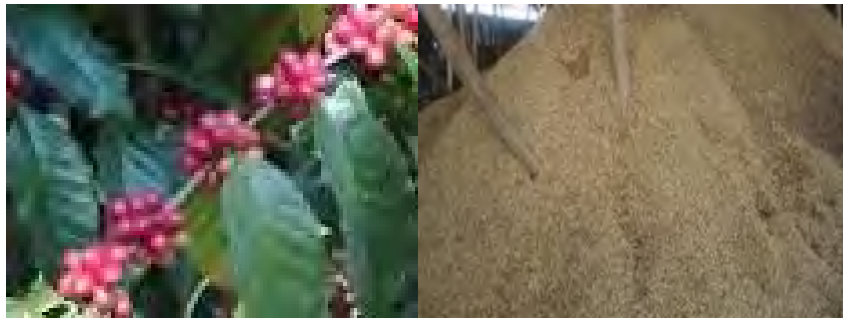


**ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCIENCE
DEPARTMENT OF MICROBIAL,
CELLULAR AND MOLECULAR BIOLOGY**



**ISOLATION AND CHARACTERIZATION OF ETHANOL, SUGAR
AND THERMO TOLERANT YEAST ISOLATES FOR BIOMASS
AND BIO-ETHANOL PRODUCTION**



**BY
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**December, 2014
Addis Ababa, Ethiopia**

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCIENCE

Isolation and characterization of ethanol, sugar and thermo tolerant yeast isolates for biomass and bio-ethanol production

By

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A thesis presented to the School of Graduate Studies of the Addis Ababa University in the Partial fulfillment of the requirement for the Degree of Masters of Science in Applied Microbiology

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ABSTRACT

The aim of the study was to isolate and characterize ethanol, sugar and thermo tolerant yeast to optimize maximal ethanol production from coffee husk. The yeasts were isolated from (Psidium guajava), grapefruit (Citrus paradise), avocado (Persea americana), papaya (Carica papaya) and gishita (Annona senegalensis pers.). All the yeast isolates were first tested for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth. Four isolates which were relatively high fermentative in Durham tube fermentation method were selected for testing of the isolates for ethanol, sugar and thermo tolerance. Further, the optimum conditions for isolation and characterization of yeast isolates were determined. The investigation revealed that the optimum conditions are 37°C temperature, 30% sugar concentration, 15% ethanol concentration, pH 5 and 15% diammonium hydrogen phosphate ((NH₄)₂HPO₄) were used as nitrogen source in M9 minimal medium. According to the morphological and biochemical characterization, the selected yeast isolates were belonged to the genus saccharomyces. The results showed that the content of sugars increased as the acid (H₂SO₄) was increased from 0.25M - 3 M and decreased as the acid concentrations increased beyond 3 M. At the optimal acid concentration (3M; H₂SO₄), the sugar yield obtained was 85% from the coffee husk. The optimum condition for acid hydrolysis was 3M H₂SO₄, 10 min hydrolysis time, pH 5 and 30°C temperature at 48 hrs fermentation time. Under the optimum conditions the maximum yield of ethanol (5.56% v/v) was obtained using AAUP₁ yeast isolate. The results indicated that locally available cheap substrate which occurs in excess and non-edible material, coffee husk will be a potential feedstock for bio-ethanol production.

Keywords: *Fermentation parameters, fruits, optical density and S. cerevisiae*

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ACRONYMS

AAU	Addis Ababa University
GC	Gas Chromatography
GHG	Greenhouse gas
HMF	hydroxymethylfurfural
Nox	Oxides of nitrogen
OECD	Organization for Economic Cooperation and Development
Psi	pound per square inch
vvm	volumes of air per volume per minute
YM	yeast extract-malt
YPD	yeast extract peptone dextrose

1. INTRODUCTION

In the 20th century, the world economy has been dominated by technologies that depend on fossil energy, such as petroleum, coal, or natural gas to produce fuels, chemicals, materials and power (Sun and Cheng, 2002; Paster, 2003). The continued use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentration of CO₂ in the atmosphere and concerns over global warming (Demirbas *et al.*, 2004). The combustion of fossil fuel is responsible for 73 % of the CO₂ emission (Wildenborg and Lokhorst, 2005).

The heightened awareness of the global warming issue has increased interest in the development of methods to mitigate greenhouse gases emission (Lombardi, 2003). Much of the current effort to control such emissions focuses on advancing technologies.

Reducing use of fossil fuels would considerably reduce the amount of CO₂ produced, as well as reduce the levels of pollutants (Demirbas, 2006). The concern about global warming and dependence on fossil fuels necessitate the search for renewable energy sources that reduce CO₂ emissions (Oliveria *et al.*, 2005). To alleviate such problems, alternative and non-edible agricultural residues such as molasses, coffee husk and cassava peels could be used as substrate for bio-ethanol production.

The use of bio-fuel to substitute fossil fuels has immense benefits for non-oil producing countries like Ethiopia. Sugar, starch and lignocelluloses are carbohydrate-rich raw materials suitable for ethanol production. Bio-fuel includes bio-ethanol, bio-butanol (where sourced from starchy crops) and biodiesel from oil crops such as *Jatropha curcas*, castor crop and palm tree which are used for ethanol production with worldwide acceptance essentially to overcome problems associated with exploitation and depletion of fossil fuels and environmental pollution. Thus, ethanol production can be effected using easily available substrates such as lignocelluloses and (or) hemicelluloses and micro-organisms which could convert them to ethanol could be very useful (Mogg, 2004).

The yeast *Saccharomyces cerevisiae* is the primary organism that is used for ethanol production and efficiency of ethanol production is mainly dependent on the choice of yeast strain and continuous improvement using modern biotechnology tools (Gera and Sharma, 1991).

The ability of yeast to produce ethanol depends on many factors such as strains, growth factors and optimum environmental conditions (Khongsay *et al.*, 2010). Tolerance of yeast to its substrate (osmo-tolerance), fermentation product (ethanol- tolerance) and temperature (thermo-tolerance) has great potential to be used in industrial scale fermentation. Isolation and characterization of ethanol, sugar and thermo tolerant yeast from fruits could promote higher yield of ethanol at higher temperature than commercial *Saccharomyces cerevisiae* like baker yeast.

1.1. Research objectives

1.1. 1. General Objectives

To isolate effective yeasts in biomass and bio-ethanol production and optimization of bio-ethanol production under different environmental condition.

1.1.2. Specific Objectives

- To isolate and characterize yeast from fruit guava (*Psidium guajava*), grapefruit (*Citrus paradise*), avocado (*Persea Americana*), papaya (*Carica papaya*) and gishita (*Annona senegalensis pers.*).
- To evaluate the isolate for ethanol, sugar and heat tolerance for bio-ethanol production.
- To optimize bio-ethanol production under different environmental condition.

2. LITERATURE REVIEW

2.1. Yeasts, sugar, thermo and ethanol tolerant yeasts

2.1.1. Yeasts

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi. Kurtzman and Fell, (2006) have described about 1,500 yeast species. They dominate fungal diversity in the oceans (Bass *et al.*, 2007). Yeasts 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. The yeast species are all characterized by a common features have-respect to morphological and physiological traits. 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: Analytical Profile Index (API) strips (BioMérieux, France) or the automated/computerized BCCM/Allev 2.00 system (Louvain-la-Neuve, Belgium) (Dechassa Tolessa, 2010).

Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, maltose, sucrose, lactose, raffinose, trehalose and xylose. With regard to fermentation of these sugars, Scheffers (1987) has argued that the anaerobic liberation of CO₂ into durham tubes is not very accurate for detecting slowly fermenting yeast species. Ethanol production assays are deemed to be more appropriate determinants for yeast characterization (Walker, 1998).

Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products. The last category includes enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, citric acid, ethanol, carbon dioxide and compounds synthesized by the introduction of recombinants DNA into yeasts. 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 used in food, beverage and fermentation industries (Jacobson and Jolly, 1989). The yeast species *Saccharomyces cerevisiae* has been used in baking and fermenting alcoholic beverages for thousands of years. It is also extremely important as a model organism in modern cell biology research and is one of the most thoroughly researched eukaryotic microorganisms. Ostergaard *et al.*, 2000 have used it to gather information about the biology of the eukaryotic cell and ultimately human biology. Other species of yeast, such as *Candida albicans*, are opportunistic pathogens and can cause infections in humans. Yeasts have recently been used to generate electricity in microbial fuel cells, and produce bio-ethanol for the biofuel industry (Haimanot Abebe, 2011).

Yeasts convert sugars (through a process known as fermentation) into alcohol and carbon dioxide. This trait is what endears yeasts to wine makers, brew masters and bread bakers. In the making of wine and beer, the yeasts' manufacture of alcohol is desired and necessary for the final product; and carbon dioxide is what makes beer and champagne effervescent. The art of bread making needs the carbon dioxide produced by yeast in order for certain dough to rise. To multiply and grow, all yeasts need is an optimal environment, which includes moisture, carbon source (in the form of sugar) and a warm, nurturing temperature is best (21.1°C to 29.4°C) (Haimanot Abebe, 2011).

Today, indicate those scientists in the form of reference have been able to isolate and identify the various yeasts that are best for wine making, beer making and baking. The two types commercially available are baker's yeast and brewer's yeast. Baker's yeast, as the name implies, is used as a leavener. It's categorized into three basic types-active dry yeast, compressed fresh yeast and yeast starters. Brewer's yeasts are special non-leavening yeasts used in beer making. These yeasts are a rich source of vitamin B-complex, can be used as a food supplement (Janse and Pretorius, 1995).

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. Criteria used in yeast identification and classification

Criteria used in yeast identification and classifications are described by Cook (1958) and Lodder (1971) as follows:

Morphological characteristics:-These include characteristics of vegetative cells, form, size and shape of cells and method of asexual reproduction. Asexual or vegetative reproduction occurs in yeasts by fission, by budding or by combination of the two. **Sexual reproductive characteristics:-** These include ascus and ascospore types and interfertility in ascomycetus yeasts are used as taxonomic criteria. The most colony morphology of yeast isolate were cream color, flat, mucoidal, large scalloped colony and circular, umbonate were identified. **Cultural characteristics: -** Cultural characteristics include ability to grow in liquid or in solid media. The shape, size and color of colonies in solid media are also criteria used in yeast identification.

Physiological characteristics:- physiological characteristics include utilization carbon compounds, fermentation sugars, oxidation of carbon compounds, splitting of arbutin, utilization of nitrogen compounds, growth in vitamin free medium, acid production, growth on media of high osmotic pressure, growth at elevated temperature, hydrolysis of urea, fat splitting, pigment formation and ester production.

Moreover, there are molecular tools can identify the species of yeast using molecular techniques can be identified using their genomic DNA. Polymerase chain reaction (PCR) techniques are used to amplify specific regions of DNA. This is accomplished by using universal primers. Universal primers anneal to a well-conserved region of DNA, generally the interspatial regions (ITS) of ribosomal DNA. The region between the two annealing sites is amplified and varies between species. The product obtained from this PCR step can then be sequenced and compared to known sequences using the Basic Local Alignment Search Tool or BLAST (Tobias, 2009). BLAST is a tool produced and run through the National Center for Biotechnology Information (NCBI), which is part of

the National Library of Medicine and the National Institute of Health ((Tobias, 2009). BLAST is used to enter unknown sequences of nucleotides or amino acids and is then compared to stock sequences of known organisms to find the closest match. BLAST ranks all results based upon similarity and in most cases identify the species and if not specifically the species, the genus (Tobias, 2009).

2.2.1. Classification and identification of yeasts

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features.

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 osmo-tolerance) 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 8µm long and 5µm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990). Alexopoulos *et al.* (1996) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction.

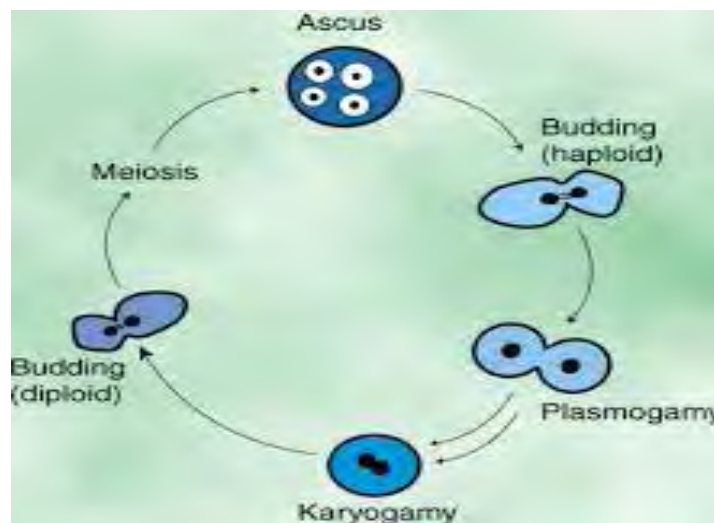
2.2.1.1. Budding

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. 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 pseudomycelium forms (Alexopoulos *et al.*, 1996). 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) is laid down somewhere 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 inward. The new wall thickens before the daughter cells separation (Conti and Naylor, 1959).

