepared PDA. The purified isolates were transferred to PDA slant and preserved at 4 ^{o}C for further study.

4.2. Cultural characterization

Cultural characteristics like (shape, color, edge, elevation, etc) of yeast isolates were performed following the methods of (Barnett *et al*., 2000) on PDA medium after 48 hrs incubation.

4.3. Major screening parameters of yeast isolates for bread leavening

4.3.1. Test of CO_2 production

The yeast isolates were selected from seven fruit samples using sugar fermentation test using Durham tube in YEPD broth medium containing; yeast extract, 5 g/L; peptone, 5 g/L; D-glucose ,10 g/L; within 24 hrs for the production of CO_2.

4.3.2. Test of H_2S production

To examine H_2S production (associated with an off-flavor and unpleasant taste), selected yeast isolates were streaked on both Bismuth Sulfite Agar(BSA), and Kligler Iron Agar (KIA) containing plates and incubated at 30ºC for 3 days. Colonies that exhibited black color on BSA plates and any blackening of the KIA along the line of inoculation or throughout the butt indicate hydrogen sulfide production (Jiranek *et al.,* 1995).

4.4. Standard culture and maintenance

The active dry baker's yeast, by the name (Saf instant) from DSM bakery ingredients, Holland was used for the investigation as standard culture. A 0.5 g of this yeast was used for the experiment by suspending in 50 ml sterile distilled water aseptically. Serial dilutions (10^{-1} -10^{-6}) were done to reduce the number of yeast cells as described in section 4.2.2. Aliquots of 0.1 ml of the suspensions were spread plated on to PDA medium. The cultures on the agar plate were incubated at 30ºC for 48 hrs. The cultures checked for purity and maintained at 4 ºC for further study.

4.5. Identification of yeast isolates

Identification of yeast isolates to genus level was carried on the basis of standard cultural, morphological and physiological/biochemical tests as described by (Harrigan and McCance, 1982; Barnett *et al.,* 2000; De Maristela *et al*., 2006).

4.5.1. Morphological characterization

In order to determine morphology of yeast cells and reproduction type, the cultures were examined microscopically (Barnett *et al.,* 2000). Vegetative cells were observed after 3 days of incubation at 30 °C in YEPD liquid containing (yeast extract, 5 g/L; peptone, 5 g/L and D-glucose, 10 g/L) medium and 1L of sterile distilled water. A sample of yeast was mixed in a droplet of sterile distilled water on glass slide and smeared until the smear dried off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope (OLYMPUS BX51,Germany) at (X100) magnification using oil immersion objective.

4.5.2. Induction of ascospore formation and observation

For production of ascospores from fruit and commercial yeasts, the methods of Lodder (1971) and Kirsop and Kurtzman (1988) were followed. Accordingly, two types of sporulation media were prepared. These were Gorodkowa agar and Macclary acetate agar media**.**

4.5.2.1. Preparation and sterilization of Gorodkowa agar medium

Ten grams of peptone, 1 g D-glucose, 5 g of NaCl and 20 g of agar were dissolved in a liter of distilled water. The contents were boiled. Thereafter, the medium was dispensed into tubes and were sterilized by autoclaving (at 121 ^{o}C for 15 minutes). After completion of the autoclaving tubes were allowed to lean to make slants.

4.5.2.2. Preparation and sterilization of Macclary acetate agar medium

A 2.5 g, of yeast extract, 1 g of D-glucose, 1.8 g of potassium chloride and 20 g of agar were dissolved in a liter of sterile distilled water. The constituents were heated and dispensed into tubes. They were autoclaved and slanted. Into both media, loopful of yeast samples (24 hrs culture) were inoculated and incubated at 25 ^{o}C for 3 weeks.

4.5.2.3. Observation of ascopores

Yeast samples were wet mounted on a glass slide to observe types of ascospores. The smears were also heat fixed and spores stained according to Lodder (1971). Accordingly, the heat fixed smears were flooded with 5% aqueous malachite green for 30-60 seconds. The excess stain was run off under running tap water for half a minute. The preparations were then counterstained with 0.5% safranin red for about 30 seconds. The excess stain was gently washed with running tap water for half a minute. The preparations were observed both under high power (X40) and oil immersion objectives (X100).

4.6. Viability determination of selected yeast isolates

One ml of each of the actively growing (24 hrs culture) yeast isolates in YEPD containing; yeast extract, 5 g/L; peptone, 5 g/L, D-glucose, 10 g/L were transferred to 9 ml of sterile distilled water and mixed thoroughly. A tenfold serial dilutions (10^{-1} -10^{-6}) were done. From appropriate serial dilutions, aliquots of 0.1 ml were spread plated on potato dextrose agar (PDA, India) plates containing (dextrose, 20 g/L; potato infusion, 200 g/L; agar, 15 g/L; pH, 5.6 and 1L sterile distilled water). The samples were incubated at 30^{o}C for 48 hrs. After incubation, colonies were counted to determine number of viable cells.

4.7. Biochemical characterization

The biochemical methods were based on the utilization of carbon and nitrogen sources, as previously described by (Harrigan and McCance, 1982; De Maristela *et al*., 2006). The ability of yeast isolates to utilize D-glucose, fructose, maltose, galactose, lactose and sucrose as a sole carbon source and production of gas was determined in Durham tubes in carbohydrate fermentation medium. A positive reaction was detected by observation of color change to yellow and gas formation in Durham tubes (for carbohydrate fermentation) in the solution.

4.7.1. Carbohydrate fermentation

A 10 g yeast extract and 10 g peptone were transferred into 1L of sterile distilled water and thoroughly mixed. The pH was adjusted to 5 and the medium was boiled. Bromocresol purple carbohydrate (2%, w/v) fermentation broth was used and added to yeast extract peptone broth and dispensed in 5 ml amount into screw capped test tubes containing inversely placed Durham tubes. The test tubes with medium were autoclaved at 121^{o}C for 15 minutes. The sugar and nitrogen solutions were sterilized similarly at 121^{o}C for 15 minutes in a separate flask. An amount of 0.5 ml of sterile sugar and nitrogen solution was added aseptically into each culture tubes containing sterile yeast extract peptone broth. The carbohydrate fermentation test was performed by inoculating (approximately 3.6 x10^{6} cells mL^{-1}) yeast cells into the tubes which contained 5 ml of yeast extract peptone broth in each along with different sugar and nitrogen (10%, w/v) for each sources (six basic sugars which included; D-glucose, fructose, maltose, galactose, lactose, sucrose, KNO3 and $(NH4)_2SO4$ and incubated at 30^{o}C for 48 hrs.

Changing of color from violet to yellow indicated the acid production from carbohydrate fermentation and the accumulation of gas bubbles in inverted Durham tube indicated CO_2 gas production. No color change indicated negative result.

4.8. Analyzing factors affecting the growth of yeast isolates

4.8.1. Growth at different temperature

For testing the ability of the isolates to grow at different temperature values, each isolate was inoculated in 50 ml yeast extract peptone D-glucose (YEPD) broth medium containing yeast extract, 5 g/L; peptone, 5 g/L; D-glucose, 10 g/L and 1L sterile distilled water. The pH of the medium was adjusted to 5 before autoclaving. Tubes containing these medium were inoculated with same number of actively growing yeast cells (approximately 3.6 $x10^6$ cells mL^{-1}) and incubated at the four different temperature values (25, 30, 35, 42^{o}C). Optical density at 550nm was determined using a spectrophotometer (6405 UV/Vis, JENWAY, United Kingdom) at intervals of 24, 48, 72, 96 and 120 hrs as a measure of growth.