2.2.1.2. 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. 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 (Fig. 1).



Source: SparkNotes LLC (2014)

Figure 1. The reproduction of yeast by sexual and asexual reproduction

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 (Alexopoulos *et al.*, 1996). Miller (1989) has 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. 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. Genetic characterization using molecular techniques such as electrophoretic karyotyping, mitochondrial DNA restriction analysis and DNA fingerprinting have been provided more powerful means of strain identification (Recek *et al.*, 2002). The identification and classification of yeasts have traditionally been based on morphological, physiological and biochemical traits. Various kits have been developed as rapid systems for yeast identification, but mostly for clinical diagnosis (Kurtzman and Robnett, 2003). In recent years, different molecular biology techniques have been developed for yeast identification, but there is no available database to identify a large number of species.

Scientific classification:-

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Ascomycotina

Class: Saccharomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Saccharomyces*

Species: *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a species of budding yeast. It is perhaps the most useful yeast owing to its use since ancient times in baking and brewing. It is believed that it was originally isolated from the skins of grapes (Ostergaard *et al.*, 2000). It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model for prokaryote. It is the microorganism behind the most common type of fermentation. *S. cerevisiae* cells are round to ovoid, 5-10 micrometers in diameter. It reproduces by a division process known as budding (Ostergaard *et al.*, 2000). "*Saccharomyces*" derives from Greek, and means "sugar mold". "*cerevisiae*" comes from Latin, and means "of beer" (Bala Subramanian and Glotzer, 2004).

2.3. Thermo tolerant yeasts

Temperature is one of the most important environmental factors affecting microbial activity. Microorganisms have generally been divided into three groups: psychophiles, mesophiles and thermophiles depending on their range of temperature for growth. The range of temperature consists of minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) temperatures. Microorganisms, which fall into overlapping categories, have been classified as thermo tolerant mesophiles or as thermophiles, depending on the point of view of the investigation. Slapack *et al.*, (1987) have reported the highest temperature at which growth is detected.

Arthur and Watson (1976) have defined psychrophilic yeast, temperature limits of growth, 2 to 20 °C; mesophilic yeast, temperature limits of growth, 5 to 35 °C; thermophilic yeast, temperature limits of growth, 28 to 45 °C; and thermo tolerant, temperature limits of growth, 8 to 42 °C. The term is usually used to describe yeast, which grows at temperature slightly above the T_{max} of most yeast. But very little yeasts have grown consistency on the optimum temperature.

The optimum temperature can be defined as the temperature at which growth rate, fermentation rate, or the cellular yield is the highest. Apiradee (2006) have indicated that the thermophilic yeasts can only grow up to 37 °C, while yeasts, which grow at temperatures exceeding 45 °C, are referred to as thermo tolerant.

2.3.1. 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 28-35⁰c (Kosaric and Vardar-Sukan, 2001). Though the initial rate of ethanol production is higher at increased temperatures the overall productivities of fermentation is decreased due to ethanol inhibition (Jones *et al.*, 1983). Sree *et al.* (2000) and Limtong *et al.* (2007) have attempted to isolate thermophilic bacteria culture which can grow and ferment at elevated temperature. Thus shifts in temperature may vary the amount of pyruvate going to ethanol, organic acid and other alcohols.

Most brewer's yeasts have a maximum growth temperature around 39-40 ⁰C. The maximum growth temperature reported for any species of yeast was 49 ⁰C for *kluveromyces marxianus*. Mesophilic strains of *saccharomyces* have optimum cell yields and growth rates between 28 and 35 ⁰C. The optimum and maximum temperature for growth of thermophilic yeast are 40 and 50 ⁰C, respectively; these strains have a high maintenance requirement and more complicated nutritional requirements (Ullmann's, 1987). In batch processing, the optimum temperature for the complete utilization of glucose and the highest final ethanol concentration is generally slightly below the optimum growth temperature (Apiradee, 2006).

This is attributed to enhanced ethanol inhibition at higher temperature. At higher temperature, the ethanol production rate through the cell membrane. The difference in these rates results in an increase of ethanol concentration in the cells, a subsequent inhibition of some enzymes and cell death. Some yeast has an optimum fermentation temperature of 40-42 ⁰C. They produce up to 12% of ethanol with yields greater than 90% of theoretical. Because sugar fermentation is exothermic (586 joule of heat produced per gram of glucose consumed), using yeasts that ferment at higher temperature substantially reduces cooling costs of fermenters (Ullmann's, 1987).

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 25-35 °C but temperature exceeds 40 °C 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 °C 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 °C (Dechassa Tolessa, 2010).

2.4. 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 yeast strain. 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. Ethanol, which is produced during fermentation, is inhibitory to cell growth than that from an exogenous source (Wayman and Parekh, 1990).

Navarro (1980) has 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. The growth of yeast was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures (Navarro, 1980). 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).

2.5. Effects of nutrients and pH on ethanol production by thermo tolerant yeasts

2.5.1. 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) suggested that nitrogen and carbon are the main nutrients in fermentation medium and 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. Amore *et al.* (2002) have reported that for *S. diastaticus* 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 resulted in a decrease in the rate and extent of glucose utilization and ethanol production.

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 suggested that magnesium has a role in relieving the detrimental effect of high temperature that may to some extent related to the requirement of some of the glycolytic enzymes cation as co-factor. In addition, increasing the cell density is resulted in an increase in ethanol production at the higher temperature. Therefore, yeast needs sugar, and sugar is turned into alcohol by yeast. But yeast needs a lot more sugar. They need amino acids to build proteins and ultimately new cells, they

need vitamins and minerals to make enzymes work correctly, and they need phosphorus to create new DNA. Exact nutrient requirements vary between yeast species and for each strain within the species. Nutrient requirements can also vary between breweries, even when they are using the same yeast strain. It depends on water supply, wort composition, brew house design, environmental conditions such as temperature and humidity, size of brew, frequency fermentation, the type of beer producing, and the season of the year. In general, yeast needs an adequate supply of sugar, nitrogen, vitamins, phosphorus, and trace metals (Wayman and Parekh, 1990).

2.5.2. Effect of pH

Hydrogen ion concentration has a significant influence on industrial fermentation this is due to its importance in controlling bacterial contamination as its affect 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). *S. cerevisiae* intracellular pH of is usually maintained between 5.5 and 5.75 during fermentation, if the external pH is varied to 3.0 or between 5.9 and 6.75 or 6.0 and 10.0. The pH variation between the extracellular (fermnetor) and the intracellular impose a stress on yeast and force them to respond by spending more energy to maintain the intracellular pH within the range that permits growth and survival of the yeast optimal. Thus, greater proportion of glucose is converted to ethanol if the pH is adjusted to 4.5 (Narendran *et al.*, 2004). The 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).

The maximum amount of ethanol (18.60 ± 0.14 g/l) was produced by *S. cerevisiae*, at pH 3 (Sathees, *et al.*, 2011). Similarly, Narendran *et al.* (2004) have reported that *S. cerevisiae* is an acidophilic organism and grow better under acidic conditions. The optimal pH range for yeast growth can be varied from pH 3-6. The intracellular enzymes of yeast work best at its optimal pH it leads to maximal conversion of sugar into ethanol.

Hydrogen ions (H⁺) in a fermentation broth affect yeast growth, ethanol production rate, by product type and amount, and types of microbes co-culture. If the pH value is less than five during fermentation, bacterial growth is severely repressed. The pH value range for growth of most strains of *S. cerevisiae* is 2.4-8.6, with an optimum of 4.5. Yeast sugar fermentation rates are relatively insensitive to pH values between 3.5 and 6 (Sathees, *et al*, 2011). Furthermore, yeasts can tolerate as low pH as two without permanent damage. Heat liberated during fermentation increases the fermenter temperature and hence cooling is required, and, therefore, it advantageous to use temperature tolerant strains.

2.6. Properties and uses of ethanol

Ethanol or ethyl alcohol, CH₃CH₂OH, has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, germicide, beverage, antifreeze, fuel, depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals (Gaur, 2006). It is a volatile, flammable, and colorless chemical compound. It is a monohydric primary alcohol and it boils at 78.5 °C. It is miscible with water in all proportions. Ethanol that is completely free of water is called absolute ethanol. Ethanol forms a constant-boiling mixture, or azeotrope, with water that contains 95 % ethanol and 5 % water and that boils at 78.15 °C. Ethanol is a psychoactive agent and it produces a variety of physiological and behavioral effects (Gaur, 2006).

2.6.1. Benefits of Ethanol

The energy market is now being influenced by the concerns associated with global warming and environmental degradation. Ethanol (ethyl alcohol, bio-ethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer (Drapcho *et al.*, 2008). It is a renewable fuel because the energy is derived from plants that can be replenished. It is an oxygenated fuel that contains 35% oxygen, which reduces particulate, CO and NO_x (x = 2 and 3) emissions from combustion (Thomas and Kwong, 2001; Balata *et al.*, 2008; Drapcho *et al.*, 2008).

This is caused by the wide flammability and oxygenated characteristic of ethanol (Pikunas *et al.*, 2003). In addition, the plant biomass used to produce ethanol absorbs

carbon dioxide (CO₂) as it grows, thus reducing the total greenhouse gas emissions. Ethanol (C₂H₅OH) is a pure substance. However, gasoline is composed of C₄–C₁₂ hydrocarbons, and has wider transitional properties. Bio-ethanol productions have socially economically and political advantages for non-oil producing countries like Ethiopia. The construction and operation of bio-fuel plants is widely recognized as a catalyst for additional job creation beyond the agricultural sector and interlink agriculture with industry (Government of Alberta, 2008; Nuez-Ortin, 2010). It has been indicated that the sugarcane the mainstay of bio-ethanol substrate in Ethiopian and Brazil context, these needs planting sugar industries which uses the sugarcane as input and then the molasses used for ethanol production (ESDA, 2005). The interlinkage absorbs thousands of job opportunity in the industry sector. Additionally, ethanol is used for production of alcoholic beverages, for industrial purposes (as a solvent, disinfectant, or chemical feedstock) (Whittington, 2006; Petrova and Ivanova, 2010; Joshi *et al.*, 2011).

2.6.2. Bio-ethanol as a sustainable fuel

In 2005, the world ethanol production was approximately 46 billion liters per year, which is expected to reach 76 billion liters per year by 2010 (Olfert and Weseen, 2007). Bio-ethanol is appropriate for the mixed fuel in the gasoline engine due to its high octane number, and its low cetane number and high heat of vaporization impede self-ignition in the diesel engine. Kim *et al.*, 2005 have observed that the, ignition improver, glow-plug, surface ignition, and pilot injection are applied to promote self-ignition by using diesel-bioethanol blended fuel.

The most popular blend for light-duty vehicles is known as E85. In Brazil, bio-ethanol for fuel is derived from sugar cane and is used pure or blended with gasoline in a mixture called gasohol (24 % bio-ethanol, 76 % gasoline) (Oliveria *et al.*, 2005). In several states of the United States, small amount of bio-ethanol (10 % by volume) is added to gasoline, known as gasohol or E10. Blends having higher concentrations of bio-ethanol in gasoline are also used, e.g. in flexible-fuel vehicles that can operate on blends of up to 85 % bioethanol—E85 (Malca and Freire, 2006).

Some countries have exercised bio-fuel program involving both form bio-ethanol–gasoline blend program, the United States (E10 and FFV E85), Canada (E10 and for

FFV E85), Sweden (E5 and for FFV E85), India (E5), Australia (E10), Thailand (E10), China (E10), Columbia (E10), Peru (E10), Paraguay (E7), and Brazil (E20, E25 and FFV any blend) (Kadiman, 2005). In Ethiopia, the blending of ethanol with benzene was started in September 2008 with 5% Ethanol and 95% benzene (MoFED, 2010).

2.6.3. Lignocelluloses biomass for bio-ethanol production

Lignocellulosic biomass such as agricultural residues (corn stover, sugarcane bagasse, wheat or rice straw, forestry), coffee pulp and husk, paper mill residues and municipal waste is abundant, domestic and renewable. It has long been recognized as a potential low-cost source that can be converted to bio-ethanol since it is the most abundant reproducible resource on the Earth. In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production is difficult because of its complex structure, which resists degradation. The basic structure of all lignocellulosic biomass consists of cellulose $(C_6H_{10}O_5)_x$, hemicelluloses $(C_5H_8O_4)_m$, and lignin $[C_9H_{10}O_3.(OCH_3)_{0.9 - 1.7}]_n$ (Wiseloge *et al.*, 1996). Cellulose is found almost exclusively in plant cell walls. It is a linear polymer of glucose, composed of thousands of molecules of anhydroglucose linked by (1, 4) to glycosidic bonds.

The basic repeating unit is the disaccharide cellobiose. The secondary and tertiary conformation of cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose resistant to hydrolysis. Cellulose can be hydrolyzed chemically by diluted or concentrated acid, or enzymatically. During hydrolysis, the polysaccharide is broken down to free sugars by the addition of water – the process is called saccharification. The combination of hemicellulose and lignin provide a protective sheath around cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur. Furthermore, the crystalline structure of cellulose makes it highly insoluble and resistant to attack. Therefore, an effective pretreatment must improve the availability of sugars, prevent degradation of carbohydrate, reduce unfavorable by-products, and be low cost (Sun and Cheng, 2002). Numerous pretreatment methods including physical, physicochemical, chemical, and biological methods have been developed for separation of lignocellulosic to cellulose, hemicellulose, and lignin.

2.6.4. Ethanol production

The *S. cerevisiae* is the primary organism used for ethanol production and efficiency of ethanol production is mainly dependent on the choice its super strain respect to pH (Gera and Sharma, 1991). The most commonly used microorganism for ethanol production is ordinary baker's yeast. In the pretreatment process some inhibitors are formed such as Furfural from xylose and HMF from glucose lead this group. It is completed by an assortment of aldehydes, acids and alcohols from lignin, sugar and protein degradation.

The increasing demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives has necessitated increased production of this alcohol. Ethanol production is usually accomplished by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products. Owing to depleting reserves and competing industrial needs of petrochemical feed stocks, there is global emphasis in ethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, 2008).

The general ethanol is toxic effect to yeast is most noticeable on the cell membrane; the major toxic effect has been postulated as membrane damage of a change in membrane properties. Ethanol inhibits both growth and ethanol production in a non-competitive manner. When ethanol is present in concentrations of up to 2%, the observed inhibition is almost negligible for most yeast, when it affects the physiology of the yeast, causes cellular dysfunctions result from inhibitory effect of high ethanol concentrations on integral glycolytic enzymes (phosphofructokinase) and the alteration in cellular membrane lipid and protein composition (Ding *et al.*, 2009). The effects of ethanol stress reflects in the fermentation process as sluggish fermentation, reduced cell growth and viability, generation of petite yeast crop which consequently affects successive fermentation performance (Stewart, 2001). Ethanol tolerance is a desirable trait in industrial yeast strain; however, slow fermenting sake yeast (*Sacchromyces sake*) can tolerate ethanol concentration around 25% w/v at low temperature as it contains lipoproteins (Pina *et al.* 2004). Ethanol inhibition is directly related to the inhibition and denaturation of important glycolytic enzymes as well as modification of membrane. Use

of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermented wash would reduce distillation costs and hence the profitability of the overall process (Chandrasena *et al.*, 2006).

2.7. Fermentation

Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as fuel, is produced by fermentation: when certain species of yeast (most importantly, *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide (Katahira *et al.*, 2006). Lignocelluloses are often hydrolyzed by acid treatment. The hydrolysate obtained is then used for bio-ethanol fermentation by microorganisms such as yeast. Because such lignocelluloses hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of bioethanol ((Katahira *et al.*, 2006).). In general, the conversion of lignocellulosic material to sugar and then ethanol is governed by equation (1) below:



According to the reactions, the theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide per kg of xylose and glucose (Hamelinck *et al.*, 2003, 2005). The overall reaction of this fermentation of hexose sugar (glucose) by yeast has been expressed by Gay-Lussac which forms the basis of calculating fermentation efficiency as:



Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethyl alcohol and other byproducts such as ATP, pyruvate, acetaldehyde, pyruvate decarboxylase, CO₂ and NADH. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, cellulosic biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to bio-ethanol. To get an efficient fermentation severe inhibition should be avoided.

There are four different strategies to do this. These are modifying the hydrolysis process, detoxification, *in-situ* detoxification and using less sensitive microorganisms to inhibitors (Taherzadeh, 2007). Microorganisms, termed ethanologens, presently convert an inadequate portion of the sugars from biomass to bio-ethanol (Demirbas, 2005).

One of the most effective bioethanol producing yeasts, *S. cerevisiae*, has several advantages owing to its high bioethanol production from hexoses and high tolerance to bioethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass. However, because wild-type strains of this yeast cannot utilize pentoses, such as xylose and arabinose, and celloligosaccharides, bioethanol production from a lignocellulose hydrolysate is inadequate (Katahira *et al.*, 2006). For xylose-using *S.cerevisiae*, high bioethanol yields from xylose also require metabolic engineering strategies to enhance the xylose flux (Hahn-Hagerdal *et al.*, 2006).

There are two pathways are prerequisites for the synthesis of a *S. cerevisiae* strain that efficiently converts xylose to ethanol: (i) expression of heterologous genes should enable *S. cerevisiae* to convert xylose to xylulose; and (ii) consumption of xylulose should be improved since *S. cerevisiae* grows on xylulose with a maximum specific growth rate 10 times lower than that on glucose. Xylulose enters the pentose phosphate (PP) pathway after phosphorylation by xylulokinase (XK), encoded by the *XKS1* gene, which is naturally present in *S. cerevisiae*, and consequently a high capacity of the PP pathway is required. A number of steps may contribute to control xylose consumption: the uptake of xylose, the conversion of xylose to xylulose, the phosphorylation of xylulose to xylulose-5-phosphate, and the conversion of xylulose-5-phosphate through the PP pathway followed by subsequent conversion to ethanol from pyruvate after glycolysis (Ostergaard *et al.*, 2000). The microorganism is capable of growing at a pH as low as 5.0 and temperatures as warm as 153 °C. Natural xylose-fermenting yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *C. parapsilosis*, can metabolize xylose via the action of xylose reductase (XR) to convert xylose to xylitol, and of xylitol dehydrogenase (XDH) to convert xylitol to xylulose.

Therefore, bioethanol fermentation from xylose can be successfully performed by recombinant *S. cerevisiae* carrying heterologous XR and XDH from *P. stipitis*, and xylulokinase (XK) from *S. cerevisiae* (Katahira *et al.*, 2006).

Microorganisms for bio-ethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas, 2004). All the recombinant strains are mesophilic organisms and function best between 150 and 155°C (Hettenhaus, 1998). An organism must maintain fairly constant balance of pH to survive. Most bacteria grow best in a narrow range of pH from 6.5 to 7.5 (Aminifarshidmehr, 1996). Yeast and fungi tolerate a range of pH 3.5–5.0. The ability to lower pH below 4.0 offers a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants. The majority of organisms cannot tolerate bio-ethanol concentrations above 10–15% (w/v) (Hettenhaus, 1998).

2.7.1. Product and solids recovery

Distillation technologies that will allow the economic recovery of dilute volatile products from streams containing a variety of impurities have been developed and commercially demonstrated (Madson and Lococo, 2000). A distillation system separates the bio-ethanol from water in the liquid mixture. Water content of bio-ethanol is generally higher than 80%. Large quantities of energy are required to concentrate the ethanol to 95.6% (azeotrope mixture of ethanol with water). The beer column separates most of the bioethanol from water (and solids, if any) and produces a top stream rich in bioethanol, and a bottom stream rich in water (Karupiah *et al.*, 2007). In this flow, bioethanol from cellulosic biomass has likely lower product concentrations (5 wt %) than in bioethanol from corn. The maximum concentration of bioethanol tolerated by the microorganisms is about 10 wt% at 153 °C but decreases with increasing temperature.

To maximize cellulase activity, the operation is rather at maximum temperature (154 °C), since the cost impact of cellulase production is high relative to distillation (Hamelinck *et al.*, 2003, 2004, 2005). The first step is to recover the bioethanol in a distillation or beer

column, where most of the water remains with the solids part. The product (37% bioethanol) is then concentrated in a rectifying column to a concentration just below the azeotrope (95%) (Hamelinck *et al.*, 2005). The remaining bottom product is fed to the stripping column to remove additional water, with the bio-ethanol distillate from stripping being recombined with the feed to the rectifier (Kwiatkowski *et al.*, 2006). The recovery of bioethanol in the distillation columns in the plant is fixed to be 99.6% to reduce bioethanol losses (Karuppiyah *et al.*, 2007).

After the first effect, solids are separated using a centrifuge and dried in a rotary dryer. A portion (25%) of the centrifuge effluent is recycled to fermentation and the rest is sent to the second and third evaporator effects. Most of the evaporator condensate is returned to the process as fairly clean condensate (a small portion, 10%, is split off to waste water treatment to prevent build-up of low-boiling compounds) and the concentrated syrup contains 15–20% by weight total solids (McAloon, 2000).

2.7.2. Microorganisms of fermentation

In fermentation, of the various ethanol producing micro-organisms yeast belonging to *S.cerevisiae* have been used most commonly. One of the most promising ethanol producing organisms is the bacterium *Zymomonas mobilis* which is used to make palm wines. Bansal and Singh (2003) did a comparative study on ethanol production from molasses using *S. cerevisiae* and *Zymomonas mobilis*. Yeast was found to be more ethanol tolerant and produced more ethanol at sugar concentration above 15% (v/v). Isolated *S. cerevisiae* from palm wine, which produced increased amounts of ethanol in yeast extract peptone dextrose medium.

2.7.3. Factors Affecting Fermentation

Microorganisms involved in ethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature, pH, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, ethanol yield, genetic stability, and inhibitor tolerance (Demirbas, 2005).

2.7.3.1. Effect of sugar concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. The amount of ethanol produced is proportional to the amount of sugar added; thus, high sugar concentrations are desired for higher ethanol yield. However, too high sugar concentrations can inhibit metabolism due to increased osmotic stress. The optimal sugar concentration could contribute in optimizing yield by reducing the effect of osmotic stress. Osmotic stress results in yeast cells slowly decreasing water activity, when their substrate is drying in the sun. Cellular water follows its concentration gradient by passive diffusion, so that the cells lose water and the concentration of biomolecules and ions in the cell increases, eventually resulting in an arrest of cellular activity: the cell suffers high osmolarity or hyperosmotic stress. Hence, each fermentation process has an optimal glucose or equivalent sugar concentration (Sofer and Zaborsky, 1981). Yeast strains were capable of fermenting up to 30% of sucrose efficiently. The efficiency of selected strains varied from 89% to 92% depending upon the utilization of total sugar available in the medium. A maximum amount of 19.7% (v/v) ethanol accumulated from fermentation of 30% sugar as compared to two reference strains, which produced 18.0 (v/v) and 15.6 (v/v).

A repeated batch fermentation system was used to produce ethanol using an osmo-tolerant *S. cerevisiae* immobilized on calcium alginate (Sree *et al.*, 2000). Fermentation was carried out with initial concentration of 150, 200, 250 g glucose per liter at 30 °C. The maximum amount of ethanol produced by immobilization osmo-tolerant *S. cerevisiae* cells using 150, 200 and 250 g/L glucose was 72.5, 93 and 83 g ethanol per liter at 30 °C after 48 hours. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%.

2.7.3.2. Temperature

Temperature has an important influence on the growth rate of the microorganisms and the rate of ethanol production. Wine and beer fermentations are generally conducted below 20 °C, whereas higher temperatures (30-38°C) are being examined for industrial alcohol production by yeast cultures (Sofer and Zaborsky, 1981). Too high temperature kills

yeast, and low temperature slows down yeast activity and growth. Thus, specific range of temperature is required (Onuki, 2005).