4.8.2. Growth at different pH Values

YEPD liquid medium containing yeast extract, 5 g/L; peptone, 5 g/L; D-glucose, 10 g /L was prepared in a separate flasks and the pH was adjusted to 4, 5 and 6 using 1N HCl and 1N NaOH which was used for detecting the ability to grow in different pH of selected yeast isolates. The isolates were inoculated (approximately 3.6 $x10^6$ cells mL^{-1}) in 50 ml YEPD broth and incubated at 30^{o}C. The growth was determined after 24, 48, 72, 96 and 120 hrs by measuring the optical densities at 550nm using a spectrophotometer.

4.8.3. Growth at different D-glucose concentration

To determine the ability of the isolates to grow at different D-glucose concentrations, yeast extract, 5 g/L; peptone, 5 g/L; 30, 40 and 50% (w/v) D-glucose broth was prepared with pH value of 5.
The isolates with same number of actively growing (approximately 3.6 $x10^6$ cells mL^{-1}) were inoculated into 50 ml flasks and incubated at 30^{o}C. The growth was determined after 96 hrs by measuring the optical densities at 550nm using a spectrophotometer.

4.8.4. Growth at different NaCl concentration

For the growth of the yeast isolates at different NaCl, yeast extract, 5 g/L; peptone, 5 g/L; 5, 10 and 15% (w/v) NaCl broth was prepared with pH value of 5. The isolates with same number of actively growing (approximately 3.6 x10^6 cells mL^{-1}) were inoculated in 50 ml flasks and incubated at 30^{o}C. The growth was determined after 96 hrs by measuring the optical densities at 550nm using a spectrophotometer.

4.9. Determination of agitation and aeration effect

In this test yeast isolates were inoculated into YEPD broth containing yeast extract, 5 g/L; peptone, 5 g/L; D-glucose, 10 g/L with pH value of 5. Same amount of actively growing yeast cells, (approximately 3.6 x10^6 cells mL^{-1}) were inoculated and incubated in both shaking incubator (at 30^{o}C and 140rpm) and unshaken incubator (at 30^{o}C). After 4 days incubation, cell mass was measured at 550nm using spectrophotometer.

4.10. Starter culture formulation of selected yeast isolates

For starter culture formulation of selected yeast isolates were growing in YEPD medium containing; yeast extract, 5 g/L; peptone, 5 g/L and D-glucose, 10 g/L for 72 hrs incubation period at 30^{o}C by inoculating the yeast isolates (approximately 3.6x10^6 yeast cells) in a separate flask of 250 ml YEPD broth medium each. Finally the cultures were harvested by centrifugation at (3000 rpm for 10 minutes) and the biomass was measured to use for leavening analysis. The type and number of yeast isolates included in the starter culture formulation was done as indicated in the Table 3.

Table 3 The type and number of yeast isolates included in the starter culture formulation

Starter culture number	Yeast isolates
1	AAUPi3
2	AAUGr5
3	AAUOr7
4	AAUOr7+ AAUPi3
5	AAUGr5+ AAUPi3
6	AAUGr5+ AAUOr7
7	AAUGr5+ AAUPi3+ AAUOr7

And the commercial yeast, SFI and negative control without yeast were used.

4.11. Leavening analysis of selected yeast isolates

Bread baking dough was prepared by mixing wheat flour (110 g), yeast cells (1g/ approximately $9x10^8$ yeast cells), table salt (1 g), table sugar (5 g) and sterile distilled water (90 ml) in 1L measuring cylinder each. The dough in each container was incubated for 2-8 hrs separately at room temperature and 30^oC in triplicate. The height of the dough was measured using graduated cylinder before and after fermentation and recorded at 2 hrs interval for 8 hrs.

4.12. Designation of yeast isolates

The yeast isolates retrieved from selected fruits were designated as AAUAv (Addis Ababa University Avocado isolate), AAUBa (Addis Ababa University Banana isolate), AAUGr (Addis Ababa University Grape isolate),AAUMa (Addis Ababa University Mango isolate), AAUOr (Addis Ababa University Orange isolate), AAUPa (Addis Ababa University Papaya isolate), and AAUPi (Addis Ababa University Pineapple isolate). ‘’ AAU’ ’ , “Av” ,” Ba” ,” Gr” ,” Ma” ,” Or” ,” Pa” , and “Pi” represents Addis Ababa University, avocado, banana, grape, mango, orange, papaya and pineapple respectively.

4.13. Data analysis

All data were presented as the average of triplicate experiments with standard deviation. Results were statistically interpreted with one-way analysis of variance (ANOVA) followed by post hoc analysis (Tukey’s test) to locate the significant differences indicated with ANOVA. The data for ANOVA were analyzed using SPSS version 20.0 at $p<0.05\%$ significance level.

5. Results

5.1. Characterization of yeast isolates

5.2. Cultural Characterization

In this study, 88 yeast isolates (13 from avocado, 14 from banana, 11 from grape, 11 from mango, 15 from orange, 14 from papaya, and 10 from pineapple were retrieved, purified and further identified (Table 4). Their cultural characteristics were compared with that of the commercial yeast. The cultural/colonial characteristics of the yeast isolates are given in Table 4.

Table 4 Cultural or colony characteristics of isolated yeast after 48 hrs of incubation

Substrates	Shape	Colour	Elevation	Margin	Number of isolates
commercial yeast	Circular	Creamy	Spread	Smooth	1
	Irregular	White creamy	Raised	Smooth	1
avocado	Circular	Creamy	Spread	Smooth	7
	Irregular	White creamy	Raised	Rough	6
banana	Circular	Creamy	Spread	Smooth	7
	Irregular	White creamy	Raised	Rough	7
grape	Circular	Creamy	Spread	Smooth	5
	Irregular	White creamy	Raised	Rough	6
mango	Circular	Creamy	Spread	Smooth	5
	Irregular	White creamy	Raised	Rough	6
orange	Circular	Creamy	Spread	Smooth	8
	Irregular	White creamy	Raised	Rough	7
papaya	Circular	Creamy	Spread	Smooth	8
	Irregular	White creamy	Raised	Rough	6
pineapple	Circular	Creamy	Spread	Smooth	5
	Irregular	White creamy	Raised	Rough	5
Total number of yeast isolates					88

5.3. Production of CO_2

A total of 6 yeast isolates were selected from 88 yeast isolates using sugar fermentation test in YEPD broth medium on the level of the production of CO_2 observed in the Durham tube within 24 hrs (Table 5).

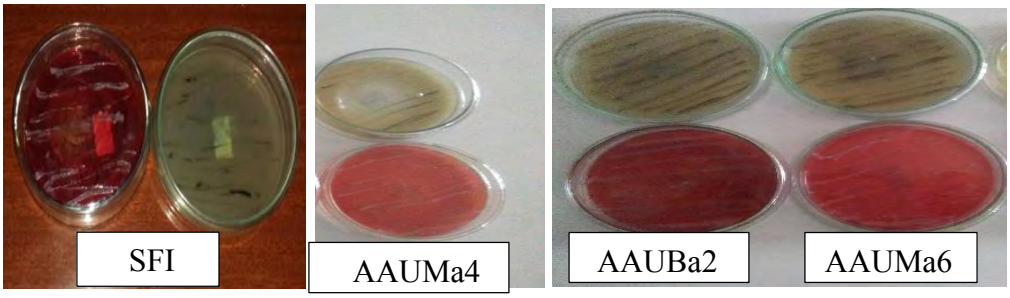
Table 5 Isolates producing more CO_2 from each substrate anaerobically

Substrate	**Total number of isolates in each**	**CO_2 production in (%) from the non-producers**	**Non CO_2 production in (%)**	**Designation of the isolates**
avocado	13	0	100	
banana	14	7	93	AAUBa2
grape	11	7	93	AAUGr5
mango	11	18	82	AAUMa4 and AAUMa6
orange	15	7	93	AAUOr7
papaya	14	0	100	
pineapple	10	7	93	AAUPi3

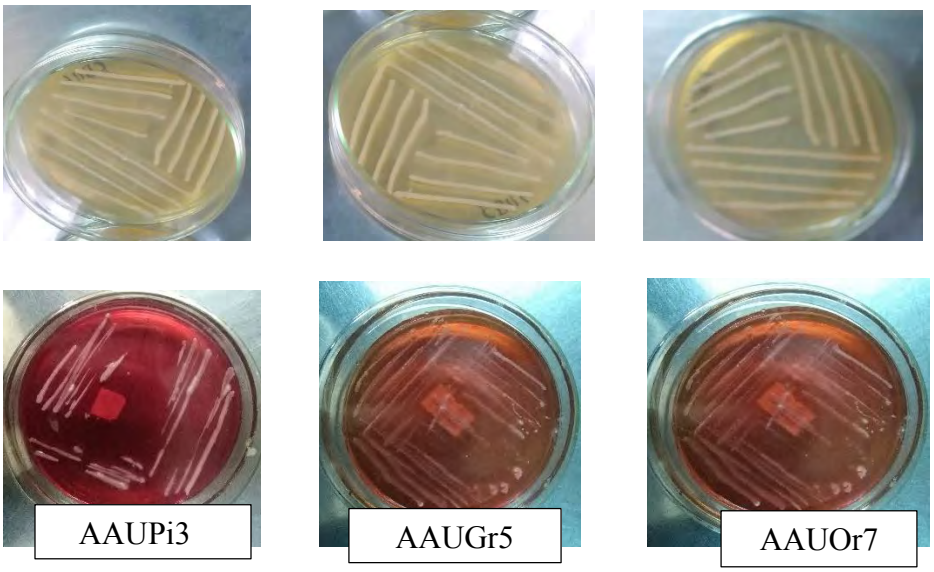
AAUBa2= banana, AAUGr5=grape, AAUMa4 and AAUMa6= mango, AAUOr7= orange and AAUPi3= pineapple

5.4. Production of H_2S

Among the 6 yeast isolates that produced higher level of CO_2, AAUMa4, AAUBa2, AAUMa6 and the commercial yeast, SFI produced H_2S gas. Yeast isolates, AAUGr5, AAUPi3 and AAUOr7 did not produce undesirable H_2S on both Bismuth Sulfite Agar (BSA) and Kligler Iron Agar (KIA) media (Fig. 5 a and b).



(a) SFI= commercial yeast, AAUMa4 and AAUMa6 = mango, and AAUBa2= banana



(b) AAUPi3 = pineapple, AAUGr5=grape and AAUOr7= orange

Fig.5. Observation of H_2S gas production by cultures on BSA (upper) and KIA media (lower)

a= H_2S producers; b= Non H_2S producers

5.5. Microscopic observation of selected yeast isolates

The cell morphology of the yeast isolates as compared with the commercial yeast under compound microscope indicated that the cells have ovoidal (AAUGr5, AAUPi3 and SFI) to spherical (AAUOr7) microscopic shape (Fig.6). And the yeast isolate from, AAUOr7 and AAUGr5 had single, paired and triple budding features. Likewise; the commercial yeast had single and paired budding pattern and the yeast isolate, AAUPi3 had single budding pattern (Fig.6).

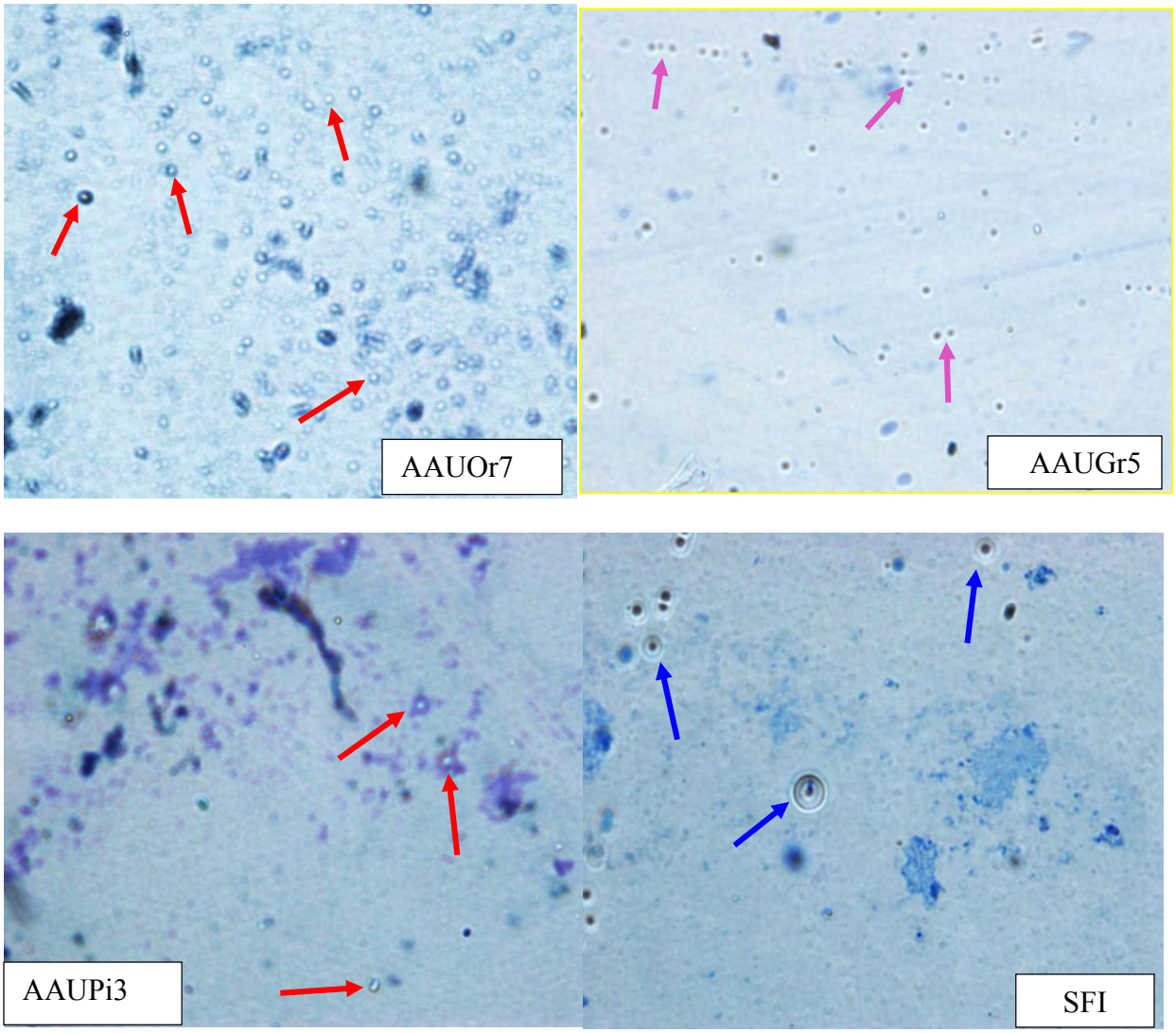


Fig.6 The cell morphology under compound microscope (OLYMPUS BX51), AAUOr7= orange, AAUGr5=grape, AAUPi3= pineapple and SFI =commercial yeast

5.6. Cell count and viability of selected yeast isolates

Viable cells of the commercial yeast and the yeast isolates that were spread plated in PDA medium for 48 hrs showed that the commercial yeast SFI had the highest cell count of 1.12 $x10^7$ cfu/ml. And the three yeast isolates; AAUGr5, AAUOr7 and AAUPi3 had the counts of 6.9x 10^6 cfu/ml, 5.2 $x10^6$ cfu/ml and 3.6 $x10^6$ cfu/ml, respectively (Table 6).