All the engineered strains are mesophilic organisms and function best between 30 to 38°C. Operating at greater temperatures is desirable for the following reasons:

- High fermentation temperature increases growth rate and productivity exponentially
- Plant capital cost is less due to higher productivity per unit volume of fermenter vessel and cooling equipment investment is lowered.
- Operating costs are less since less energy is required to maintain desired fermentation temperature and recover the ethanol.
- Contamination risk is less as fewer organisms exist at high temperatures.

The tolerant ability of yeasts under the conditions of high temperature and high ethanol levels Osho *et al.* (2005) during fermentation process is becoming an increasingly important characteristic that attracts many researchers as there has been a mixed variety of benefits which could be exploited through the use of thermophilic yeasts for ethanol production. Thermo-tolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries and under tropical climates as well. Cooling costs during the process of ethanol production are expensive; hence, by using thermo-tolerant yeasts, cooling and distillation costs can be reduced. Besides, higher saccharification and fermentation rates, continuous ethanol removal and reduced contamination have stimulated a search for routes to thermo-tolerant yeasts.

2.7.3.3. Effect of pH

One of the factor affect cellular growth is external pH, the physiological status of an organism is the result of evolution which is the outcome of adaptation where their enzymes works best at optimal pH condition. Most alcoholic yeast fermentations are conducted below pH 4.5, although this may not be the optimal pH for growth or ethanol production. Yeast cultures can grow over a wide range from 3 to 8 with an optimum for growth generally in the slight acidic range. Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures (Goksungur and Zorlu, 2001). Thus,

the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth, and physicochemical effect on waste product pathways. Low pH values in yeast fermentation help to inhibit growth of contaminating bacterial cultures. Bacterial cultures generally have a pH optimum around 7-7.5, with less tolerance than yeast to acid conditions (Sofer and Zaborsky, 1981). Thus, optimizing pH condition where an optimal ethanol production obtained is necessary to exploit the full potential of yeast and substrate.

2.7.3.4. Ethanol concentration

The concentration of ethanol in the fermentation broth can directly affect the growth rate of the culture and its ability to convert sugar to ethanol. Inhibitory and toxicity level of ethanol vary from culture to culture. Higher temperature lowers the tolerance of the organism. At temperatures above 35 °C, current strains lose viability at ethanol concentrations of 10 % (w/v) (Hettenhaus, 1998). Successful fermentations to produce ethanol using yeast require tolerance to high concentrations of both glucose and ethanol. These cellular characteristics are important because of high gravity fermentations, which are common in the ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation.

2.7.3.5. Osmotic tolerance

The semi-permeable membrane surrounding the cell must be able to withstand wide osmotic pressure changes in extracellular fluids that impact the relative osmotic pressure difference. If not, the cells may be severely damaged or even killed. The cells may burst in a hypotonic solution, when the solution becomes more dilute than the intracellular fluid. If hypertonic, the cells will shrink from the osmotic pressure difference. Osmotic pressure limits can be one of the factors that restrict maximum substrate concentration (Sofer and Zaborsky, 1981).

2.8. Potential of coffee residues for bio-ethanol production in Ethiopia

Currently, the coffee pulp is discharged into local streams and rivers where it tends to clog, forming a putrescent mass and producing a highly acidic effluent which pollutes the water, destroying aquatic life and generating an offensive odor (Yisehak Seboka *et al.*,

2009). Recovery of this pulp for industrial fuel use would require collecting the residues as they are discharged from the pulping machine and processing them to greatly reduce the moisture content. Table 1 shows the regional distribution of coffee residues in Ethiopia (Kebede Dawit, 2001).

Table 1. Regional distribution of coffee residues

Process	Location	Green Coffee (tonnes/yr)	Coffee Residue (tonnes/yr)	No. of Processing Plants
Dry process	SNNPR	35,060	49,496	113
	Oromia	94,145	132,911	273
	Gambela	1,033	1,458	2
	Others	112	158	-
Wet process	SNNPR	16,533	20,006	309
	Oromiya	6,959	8,421	189
	Gambela	1,519	1,838	6
	Others	8	10	-
Grand Total		155,369	214,299	892

Source: Kebede Dawit (2001)

2.9. Production of Bio-ethanol from Lignocelluloses Biomass

Ethanol can be produced from lignocelluloses biomass by the hydrolysis and sugar fermentation processes. In order to produce sugars from the biomass, the biomass is pre-treated in to reduce the size of the feedstock and to open up the plant structure. The cellulose and the hemicelluloses portions are broken down (hydrolysed) by enzymes or dilute acids into glucose or sucrose sugars and then fermented into ethanol. Processing lignocelluloses to ethanol consists of four major unit operations: 1, Pre-treatment 2, Hydrolysis 3, Fermentation and 4, Distillation /Purification.

2.9.1. Pre-treatment

Pre-treatment is a vital step in production of ethanol from lignocellulosic materials. It is required in order to change the structure of cellulosic materials and to make cellulose more accessible to the enzymes or dilute acids, which convert the carbohydrate polymers

into fermentable sugars (Dehnavi, 2009). A successful pre-treatment must meet the following requirements: (i) improve formation of sugars or the ability to subsequently form sugars by hydrolysis, (ii) avoid degradation or loss of carbohydrate, (iii) avoid formation of by-products inhibitory to subsequent hydrolysis and fermentation processes, and (iv) be cost effective (Balata *et al.*, 2008). Additionally, main part of hemicellulose should be hydrolyzed and lignin should be released or even degraded. Pre-treatment methods are classified in different groups: physical such as milling/grinding, physicochemical like steam pretreatment/auto-hydrolysis, hydro-thermolysis/wet oxidation, chemical such as alkali/dilute acid/oxidizing agents/organic solvents, biological, electrical and combination of them (Dehnavi, 2009).

2.9.2. Hydrolysis

After pre-treatment of Lignocellulosic biomass, the cellulose is prepared for hydrolysis, which means, that a molecule is cleaved by the addition of a water molecule (Idi and Mohamad, 2011): $(C_6 H_{10} O_5)_n + n H_2O \rightarrow n C_6 H_{12} O_6$ (2) Hydrolysis involves cleaving the polymers of cellulose and hemicellulose into their monomers. Complete hydrolysis of cellulose results in glucose, whereas the hemicellulose gives rise to several pentoses and hexoses. While softwood hemicelluloses are mainly composed of mannose, the dominant sugar in hemicellulose derived from hardwood and crop residues is usually xylose (Taherzadeh *et al.*, 1997; Karimi *et al.*, 2006). The hydrolysis can be carried out chemically or enzymatically (Joshi *et al.*, 2011).

2.9.3. Acid hydrolysis

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicellulose polymers. Acids are predominantly applied in chemical hydrolyses. Sulfuric acid is the most investigated acid, although other acids such as hydrochloric have also been used. Acid hydrolyses can be divided into two groups: (a) concentrated-acid hydrolysis and (b) dilute-acid hydrolysis (Taherzadeh and Karimi, 2007; Joshi *et al.*, 2011).

Lignocelluloses can be degraded by physical, chemical and biological procedures. Physical methods involving milling and radiation treatment that decreases particles sizes,

surface area and degree of polymerization. In chemical procedures, lignocelluloses are treated with acid, alkalin, ammonia, steam; hypochlorite etc to remove lignin. But the main problem is low yields because of sugar degradation products and inhibits fermentation process. Biological or enzymatic degradation of lignocelluloses yields maximum and provides promising aspect of bioconversion products because of high specificity of enzyme substrate reaction. Crystalline structure and microstructure and porosity of cellulose are also not degraded by biological degradation. Moreover, the enzyme cleaves the cellulose bond precisely and product conversion rate is higher.

2.9.3.1. Dilute-Acid Hydrolysis

Dilute-acid hydrolysis is probably the most commonly applied method among the chemical hydrolysis methods. It is a method that can be used either as a pre-treatment preceding enzymatic hydrolysis, or as the main drawback of dilute-acid hydrolysis processes, particularly in one stage, is degradation of the sugars in hydrolysis reactions and formation of undesirable by-products potential inhibitors are furfural, 5 hydroxymethylfurfural (HMF), levulinic acid, acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, formaldehyde, etc (Taherzadeh and Karimi, 2007 ; Joshi *et al.*, 2011). These are not only lowers the yield of sugars, but also severely inhibit the formation of bio-ethanol during the fermentation. Some inhibitors, such as terpene compounds, are initially present in the wood, but apparently most of the inhibitors are formed during the hydrolysis process. In order to avoid degradation of monosaccharide's at high temperatures and formation of the inhibitors, dilute-acid hydrolysis is carried out in two (more) stages. Stage one is performed at low temperatures, to maximize the yield from hemicelluloses, as well as to recover the pentose. Stage two is done at higher temperatures, to optimize the cellulose portion of the feedstock, as well as to recover the hexose (Idi and Mohamad, 2011). In a one-stage pre-treatment, a temperature between 140 and 170 °C can be used, but two treatments at about 120 °C for a longer time may also be applied (Taherzadeh and Karimi, 2007; Idi and Mohamad, 2011). Ethanol production from lignocellulosic biomass includes pretreatment of biomass, enzymatic hydrolysis of cellulose, fermentation of hexose/pentose sugars, and recovery of ethanol. Intensive efforts have been made in recent years to develop efficient technologies for the pretreatment of sugarcane bagasse,

developments of enzymes for enhanced cellulose/hemicellulose saccharification, and suitable technologies for the fermentation of both C6 and C5 sugars (Soccol *et al.*, 2010). Hemicellulose hydrolysate typically contains primarily pentose sugars (xylose and arabinose) and some amounts of hexose sugars (mannose, glucose and galactose) (Singh, *et al.*, 2011). A variety of yeast, fungi, and bacteria are capable of assimilating pentose, but only a few are promising candidates for the efficient xylose fermentation into ethanol (Singh, *et al.*, 2011).

In yeasts, the assimilation of D-xylose follows the pathway where the sugar passes through a pool enzymatic to enter in the phosphopentose pathway (Bettiga *et al.*, 2008). There are several microorganisms capable of assimilating pentose sugars, but only few species are capable of assimilating sugars to produce ethanol at industrial scale.

2.9.3.2. Enzymatic hydrolysis

Enzymatic hydrolysis is a method in which cellulases are utilized for the hydrolysis. This is a quite new approach compared to concentrated-acid and dilute-acid hydrolysis. Cellulolytic enzymes were discovered during World War II when American scientists found the agent that was responsible for army clothing deterioration in the jungles of the South Pacific (Alriksson, 2006). This process occurs by an enzyme called cellulase. The cellulase consists of three consortiums of enzymes: Endoglucanase (endo-1,4-D-glucanohydrolase) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends, exoglucanase or cellobiohydrolase, which degrades the molecule further by removing cellobiose units from the free chain-ends and β -glucosidase (cellobiase) which hydrolyzes cellobiose to produce glucose. These are usually come from the fungus known as *Trichoderma reesei* (Alriksson, 2006). Hemicelluloses can also be hydrolyzed by hemicellulase or acid to its monomeric sugars including arabinose, galactose, xylose, and glucose. Using enzymes for the hydrolysis of lignocelluloses is considered more advantageous than other chemical conversion. It offers low energy requirements, mild operating conditions, higher yields, minimal by-products formation, known end product, and environmentally friendly. The disadvantages are the slow reaction rate of the enzymes and the high enzyme cost. (Alriksson, 2006; Idi and Mohamad, 2011; Joshi *et al.*, 2011). To overcome this problem, enzymatic hydrolysis of

cellulose is carried out by *cellulase* enzymes which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50 °C) and does not have a corrosion problem (Idi and Mohamad, 2011). Both bacteria and fungi can produce *cellulases* for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases (Joshi *et al.*, 2011). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulose production.

2.10. Current status of ethanol and sugar production in Ethiopia

In Ethiopia, the large energy utilization in the form of imported petroleum poses different socio-economic and environmental problems. Therefore, dealing with the conditions and reducing the socio-economic and environmental challenges of the country by developing alternative sustainable energy supply and use systems such as use of locally produced ethanol blended fuel in transportation are a very important means that would apparently reduce some of these burdens of the country.

There is only one sugar mill producing ethanol and few distilleries participating in downstream chemicals from alcohol in the country at present due to poor economy of scales (ESDA, 2005), which sugar factory is doing that Finchaa, Wonji/Shoa sugar and Metehara sugar factories but Finchaa sugar factory among molasses derived products ethanol takes the largest part, but its utilization must attract the attention of the government policy makers in order to utilize as a bio-ethanol. Bio-ethanol or biofuel (bio-ethanol is not equivalent to bio-ethanol, it includes biodiesel) is ethanol-based products that can be processed into liquid fuels for either transport or heating purposes from starch crops sugar cane, cassava and sugar beet. With the coming into being of the sugar sector expansion and modernization in the country, implementation of the different domestic measures for bio-ethanol fuels utilization has to take place.