Table 6 Colony counts on PDA

Isolate	Yeast counts (cfu/ml)
SFI	1.12 $x10^7$
AAUGr5	6.9x 10^6
AAUOr7	5.2 $x10^6$
AAUPi3	3.6 $x10^6$

SFI= commercial yeast, AAUGr5= grape, AAUOr7 = orange and AAUPi3 = pineapple

5.7. Biochemical characteristics of selected yeast isolates

Results of sugar fermentation ability of the yeast isolates indicated that almost all isolates have fermented the sugars except lactose. The abilities of fermenting sugar sources were indicated by change color from violet to yellow (appendix, Fig.h). And also released CO_2 gas bubbles (observed in Durham tube) (Table 7). The selected three yeast isolates were grossly identified as genus *Saccharomyces* on the basis of comparison of their biochemical characteristics to that of commercial yeast and designated as AAUGr5, AAUOr7 and AAUPi3 (Table 7).

Table 7 Biochemical characteristics of isolated yeast isolates anaerobically

Isolate	D-glucose	fructose	maltose	galactose	lactose	sucrose	KNO3	$(NH4)_2$ SO4	Remark
SFI	+	+	+	+	-	+	-	+	++
AAUGr5	+	+	+	+	-	+	-	+	++
AAUOr7	+	+	+	+	-	+	-	+	++
AAUPi3	+	+	+	+	-	+	-	+	++

+=The isolate fermented the carbon source and utilized the nitrogen source, - = the isolates didn't ferment the carbon source and didn't utilize the nitrogen source, ++= recommended as *Saccharomyces*

5.8. Effect of Temperature

The biomass yield of the isolates from the reading of spectrophotometer at 550nm was observed ranging from 0.004 to 1.53 OD at different incubation temperature values (Fig.7). All the yeast isolates showed slow growth at 25°C and 35°C. Extended incubation period did not show increase in biomass (Fig. 7). The highest biomass was observed at 30°C by yeast isolates; AAUPi3 (1.53), AAUOr7 (1.50), AAUGr5 (1.07) and SFI (1.14) at 96 hrs. There was a rapid decrease in cell number for all yeast isolates after 96 hrs of incubation (Fig.7).

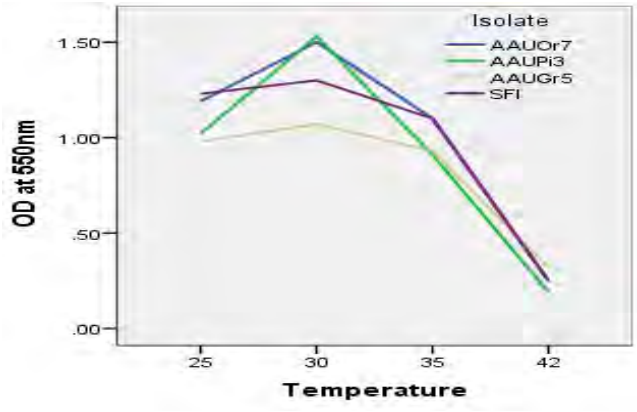


Fig.7 Effect of temperature on the growth of yeasts in YEPD at 96 hrs of incubation

5.9. Effect of pH

The maximum OD readings of the yeast growth at 550nm at pH 5 for all yeast isolates at the end of 96 hrs incubation period were; AAUPi3 (0.95), AAUOr7 (0.89), AAUGr5 (0.88) and SFI (0.99) (Fig.8). The optimum pH value was obtained at pH 5 for all yeast isolates (Fig.8). Generally, there was a steady decrease in biomass yield after 96 hrs by all yeast isolates (Fig.8).

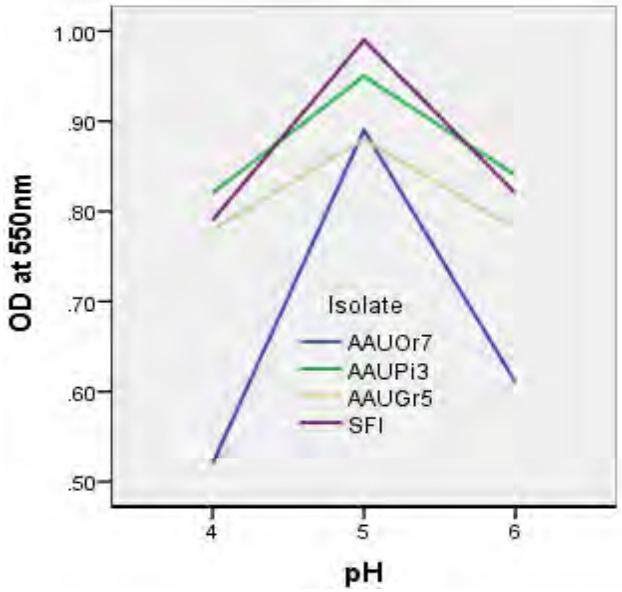


Fig.8 Effect of pH on the growth of yeasts in YEPD at 96 hrs of incubation

5.10. Effect of D-glucose

The maximum mean growth of the yeast isolates at the end of 96 hrs growth period for all yeast isolates were; AAUPi3 (1.81), AAUOr7 (1.74), AAUGr5 (1.70) and SFI (1.78) (Table 8). All yeast isolates had their highest growth in 30% D-glucose containing media and gradually lowers in 40% and then further down in 50% D-glucose containing media respectively (Table 8). The effect of D-glucose on the growth and biomass yield of yeast isolate is shown in Table 8.

Table 8 Effect of D-glucose on the growth of yeast isolates aerobically

D-glucose (%)	**Growth (OD at 550nm)of the isolates at different D-glucose concentrations**			
	AAUPi3	AAUOr7	AAUGr5	SFI
30	1.81±0.03[a]	1.74±0.05[a]	1.70±0.04[a]	1.78±0.01[a]
40	1.67±0.05[b]	1.63±0.02[b]	1.59±0.07[ab]	1.72±0.01[b]
50	1.56±0.00[c]	1.51±0.02[c]	1.38±0.00[b]	1.58±0.00[c]

AAUPi3=pineapple, AAUOr7=orange, AAUGr5=grape and SFI=commercial yeast

Means followed by the same letters with in a column are not significantly different (Tukey's HSD test, $p<0.05\%$)

5.11. Effect of NaCl

All yeast isolates had their highest growth in 5 %(w/v), NaCl containing media and gradually lowers in 10% and then further down in 15 %(w/v), NaCl containing media. The maximum mean growth for all yeast isolates were; AAUPi3 (1.02), AAUOr7 (0.97), AAUGr5 (1.38) and SFI (1.73) (Table 9). The results showed all yeast isolates are resistant against higher osmotic pressure.

Table 9 Effect of NaCl on the growth of yeast isolates aerobically

NaCl (%)	**Growth(OD at 550nm) of the isolates at different NaCl concentrations**			
	AAUPi3	AAUOr7	AAUGr5	SFI
5	1.02±0.08[a]	0.97±0.02[a]	1.38±0.06[a]	1.73±0.01[a]
10	0.90±0.01[ab]	0.87±0.00[b]	1.11±0.01[b]	1.63±0.01[b]
15	0.80±0.02[b]	0.72±0.01[c]	0.94±0.01[c]	1.12±0.01[c]

AAUPi3=pineapple, AAUOr7=orange, AAUGr5=grape and SFI=commercial yeast

Means followed by the same letters with in a column are not significantly different (Tukey's HSD test, $p<0.05\%$)

5.12. Effect of shaking

All the yeast isolates that were growing in shaking condition at 140rpm for 96 hrs in YEPD medium showed better biomass production than yeast isolates that were growing in unshaken condition in YEPD medium for 96 hrs incubation period (Fig. 9). The yeast isolate had the maximum and minimum mean growth of, SFI (3.182^{a}-1.612^{b}), AAUGr5 (2.911^{a}-1.588^{b}), AAUOr7 (2.47^{a}-1.48^{b}) and AAUPi3 (2.002^{a}-1.008^{b}) respectively.

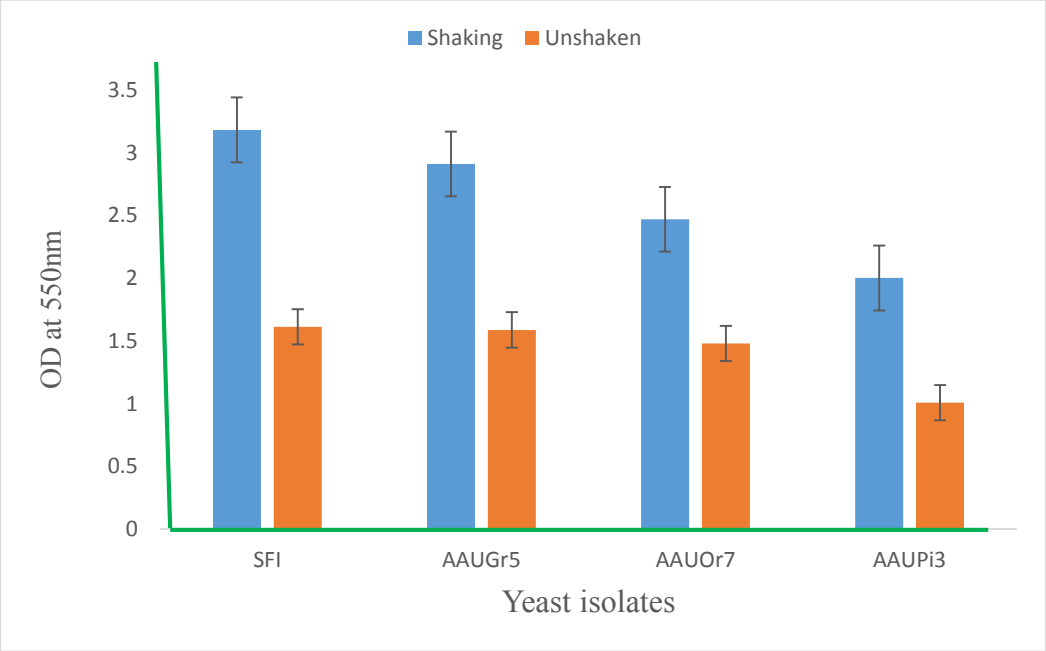


Fig.9 Effect of shaking condition on biomass production by the yeast isolates

Error bars depict standard error of mean (n = 3, p<0.05%).

SFI= commercial yeast, AAUGr5=grape, AAUOr7=orange and AAUPi3=pineapple

5.13. Leavening Action

As shown in Table 10, dough volume increased by the commercial yeast (SFI) both at room temperature and 30ºC showed the best leavening potential followed by combination of the three selected yeast isolates compared to the two combinations or individual isolate. AAUGr5 had the highest leavening potential from the single isolate both at room temperature and 30ºC after 8 hrs (Table 10).

Table 10 Comparison of leavening action of yeast isolate from fruits and commercial baker's yeast both at room temperature and 30ºC at different time.

Yeast Isolates	**Volume increment(ml) at 30ºC**			
	2 hrs	4 hrs	6 hrs	8 hrs
SFI	52±2.00^{a}	61±4.73^{a}	73±3.51^{a}	135±3.51^{a}
AAUGr5+AAUPi3+AAUOr7	37±2.00^{b}	43±1.53^{b}	49±2.52^{b}	92±2.08^{b}
AAUGr5+ AAUPi3	29±2.00^{c}	34±1.00^{c}	41±2.00^{c}	84±3.21^{c}
AAUGr5+ AAUOr7	26±3.00^{cd}	32±1.52^{c}	38±2.01^{cd}	77±1.53^{cd}
AAUPi3+ AAUOr7	21±2.52^{de}	28±2.52^{cd}	35±2.12^{cde}	72±3.00^{d}
AAUGr5	17±2.00^{ef}	24±2.50^{de}	32±2.00^{def}	61±2.08^{e}
AAUOr7	14±2.00^{f}	22±3.00^{de}	29±2.51^{de}	53±2.08^{f}
AAUPi3	12±3.00^{f}	19±2.00^{e}	26±2.50^{e}	44±3.51^{g}
without yeast	0±0.00^{g}	0±0.00^{f}	0±0.00^{f}	0±0.00^{h}

Yeast Isolates	**Volume increment(ml) at room temperature**			
	2 hrs	4 hrs	6 hrs	8 hrs
SFI	43±3.00^{a}	49±2.52^{a}	63±4.00^{a}	115±2.52^{a}
AAUGr5+AAUPi3+AAUOr7	33±3.00^{b}	39±2.00^{b}	46±3.06^{b}	87±1.00^{b}
AAUGr5+ AAUPi3	27±2.00^{bc}	32±1.53^{c}	41±3.00^{bc}	79±2.08^{c}
AAUGr5+ AAUOr7	23±2.52^{cd}	30±2.00^{cd}	36±2.00^{cd}	70±2.52^{d}
AAUPi3+ AAUOr7	18±2.08^{de}	25±2.52^{de}	32±2.52^{de}	62±2.52^{e}
AAUGr5	13±2.00^{ef}	21±2.00^{ef}	28±2.52^{ef}	52±2.00^{f}
AAUOr7	9±2.52^{f}	17±3.00^{f}	25±2.00^{ef}	44±2.52^{g}
AAUPi3	7±2.52^{f}	16±3.00^{f}	24±2.52^{f}	33±2.08^{h}
without yeast	0±0.00^{g}	0±0.00^{g}	0±0.00^{g}	0±0.00^{i}

SFI = commercial yeast, AAUGr5= grape, AAUOr7 = orange, and AAUPi3= pineapple

Means followed by the same letters with in a column are not significantly different (Tukey's HSD test, $p<0.05\%$).

6. Discussion

In this study, 88 yeast isolates from local fermented fruits were isolated and compared with the commercial baker's yeast. The isolates had, white and creamy colour, ovoid and spherical microscopic shape, 4- ascospores in ascus and budding. These results were consistent with the previous findings that indicated yeasts with similar features are grossly identified as *Saccharomyces* (Birhanu Abegaz *et al.,* 1982; Samuel Sahle and Bihanu Abegaz, 1991).The morphological characteristics of the three yeast isolates observed by using microscope (X100 magnifications) showed that the three yeast isolates were unicellular with spherical or oval cell shape, comparable to commercial yeast. This result is in agreement with (Greame, 2005) who confirmed the characteristic of *Saccharomyces*.