At present there was about 8000 cubic meter annual production of ethanol, but there are projects towards increasing the product to over 142000 cubic meter (ESDA, 2005). Therefore, this study has initiated to reduce the net contribution of greenhouse gases to the atmosphere, bio-ethanol and any other biofuel has been recognized as a potential alternative to petroleum-derived transportation fuels and cooking fuels. Biomass, which is the source of bio-ethanol and other biofuels, is a potential renewable energy source that could replace fossil energy for transportation. There has not been any significant exploration of coffee as a substrate for ethanol production in Ethiopia and therefore its potential can be estimated from other often-used substrates like sugarcane bagasse. Now a day sugarcane bagasse had the leading potential for ethanol production in Ethiopia. Ethiopia being a leading producer of coffee could yield substantial amounts from coffee waste conversion processes.

2.10.1. Development status of bio-fuels in Ethiopia

Ethiopia are currently looking at growing high-yielding crops for the production of bio-fuels (Jatropha) as alternatives to traditional fuels (bio-diesel) to address imminent shortages and reduce impacts of climate change. It is also aimed in alleviation dependency on importation of fossil fuel that could severely dependant on political stability of OPEC countries. Moreover, it drains most of the foreign currency earning. Owing to such phenomenon, and indeed in view of the recent trends in the escalating price of the traditional petro-fuel, biofuel has been gaining greater attention by the Ethiopian government. Bio-fuel development is aimed for, import substitute of petroleum products and export earning and enhance agricultural development and agro processing; job creation and improve income of the people improve agricultural land productivity through integrations of bio-fuel development program with land use plan decrease environmental pollution through the promotion of bio-fuel utilization. The current Bio-fuel development in the country emphasizes on the production of: Bio-ethanol from (sugar cane and others) and bio-diesel from (Jatropha, castor bean plants, and palm) (ESDA, 2005).

3. MATERIALS AND METHODS

3.1. Sampling site and sample collection

Twenty seven different sample of fruits, guava (*Psidium guajava*), grapefruit (*Citrus paradise*), avocado (*Persea americana*), papaya (*Carica papaya*) and *gishita* (*Annona senegalensis pers.*) were collected from Southwestern Ethiopia, Jimma ,Sheka, Kefa, Gurage and Bench-Maji Zone and Addis Ababa fruit market (*Atikilt Tera*) using sterile plastic bags and brought to the Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University and the samples were kept at 4 °C for farther study.

3.2. Isolation and screening of ethanol, sugar and thermo tolerant yeasts

The yeasts were isolated from guava (*Psidium guajava*), grapefruit (*Citrus paradise*), avocado (*Persea Americana*), papaya (*Carica papaya*) and *gishita* (*Annona senegalensis pers.*) samples after dilution followed by plating aliquots of appropriate dilution of samples on yeast extract peptone dextrose agar (YPDA). 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 (10^{-3} and 10^{-4}) were spread onto YPDA (Pons *et al.*, 1986). The YPDA medium contains g/l of yeast extract 10, peptone 20, dextrose (glucose) 20, and agar 20. It supplemented with 0.1 mg/ml streptomycin sulphate antibiotics to inhibit bacterial growth (Osho, 2005). The plates were incubated at 37°C for 24 and 48 hrs. Morphologically distinguished colonies were then selected under a dissection microscope. Yeast isolates were purified by sub culturing on YPD medium by streaking. The Pure culture was kept on YPD slant agar and stored at 4°C for further study.

3.3. Identification of ethanol, sugar and thermo tolerant yeast isolates

3.3.1. Morphological characterization

The yeast isolate from grapefruit were designated by AAUD, the yeast isolate from guava were designated by AAUZ. The yeast isolates from avocado were designated by AAUAV. The yeast isolates from papaya were designated by AAUP (AAUP₁, AAUP₂ and AAUP₃) and the yeast isolates from *gishita* (*Annona senegalensis pers.*) were

designated by AAUG (AAUG₁, AAUG₂ and AAUG₃). The morphology of the vegetative cells determined by growing in liquid and on solid culture media YPD (Kreger-van Rij, 1984; Kurtzman and Fell, 1997). Yeast cells were examined under microscope for size, shape and methods of vegetative reproduction.

3.3.1.1. Growth on solid medium

According Apiradee (2006) the morphology of cells of ethanol, sugar and thermo tolerant yeasts and their appearance on solid medium, on YPD agar and YPD liquid medium was examined, after incubating at 37°C for 3 days. The cultural features recorded were texture, color and surface of colonies. Moreover, ascospore and pseudo mycelium formation were recorded.

3.3.1.2. Ascospore formation

The selected yeast isolates were examined for ascospore formation using presporulation and sporulation according to Kurtzman and Fell (2005). The presporulation medium composed 20 g of glucose, 2 g of ammonium sulfate ((NH₄)₂SO₄), 2 g of potassium dihydrogen phosphate (KH₂PO₄) and 5 g of yeast extract in 1000 ml of distilled water and the medium was kept in sterile state for 7 days. The medium was inoculated with a loopful young culture of 48 hrs old and incubated at 25°C, on shaker for 3 days. The sporulation medium consisted of 1 g of glucose, 8.2 g of potassium acetate, 2.5 g of yeast extract and 1.86 g of magnesium sulfate heptahydrate (MgSO₄.7H₂O) in 1000 ml of distilled water. The medium was inoculated with a drop of yeasts from the presporulation medium and incubated at 25°C. The culture was examined microscopically for ascospores production at weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation with carbol-fuchsin (Kreger-van Rij, 1984). Slides were decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. They were slide was rinsed in water and counter stained with 1% methylene blue to observe the mature ascospores stain red and blue stains of the vegetative cells.

3.3.1.3. Pseudo mycelium formation

The formation of pseudo mycelium was investigated by slide culture technique, according to Kreger-van Rij (1984). 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 sterile Petri dish. The glass slide was quickly removed from the glass rod dipped into the medium 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 the 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.

3.4. Testing of yeast isolates for carbohydrate fermentation using Durham tube method

Durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast fermentation YP broth was used characterization of the yeast isolates based on fermentation of specific carbohydrates. The carbohydrates used were; glucose, galactose, maltose, sucrose, lactose trehalose, fructose and xylose. Yeast fermentation broth was modification of media developed by Wickerham for the determination carbohydrate to detect the color of the medium and gas formation (Warren and Shadomy, 1991). Yeast fermentation broth with carbohydrate and Durham tube composed of 4.5 g of yeast extracts, 7.5 g of peptone, 80 g of lactose, 120 g of raffinose, 60 g other carbohydrates and 17 g of bromcresol blue per liter deionized filtered water and final pH 7.1 ± 2 at 25°C.

3.5. Optimization of temperature for ethanol, sugar and thermo tolerant yeast isolates growth

Fifty ml of YPD medium was distributed into 125 ml screw cap Erlenmeyer flask and inoculated with actively growing culture according to Sree *et al.* (1999). All cultures were incubated at 30 °C, 37 °C, 40 °C and 45 °C for 72 hrs. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one $OD_{660\text{ nm}} = 1.85 \times 10^7 \text{ cell/ml}$).

3.6. Detection of ethanol tolerance of the yeast isolates

The medium for the detection of ethanol tolerance of ethanol, sugar and thermo tolerant yeast was modified from Osho (2005). YPD liquid medium was used for the tests. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was taken from 5 to 25% (v/v), and transferred to different flasks (125ml). Forty ml of the medium was distributed into each flask and then inoculated with selected isolates. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one OD_{660 nm} = 1.85×10^7 cell/ml). All cultures were incubated at 37°C for 3 days. The increase in optical density in a flask was recorded as evidence of growth.

3.7. Optimizations of glucose tolerance of the yeast isolates

Testing of glucose tolerance, YPD broth was prepared containing 10%, 15%, 30%, 45% and 50% of different sugar concentrations according to Fakruddin (2013). The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one OD_{660 nm} = 1.85×10^7 cell/ml). All cultures were incubated at 37°C for 48 hrs. The increase in optical density in a flask was recorded as evidence of growth.

3.8. Optimization of the yeast isolates growth for ethanol production

3.8.1. Nitrogen sources

The M9 minimal basal medium was used to determine appropriate nitrogen sources for ethanol production according to Sambrook and Russell (2001) (Table 2). Five sources of nitrogen; sodium nitrate (NaNO₃), ammonium chloride (NH₄Cl), ammonium sulphate ((NH₄)₂SO₄), diammonium hydrogen phosphate ((NH₄)₂HPO₄) and ammonium dihydrogen phosphate (NH₄H₂PO₄) were used as substituted nitrogen sources in 5X M9 salts was tried at 0 to 25 g/l, differing by 5 g/l from one flask to the other. Fifty ml of the

medium was distributed into 125 ml screw cap Erlenmeyer flask and adjusted to pH 5.5 with 1 N HCl and with NaOH. The inoculums were prepared from the yeast isolates which were grown aerobically in YPD medium and incubated at 37°C for 24 hrs. A ten percent inoculum were centrifuged at 4,000×g for 15 min and the pellet washed with sterile distilled water before inoculated into the medium. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer (Norrell and Messley, 1997). The treatments were replicated three times and the blank was made of YPD medium. All cultures were incubated at 37°C for 3 days.

Table 2. The composition of M9 minimal medium

The composition of M9 minimal medium per liter	
5X M9 salts	200 ml
1M MgSO ₄ 2 ml	
20% solution of carbon source	20 ml
1M CaCl ₂	0.1 ml
5X M9 salts (composition per liter)	
Na ₂ HPO ₄ .7H ₂ O	64 g
KH ₂ PO ₄	15 g
NaCl	2.5 g
NH ₄ C	15 g

Source: Sambrook and Russell (2001)

3.8.2. Glucose concentration

The basal medium and methodology to examine the optimum glucose concentration was described in section 3.8.1 except the addition of different glucose concentration, and nitrogen source in 5X M9 salts under different incubation times. Glucose concentrations were prepared from 0 to 25% (v/v), differing by 5% (v/v) from one flask to the other. Ten g/l (NH₄)₂SO₄ was used as a source of nitrogen. All cultures were incubated at 37°C for 3 weeks. Similar to section 3.7.1, the highest dry mass of yeast isolates was selected for further analysis at various glucose concentrations.

3.8.3. Initial pH

The basal medium and methodology were similar to section 3.8.2., five percent (v/v) glucose solution and 10 g/l $(\text{NH}_4)_2\text{SO}_4$ in 5X M9 salts were used to determine the optimum pH for yeast growth and ethanol production. The pH was adjusted in the range between 2.0 to 8.0 with 1 N HCl or 1N NaOH. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer (Norrell and Messley, 1997). The treatments were replicated three times and the blank was made of YPD medium. All cultures were incubated at 37°C for 3 days.

3.9. Determination of alcohol production by yeast isolates

Ethanol assay from sample was tested by the method of Caputic *et al.* (1986). One ml of culture supernatant was taken from YPD broth and made up to the volume to 5ml with distilled water and followed by the addition of 1ml of $\text{K}_2\text{Cr}_2\text{O}_7$ solution and 4ml of concentration H_2SO_4 solution. The intensity of colour was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. Blank was prepared in the same manner without ethanol. Alcohol standard was prepared by dissolving absolute ethanol in water and constructed according Satish *et al* (2010).