Baker's yeast acts as a natural leavening agent, or rising agent of baked bread by producing CO_2 gas during the time of dough fermentation. In this experiment, yeast isolates; AAUPi3, AAUOr7 and AAUGr5 were found to be the best gas producer observed in the Durham tube among the 88 yeast isolates. In dough leavening, fermentative capacity using carbon source with the production of carbon dioxide is exhibited as one of the important parameters for baker's yeasts (Benitez *et al*., 1996). The results showed that all the yeast isolates were able to ferment the six sugars provided except lactose and released carbon dioxide gas as observed in Durham tube. This could be an important indication of invertase activity exhibited by yeast isolates used in this study. All the yeast isolates including the commercial yeast did not ferment lactose.
Tarek (2001) reported that *Saccharomyces* which were unable to ferment lactose lack lactase or β-galactosidase system.

The three yeast isolates including the commercial strain grew on $(NH4)_2$ SO4 as a source of nitrogen. But KNO3 had inhibitory effect on the growth of the yeast isolates including the commercial strain. 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 this study, some of the yeast isolates produced black color in BSA and KIA media including even the commercial yeast which is packed and sold to the public compromising bread quality. But in this study, the three yeast isolates with the best fermentative abilities were found not producing black color on both BSA and KIA medium, and this trait makes them the best alternative starter cultures to be used for bread baking. Therefore; AAUPi3, AAUGr5 and AAUOr7 were subjected to further test. H_2S is undesirable compound associated with an off-flavor and unpleasant taste that must be avoided in processed foods (Ribeiro and Horii, 1999). Yeasts with elevated production of hydrogen sulfide are undesirable for bread baking because it contributes to flavor and taste that compromise the quality of the bread (Vicente *et al.*, 2006). Gastrointestinal disorders have been attributed to consumption of foods spoiled by *Saccharomyces* yeasts that produced H_2S (Muzikar, 1984).

The effect of temperature on the growth and maximum biomass yield showed that the optimum biomass was observed at 30ºC by all yeast isolates. The result of this study is in consistent with the findings of other investigators (Eroshin *et al*., 1976; Parveen, 1991) who obtained maximum cell mass production at 30°C.There was a rapid decrease in cell number for all yeast isolates after 96 hrs of incubation at all tested temperatures since the synthesis of enzyme is affected by the growth temperature (Knox, 1955).

Regarding pH of growth and maximum yield development, pH 5 was found to be the optimum for the growth of the yeast isolates including the commercial yeast. In a similar study, yeasts, specifically *Saccharomyces* were found to grow optimally at pH of 5 (Azmuda *et al*., 2006). Qureshi *et al*., (2007) reported that low pH to be helpful for inhibiting the growth of undesirable microorganisms in large scale fermentation. In general, the three yeast isolates from the fruits were found to perform close to the commercial yeast with respect to pH and temperature tolerance. In addition, the screened yeast isolates were superior to the commercial strain since they did not produce undesirable substance like H_2S.

In this study the yeast isolates were able to grow on medium (yeast extract peptone broth) containing different D-glucose and NaCl concentrations. The optimum growth was obtained at 30% D-glucose and 5% NaCl. This finding is in agreement with Pataro *et al*., (2000) who reported that most of *Saccharomyces* isolated from conventional fermentation processes were physiologically adapted to extreme conditions.

All the yeast isolates showed more than one fold log increase in biomass production under shaking condition than when they were unshaken. The biomass and viable cell production rate by the commercial yeast was significantly higher than the screened wild yeast isolates. This might be happened as shaking ensures the availability of nutrients and oxygen for all the cells and thus confirms maximum biomass production within minimum period of time (Narang and Satyanayarana, 2001).

The screened wild yeast isolates showed relatively lower baking potential than commercial yeast. The yeast isolates combined in three was found superior to any other combinations. Gobbetti (1998) indicated that the importance and synergistic interactions between yeasts are used for the metabolism of carbohydrates and the production of carbon dioxide which ultimately uses for the leavening of bread. On the other hand, AAUGr5 showed better result of dough leavening than the other yeast isolates when tested separately.

7. Conclusion

In the experiment, 88 yeast isolates were collected from seven fruit types and purified. The cultural characteristics indicated that the isolates were either creamy or white creamy in color, rough or smooth margin and either spread or raised elevation. Morphologically they were unicellular spherical, oval produced ascospore and buds that are similar to genus *Saccharomyces*. The selected yeast isolates including the commercial yeast were also utilized five sugars except lactose and $(NH4)_2SO4$ from the nitrogen source but failed to utilize KNO3 and in general the three selected yeast isolates were recommended to be *saccharomyces* on the basis of the comparison of their biochemical characteristics to that of the commercial strain.

The study also indicated that the yeast isolates; AAUPi3, AAUOr7 and AAUGr5 with the best feature of wheat dough leavening abilities, (not producing H_2S which the commercial yeast produces, growth at 30^oC, pH 5, 5% NaCl, 30% D-glucose optimally and good CO_2 production) as potential candidates to be used as baker's yeast. The combination of the three yeast isolates were found relatively better and closer to leavening capacity of the commercial yeast.

8. Recommendation

The supplementation of additional nitrogen sources like aqueous ammonia or anhydrous ammonia to the molasses and other cheap raw materials medium should be done in order to increase the cell mass of the selected yeast isolates.

A further study on the physicochemical properties and molecular biology of the local yeast isolates, AAUGr5, AAUOr7 and AAUPi3 is needed to be carried out in order to improve the performance in biomass propagation and the leavening action of the wheat dough. It will help to overproduce the enzymes responsible for breaking down dough sugar into CO_2 and ethanol and thereby increasing its bread baking efficiency. So that the yeast isolates can replace the commercial yeast and the country does not lose such a high amount of foreign currency every year.

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Appendices

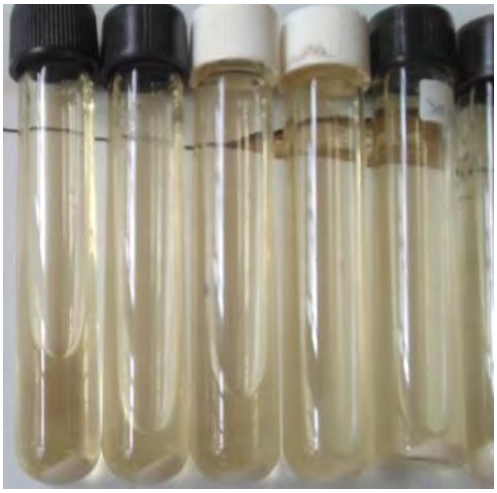

Fig.a: Yeast biomass after centrifugation, pellet at the bottom of the test tube

Fig.b: Purified yeast isolates in the slant

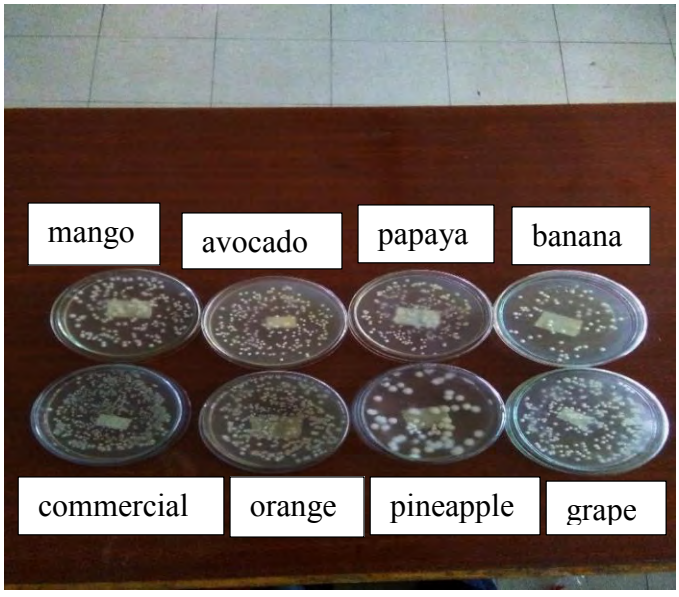

Fig. c: Colonies of yeast isolates on solid medium (PDA)

Fig.d: Durham tube carbohydrate fermentation set up

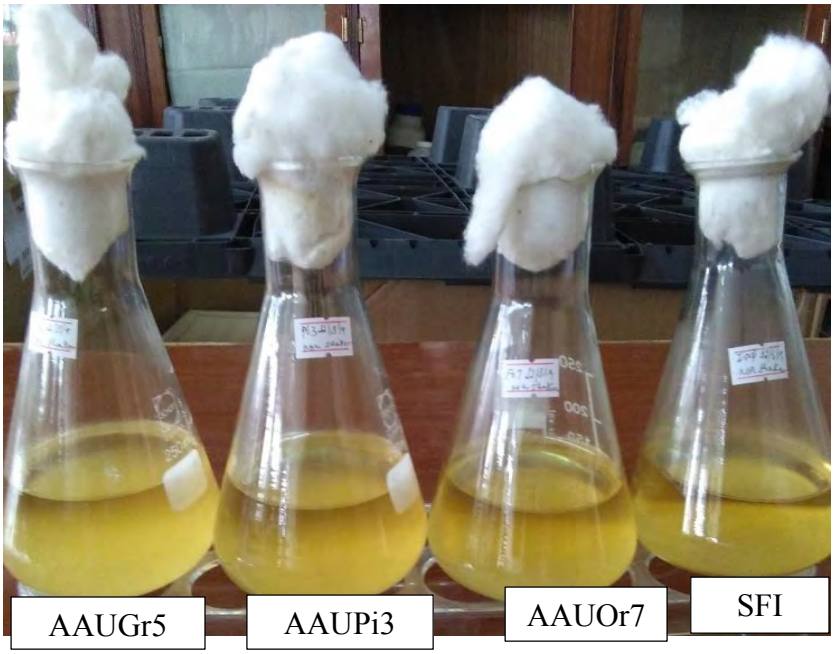

Fig.e: Submerged fermentation of grape, pineapple, orange yeast isolates and commercial yeast

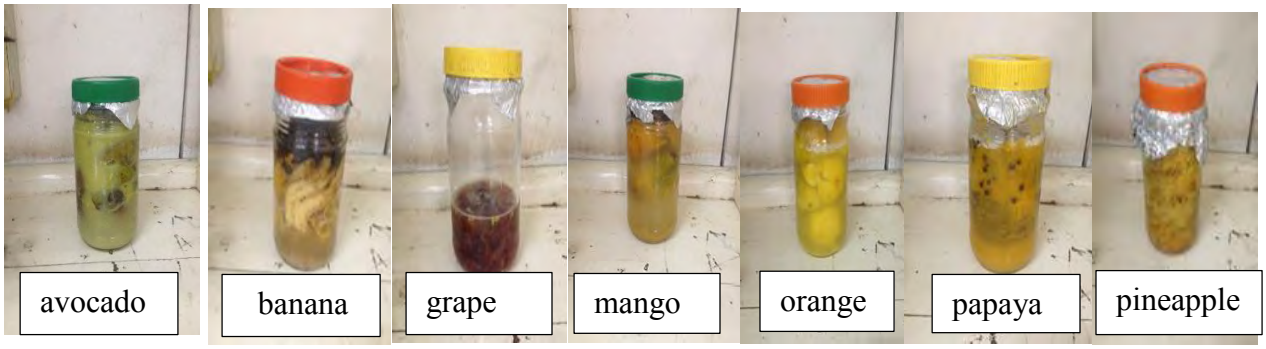

Fig.f: The selected fruits used to isolate the yeast while fermenting at room temperature.