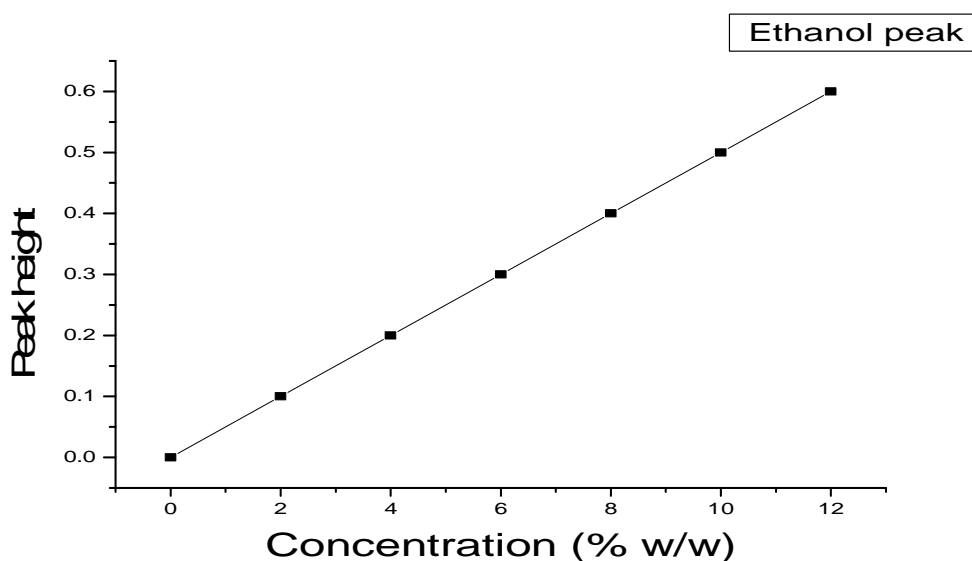


Figure 2: Calibration curve of ethanol standard solution

3.10. Coffee husk preparation for acid hydrolysis

Coffee residues (coffee husks) of *Coffea arabica* was collected from a pulping centers of Jimma Zone and the samples were stored in hermetically closed plastic containers, at room temperature, until required for treatments and then taken to Addis Ababa University, Department of Chemistry laboratory for analysis. The husk was oven-dried at 60°C for 48 hrs (moisture content of 7.24 %), grinded by coffee grinder and sieved (Urbaneja *et al.*, 1996).

3.11. Determination coffee husk moisture content

The moisture content of the samples was determined by oven drying method. The sample was weighed with glass crucible and placed in the air drying oven for 48 h at 60°C and cooled to room temperature in desiccators and weighed. The process was repeated until a constant weight was achieved and thus making it free of moisture content. The moisture content was calculated using the formula (Thuong and Phu, 2014):

$$\% \text{ Moisture content} = \frac{w_1 - w_2}{w_1} \times 100$$

Where:

W1= Weight of the sample before drying, in g.

W2= Weight of the sample after drying, in g.

3.12. Coffee Husk for Fermentation:

The dried coffee husk samples were acid hydrolyzed using H₂SO₄ at different concentrations, 5, 10 and 15 minute hydrolysis times and constant temperature (90°C) to determine the maximal sugar released and ethanol production from the substrate. The fermentation of coffee husk conducted for three times in monthly interval with three treatment replication.

Treatment details: A 25 g of dried coffee husk was weighed and then saccharified in volume of 250 ml H₂SO₄ at molar concentrations of 0.25, 0.5, 1, 2, 3 and 4 and 12.5, 25,50,100,150 and 200 ml distilled water was added respectively using reflux setup. The saccharified hydrolysates were filtered for fermentation.

3.13. Fermentation

3.13.1. Preparation yeast inoculums

The yeast *S. cerevisiae*, purchased from local market in Addis Ababa, Ethiopia and the four yeast isolates used in all experiments. *S. cerevisiae*, purchased from local market was first 3 g/l rejuvenated in lukewarm water for 10 minutes. Then the prepared inoculums were taken into the flask containing coffee husk hydrolysate sample was adjusted to pH 5 with 1NaOH to remove the acid in the form of salt and water. A 10 ml of yeast isolates were taken from their 48 hrs old broth for inoculums.

3.13.2. Ethanol Fermentation

Fermentation conditions of the experimental setup were the following; the substrates which were saccharified with different molar concentration of H₂SO₄ were kept for different period of fermentation (24, 48 and 72 hrs), hydrolysis time (5, 10, 15 min), temperature (25, 30 and 37°C) and pH (4, 5, and 6) and the experiments replicated three times to identify the maximal ethanol production from the substrate.

3.14. Ethanol recovery

Distillation method: Fermented solution 250 ml flask was heated at constant temperature (90°C) to force the lowest boiling material into the vapour phase. The vapour was passed over fractional column and the bulb of a thermometer at which point vapour was determined (El-diwany *et al.*, 1992). The vapour was condensed to a liquid in the horizontal condenser that was cooled with a flow of cold water. The distillate was collected in a receiver. The volume of the distillate was measured.

3.15. Analytical Methods

3.15.1. Determination of sugar content

The amount of sugar in the hydrolyzed samples was determined by Fehling method. Fifty ml of hydrolyzed sample solution was dissolved in 10 ml of distilled water and 2 ml of concentrated 1HCl was added and boiled. The obtained sample was neutralized with 1NaOH and the solution was made up to a volume of 300 ml and taken into the burette. The 5 mL of Fehling A and 5 ml of Fehling B were taken and mixed with 90 ml of distilled water in 250 ml Erlenmeyer flask and two drop of Methylene blue indicator was

added. The solution in the flask was titrated with burette solution in boiling conditions until disappearance of blue color and the volume at which brick red color observed were recorded. For each sample the sugar content was calculated by using the formula given below (Periyasamy *et al.*, 2009):

$$\text{Sugar content (\%)} = \frac{300\text{mL} \times f}{v} \times 100$$

Where: f= Fehling factor (0.051); v= volume used in the titration (titrate value) (ml).

3.16. Determination of bio-ethanol concentration

The Fourier Transform Infrared (FTIR) response was calibrated using different concentrations of 99.9% pure ethanol. The measurement was carried out in reflectance mode where ZnSe (Zinc selenide) window was used as a sample holder. After the absorbance the concentration of pure ethanol was recorded against the calibration curve and then converted to volume-to-volume concentration units.

3.17. Data analysis

All data were analyzed using one way analysis of variance (ANOVA) among treatment means at 5% level of significance were compared using the least significant difference (LCD) by SPSS software version 16 for windows.

4. RESULTS

4.1. Isolation and screening of ethanol, sugar and thermo tolerant yeasts

A total of nine yeast isolates were retrieved from five different fruit samples collected from Southwestern Ethiopia and Addis Ababa fruits markets, each from guava (*Psidium guajava*), grapefruit (*Citrus paradise*) and avocado (*Persea americana*) and three each from papaya (*Carica papaya*) and *gishita* (*Annona senegalensis pers.*). Most colonies were creamy (whitish) oval, convex and dome shaped except one isolate from papaya, which was pink after ten days of fermentation. All yeast isolates were reproduced by budding (Table 3).

4.2. Identification of the yeast isolates

4.2.1. Morphological and physiological characteristics of the yeast isolates

All of yeast isolates were observed under compound microscope and cell morphology was observed after 3 days of incubation, at 37°C, heavy, dry climbing pellicles were formed on the surface of YPD broth medium. The growth was butyrous with white cream color on YPD agar (Table 3). The cell morphology of AAUP₁, AAUD, AAUG₃ and AAUAV isolates was ovoidal to elongate and had single, pairs, or triple budding cells and Pseudo mycelia were also developed. Ascospore formed in ascospore forming media after incubating for 3 weeks at 25⁰C.

Table 3. Summary of morphological features of yeast isolates

Character	AAUP ₁	AAUD	AAUG ₃	AAUAV	S.cer.(commercial)
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Slightly crispulate	Crispulate	Crispulate	Slightly crispulate	Crispulate
Color	Cream,white	Cream,white	Cream, white	Cream, white	Cream, white
Elevation	Convex	Slightly Convex	Convex	Convex	Convex
Cell	spheroidal,ellipsoidal Multilateral budding	spheroidal, ellipsoidal Multilateralbuding	spheroidal, ellipsoidal Multilateral budding	spheroidal, ellipsoidal Multilateral budding	spheroidal, ellipsoidal Multilateral budding
Ascospore	+	+	+	+	+
Pseudomycelium	+	+	+	+	+

4.3. Testing of yeast isolates for carbohydrate fermentation using Durham tube method

In this study, the yeast isolates showed variation in utilization of eight different sugars. Almost all isolates utilized glucose, galactose, sucrose, maltose, fructose and trehalose. All isolates failed to grow on xylose and lactose. The most vigorous isolates were AAUP₁, AAUD, AAUAV and AAUG₃ growing on several of the test carbohydrates (Table 4).

Table 4. Comparison and selection of yeast isolates by Durham tube yeast extract peptone dextrose (YPD) liquid medium fermentation method

YPD liquid medium for fermentation									
Isolates	Glucose	Galactose	Maltose	Lactose	Sucrose	Fructose	Trehalose	Xylose	Total
AAUP ₁	++	++	+++	-	+++	+	++	-	6
AAUD	+++	++	++	-	++	+	+	-	6
AAUG ₃	++	++	++	-	++	++	++	-	6
AAUAV	++	++	+	-	+	+	-	-	5
S.cer	+	+	+	-	+	+	+	-	6
Total	5	5	5	0	5	5	4	0	

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

4.4. Optimization of temperature for the growth of ethanol, sugar and thermo tolerant yeast isolates

The effect of temperature on the growth of yeast isolates was study under the range of 30-45⁰C. The highest cell dry weight was at 37⁰C but for commercial yeast 30⁰C (Figure 3). Accordingly AAUP₁, AAUD, AAUG₃ and AAUAV were relatively the highest thermo tolerant than the other yeast isolates. The four yeast isolates were resistant to high temperature in the broth media and high fermentative when compared to the other isolates (Figure 3). Four isolates identified as thermo tolerant, they are AAUP₁, AAUD, AAUG₃ and AAUAV. The most thermo tolerant isolates were AAUD tolerant to 45°C, followed by isolate AAUG₃ which was tolerant to 40°C temperature in the broth media (Figure 3).

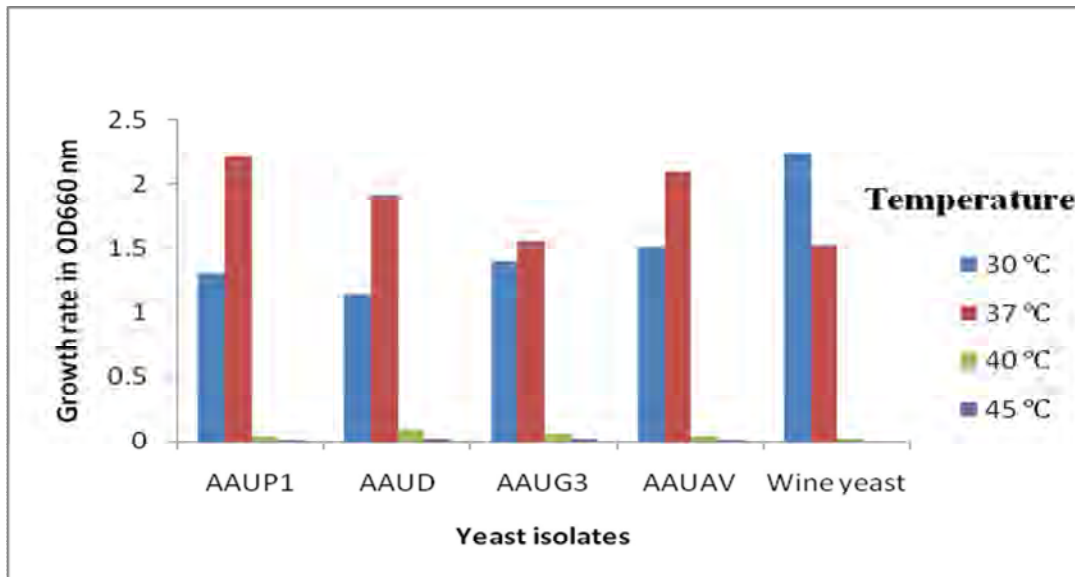


Figure 3. The effect of temperature on the growth of thermo tolerant yeast isolates cultivated in YPD broth for 3 days (Dry weight in OD₆₆₀ nm)

4.5. Ethanol tolerance of the yeast isolates

Yeast isolates (AAUP₁, AAUD, AAUG₃ and AAUAV) were able to grow in $\geq 5\%$ (v/v) ethanol concentration (Figure 4). There were slight differences observed in growth rates while increasing the ethanol concentration from 5 to 10 % (v/v) and 15 to 25 % (v/v). The most ethanol tolerant yeast isolates (AAUG₃) that was isolated from *gishita* (*Annona senegalensis pers.*) with ethanol tolerance to 15% (v/v) followed by the yeast isolate (AAUD) from grape with ethanol tolerant of 10% (v/v) ethanol in the broth medium (Figure 4). The isolates AAUAV was tolerant to ethanol up to 10% (v/v). Accordingly, AAUP₁, AAUD, AAUG₃ and AAUAV were relatively rapidly fermentative and the highest ethanol tolerant.