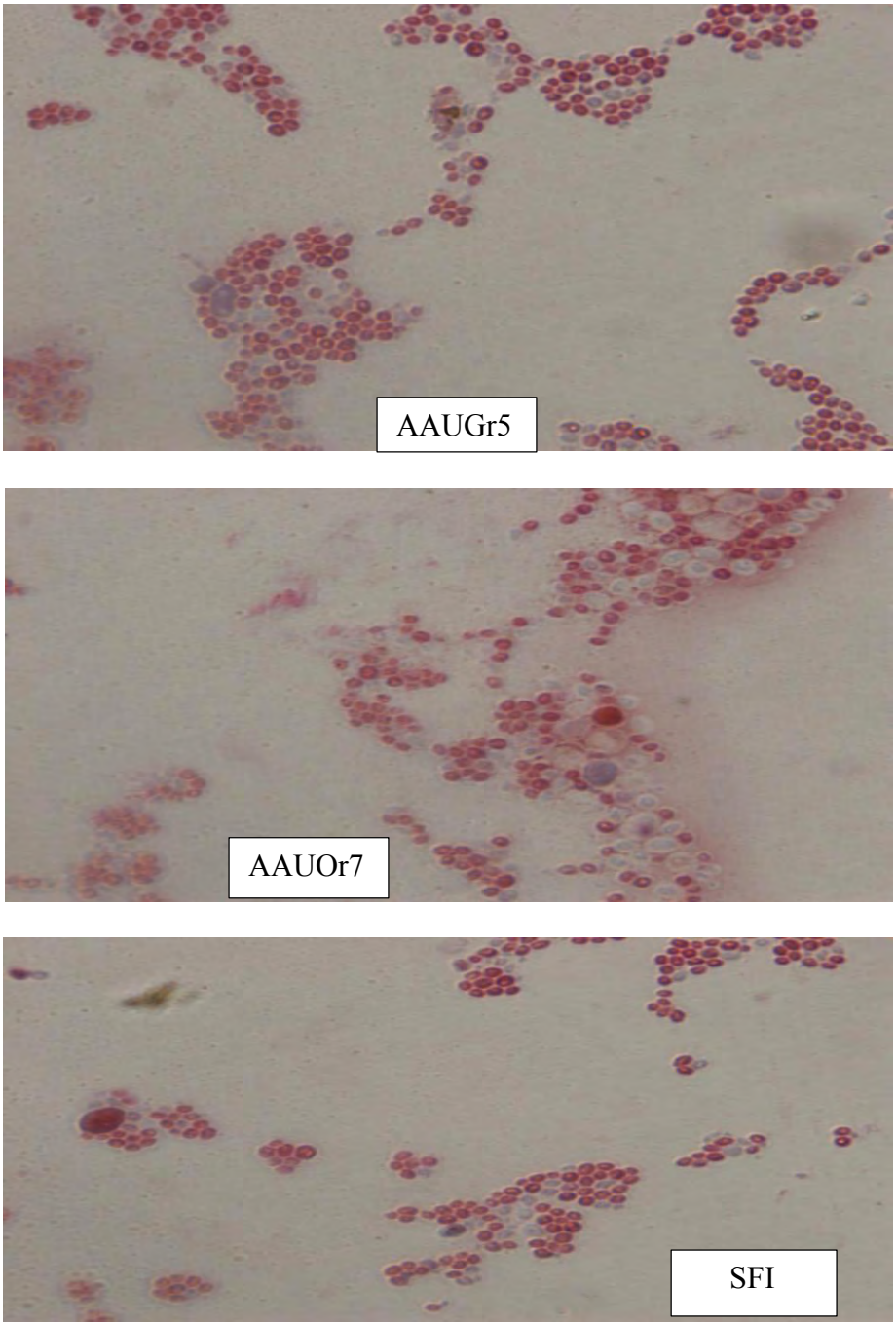

Fig.g: Ascospore formation of the yeast isolates

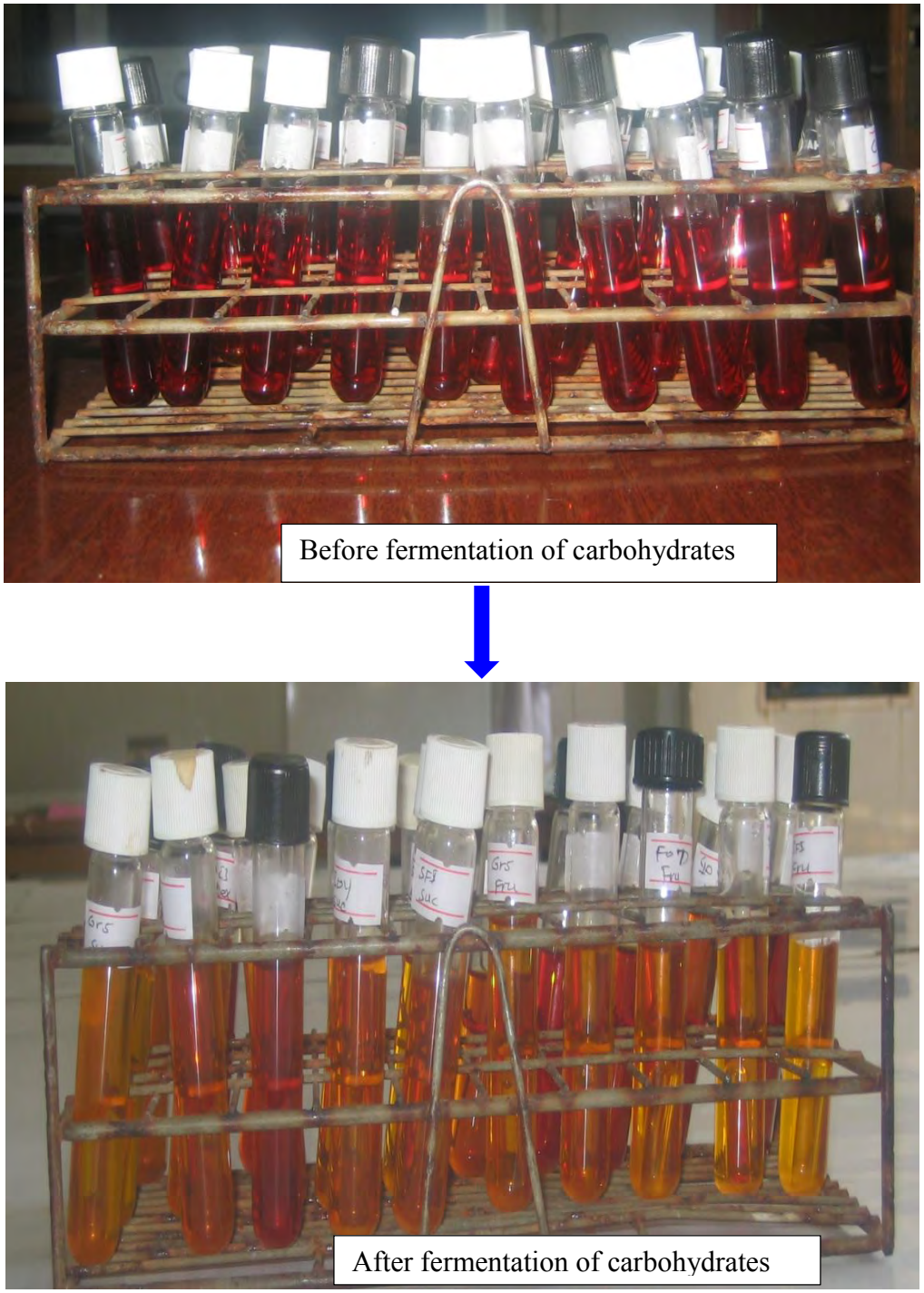

Fig.h. Carbohydrate fermentation at the end of 48hrs.

Table 1 Data analysis showing leavening of wheat dough both at room temperature and 30ºC (a &b) respectively.

(a)

Isolate	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
SFI	3	114.6667	2.51661	1.45297	108.4151	120.9183	112.00	117.00
AAUGr5+AAUPi3+AAUOr7	3	87.0000	1.00000	.57735	84.5159	89.4841	86.00	88.00
AAUGr5+AAUPi3	3	78.6667	2.08167	1.20185	73.4955	83.8378	77.00	81.00
AAUGr5+ AAUOr7	3	70.3333	2.51661	1.45297	64.0817	76.5849	68.00	73.00
AAUPi3+ AAUOr7	3	62.3333	2.51661	1.45297	56.0817	68.5849	60.00	65.00
AAUGr5	3	53.0000	2.00000	1.15470	48.0317	57.9683	51.00	55.00
AAUOr7	3	44.3333	2.51661	1.45297	38.0817	50.5849	42.00	47.00
AAUPi3	3	33.3333	2.08167	1.20185	28.1622	38.5045	31.00	35.00
Without yeast	3	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	27	60.4074	31.87989	6.13529	47.7961	73.0187	.00	117.00

(b)

Isolate	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
SFI	3	135.3333	3.51188	2.02759	126.6093	144.0573	132.00	139.00
AAUGr5+AAUPi3+AAUOr7	3	92.3333	2.08167	1.20185	87.1622	97.5045	90.00	94.00
AAUGr5+AAUPi3	3	84.3333	3.21455	1.85592	76.3479	92.3187	82.00	88.00
AAUGr5+ AAUOr7	3	77.3333	1.52753	.88192	73.5388	81.1279	76.00	79.00
AAUPi3+ AAUOr7	3	72.0000	3.00000	1.73205	64.5476	79.4524	69.00	75.00
AAUGr5	3	61.3333	2.08167	1.20185	56.1622	66.5045	59.00	63.00
AAUOr7	3	52.6667	2.08167	1.20185	47.4955	57.8378	51.00	55.00
AAUPi3	3	43.6667	3.51188	2.02759	34.9427	52.3907	40.00	47.00
Without yeast	3	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	27	68.7778	35.66763	6.86424	54.6681	82.8874	.00	139.00

Declaration

I, the undersigned, declared that this is my own original work, has not been presented for a degree to any other University and that all sources of materials used for the thesis have been fully acknowledged. I also confirm that this work has not been submitted anywhere else for the same purpose.

Eshet Lakew Tesfaye

Signature _____

Date_____

This thesis has been submitted for examination with approval as the University advisors.

Dr. Diriba Muleta Dr.Anteneh Tesfaye

Signature _____ Signature _____

Date_____ Date _____