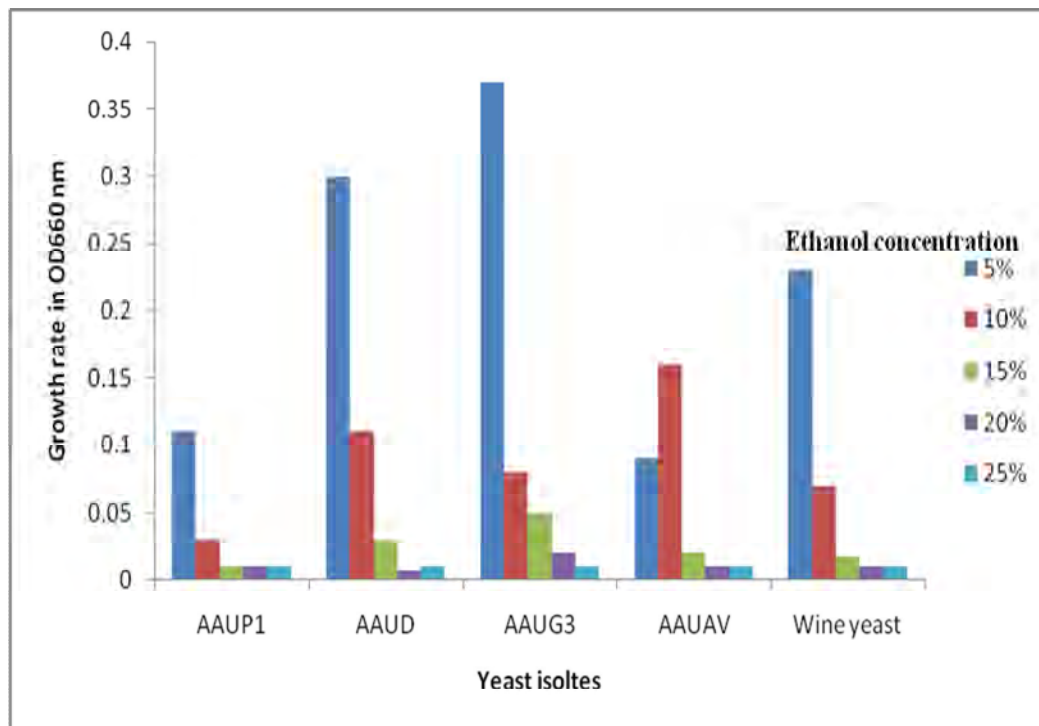


Figure 4. The growth response of yeast isolates in relation to ethanol concentrations (% v/v) in YPD medium (Dry weight in OD₆₆₀ nm)

4.6. Sugar tolerance of the yeast isolates

Sugar tolerance the yeast isolates (AAUP₁, AAUD, AAUG₃ and AAUAV) increased in growth at an increasing concentration up to 30% but decreased slightly at concentration up to 50% (Figure 5). The most sugar tolerant was AAUAV with sugar tolerance capacity of up to 50% (v/v) followed by isolates AAUG₃ which was tolerant to 45% (v/v) sugar concentration in the broth media. In general AAUP₁, AAUD, AAUG₃ and AAUAV were the highest sugar tolerant than other yeast isolates (Figure 5). These four yeast isolates were identified as resistant to high sugar concentration in the broth medium.

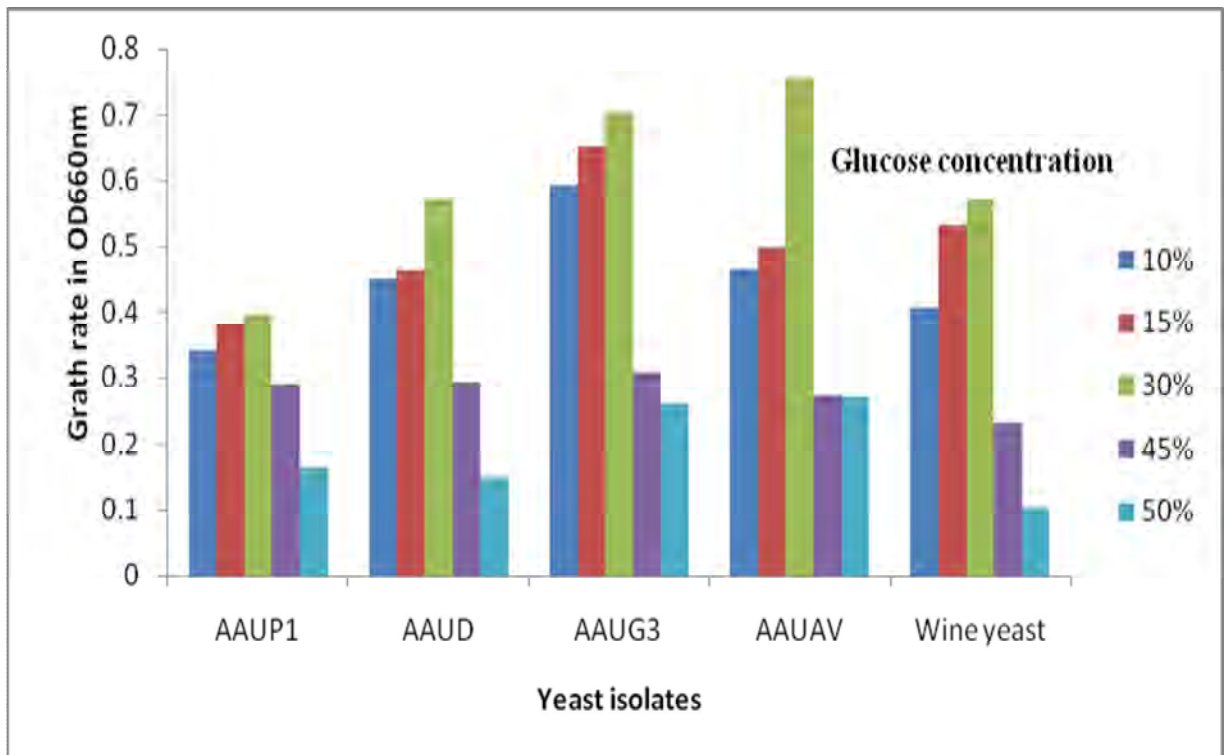
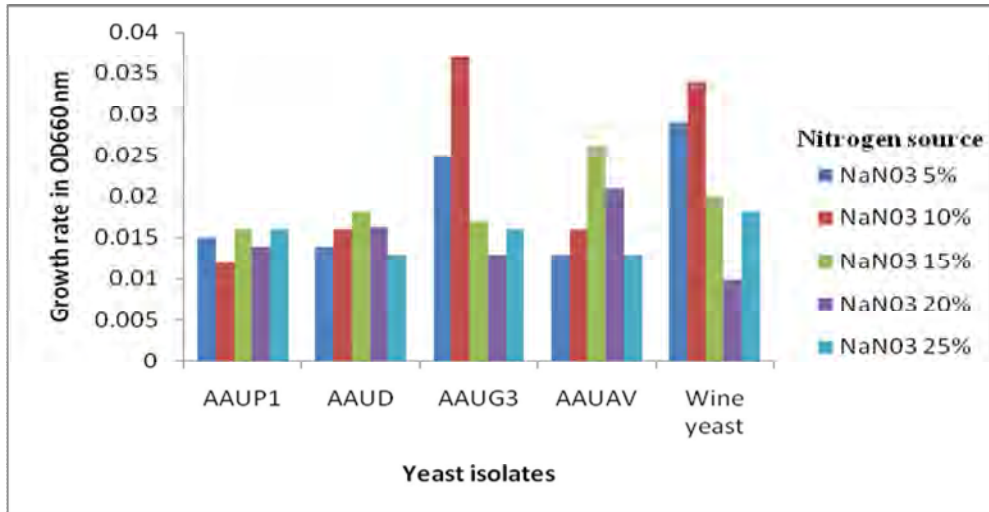


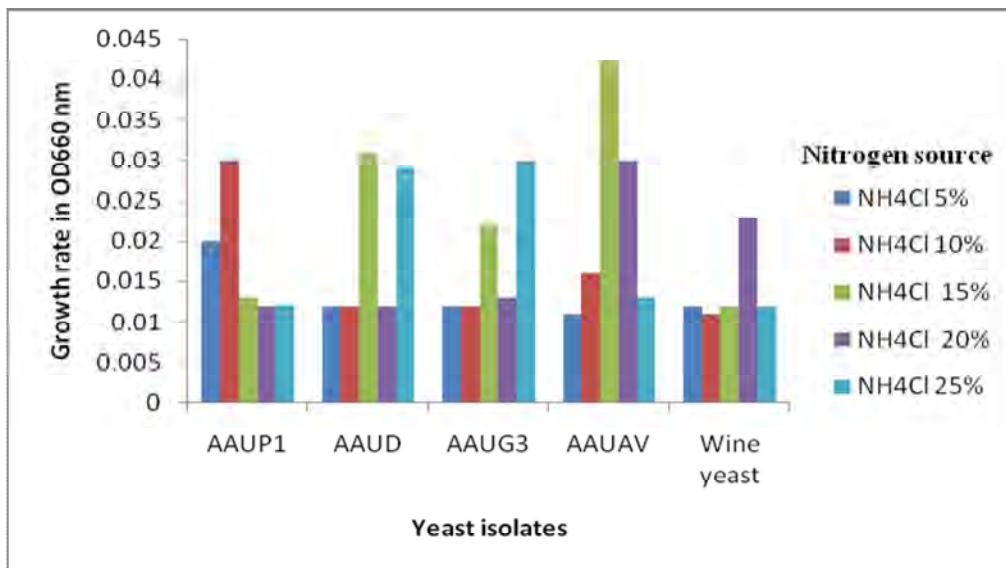
Figure 5. Summary of yeast isolates growth rate response for various glucose concentrations (% w/v) in YPD medium (Dry weight in OD₆₆₀ nm)

4.7. Optimization of nitrogen sources for growth of the yeast isolates

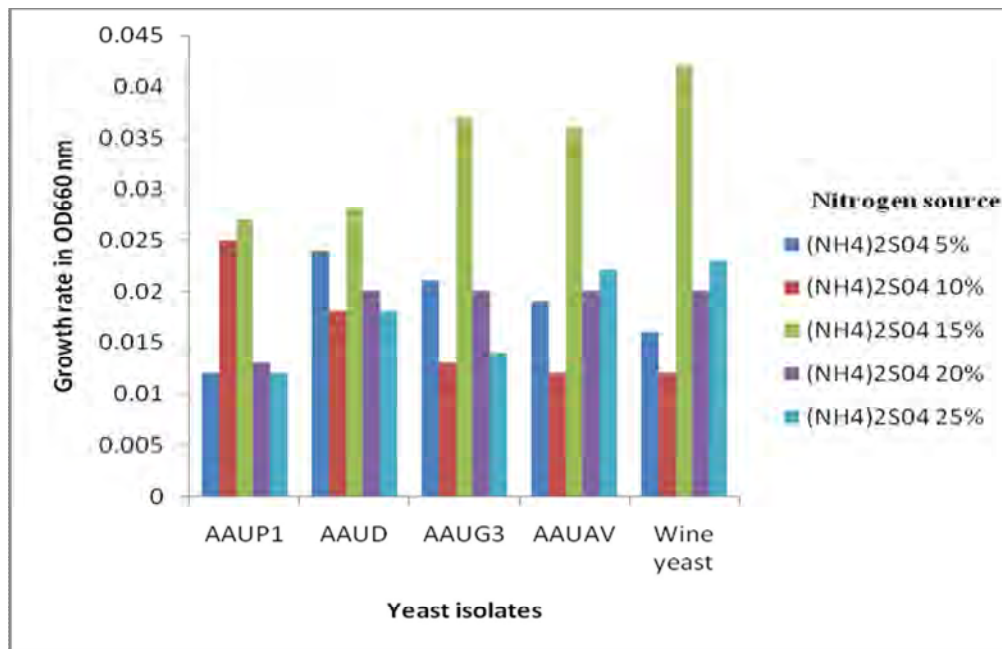
The isolates preferred $(\text{NH}_4)_2\text{HPO}_4$ as the nitrogen source for growth, to other nitrogen sources such as $\text{NH}_4\text{H}_2\text{PO}_4$, NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$ and NaNO_3 (Figure 6).



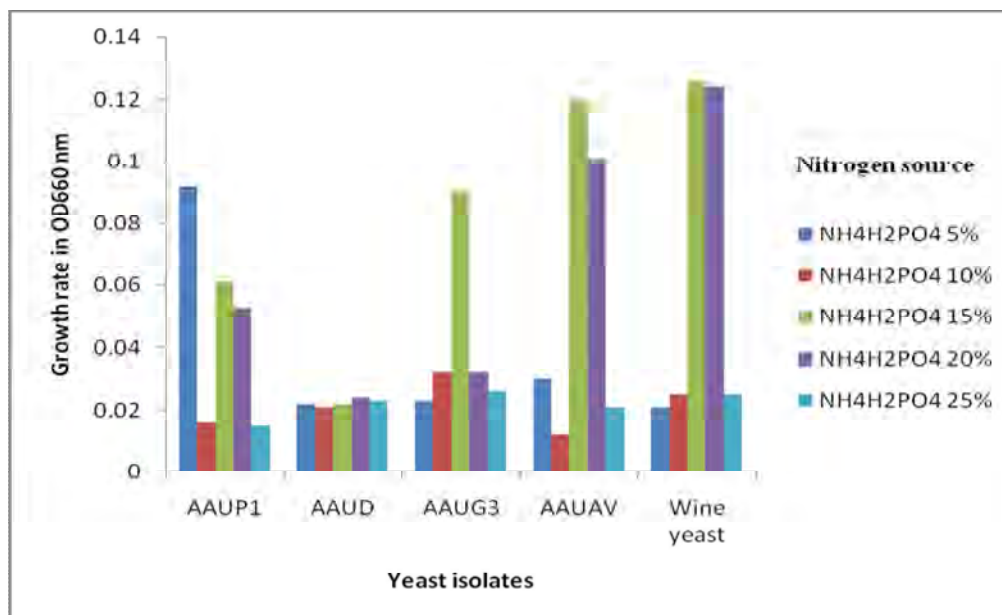
(a)



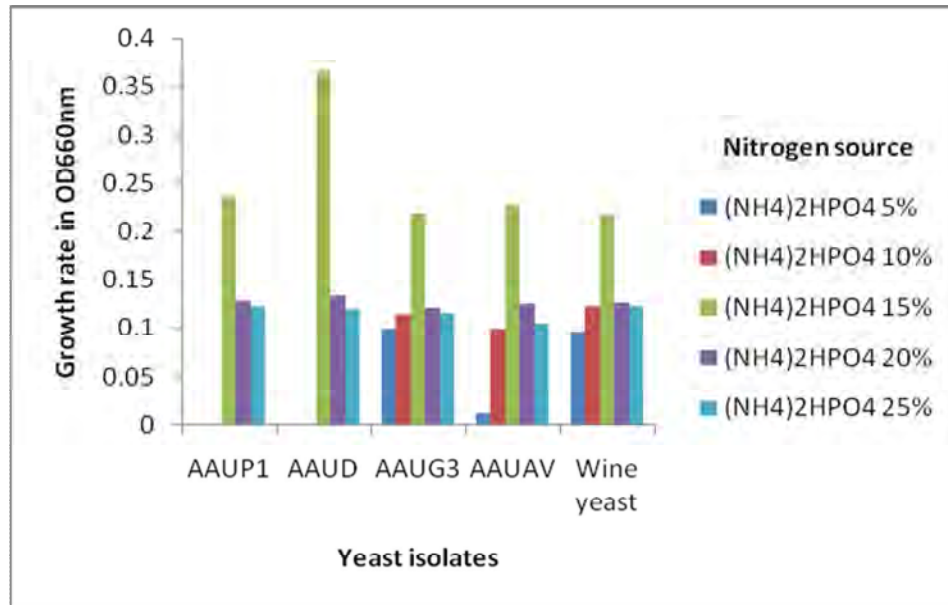
(b)



(c)



(d)



(e)

Figure 6. Effect of various sources and concentration of nitrogen for growth of yeast isolates at 37°C for 3 days in M9 minimal medium (Dry weight in OD₆₆₀ nm) a) NaNO₃, b) NH₄Cl, c) (NH₄)₂SO₄, d) (NH₄)₂HPO₄ and e) NH₄H₂PO₄

4.8. Optimization of pH for growth of the yeast isolates

The growth rate of yeast isolates increased along with the pH level increase to 3.0. The pH of the medium did not show different effect on growth rate at pH 3.0 to 6.5, beyond which the growth was decreased. At medium pH 7.0 to 8.0, the final dry mass was slightly decreased (Figure 7). The highest dry mass was observed in the medium where the pH was 5.0 for all yeast isolates (Figure 7).

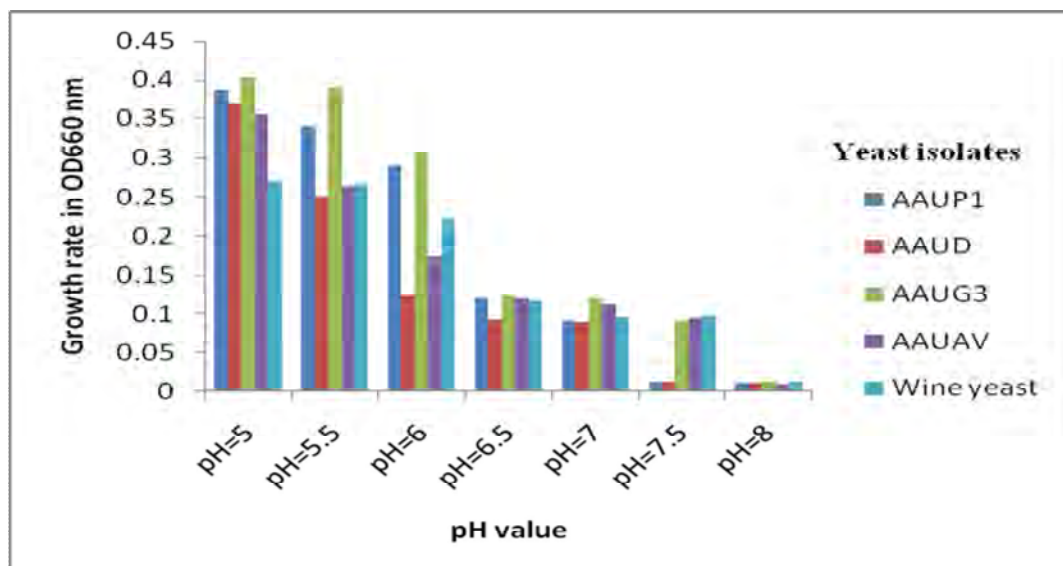
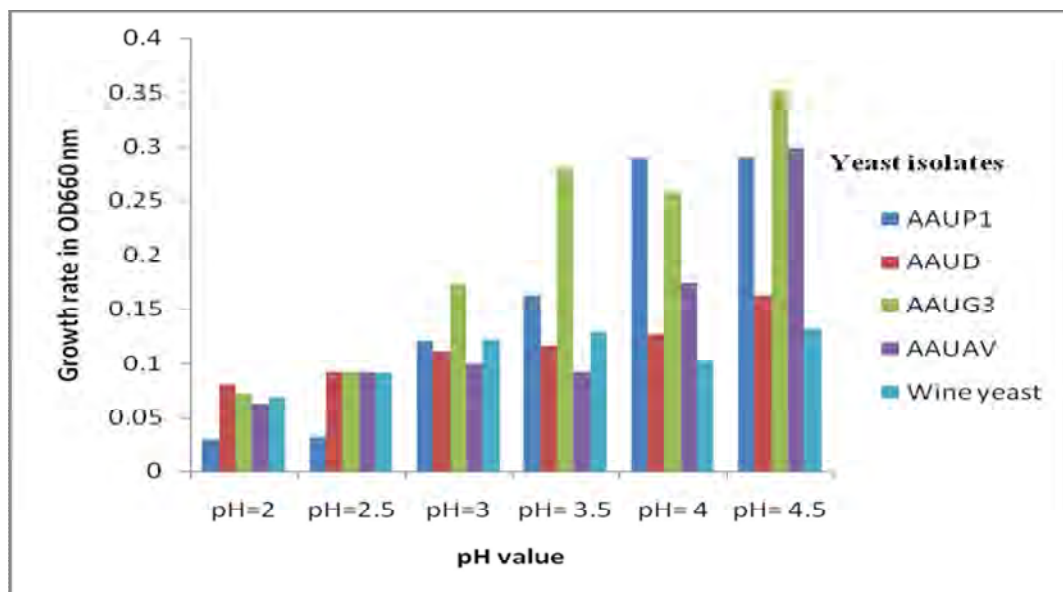


Figure 7. Summary of the effect of pH on the growth of the yeast isolates (Dry weight in OD₆₆₀ nm)

4.9. Glucose concentration optimization and fermentation for growth of yeast isolates

The effect of glucose concentration on growth of yeast is presented in Figure, 8. The yeast isolates growths were slowly increased with increasing the periods of incubations. The growth of yeast isolates increased at first week of incubation periods and reached the maximum growth rate at second week and then after decreased. Maximum specific growths of yeast isolates were increased with an increasing the glucose concentration 15% (v/v) then after the yeast isolates were decreased when the glucose concentration greater than 20% (v/v) (Figure, 8).

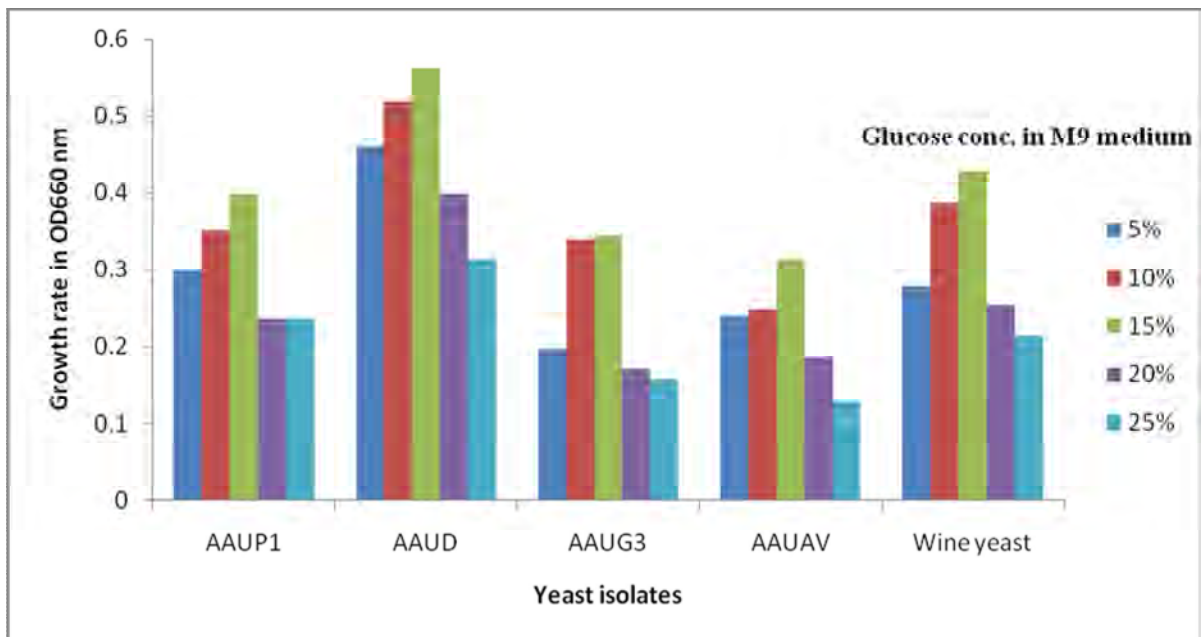


Figure 8. Summary of the effect of glucose concentration on the growth of yeast isolates on M9 minimum media for three weeks (Dry weight in OD₆₆₀ nm)

4.10. Determination of alcohol production by yeast isolates

Determinations of alcohol production by yeast isolates compared to standard alcohol were more linear with optical density reading (Figure 9). Ethanol production ability of yeast isolates is indicated in Figure 9. The yeast isolates ethanol producing ability was slowly increased with increasing the standard alcohol concentration. The yeast isolates AAUP₁ and AAUAV higher ethanol producing ability than the other isolates but the yeast isolates AAUD lower ethanol ability. Four yeast isolates (AAUP₁, AAUD, AAUG₃ and AAUAV) and showed high potential for ethanol production.

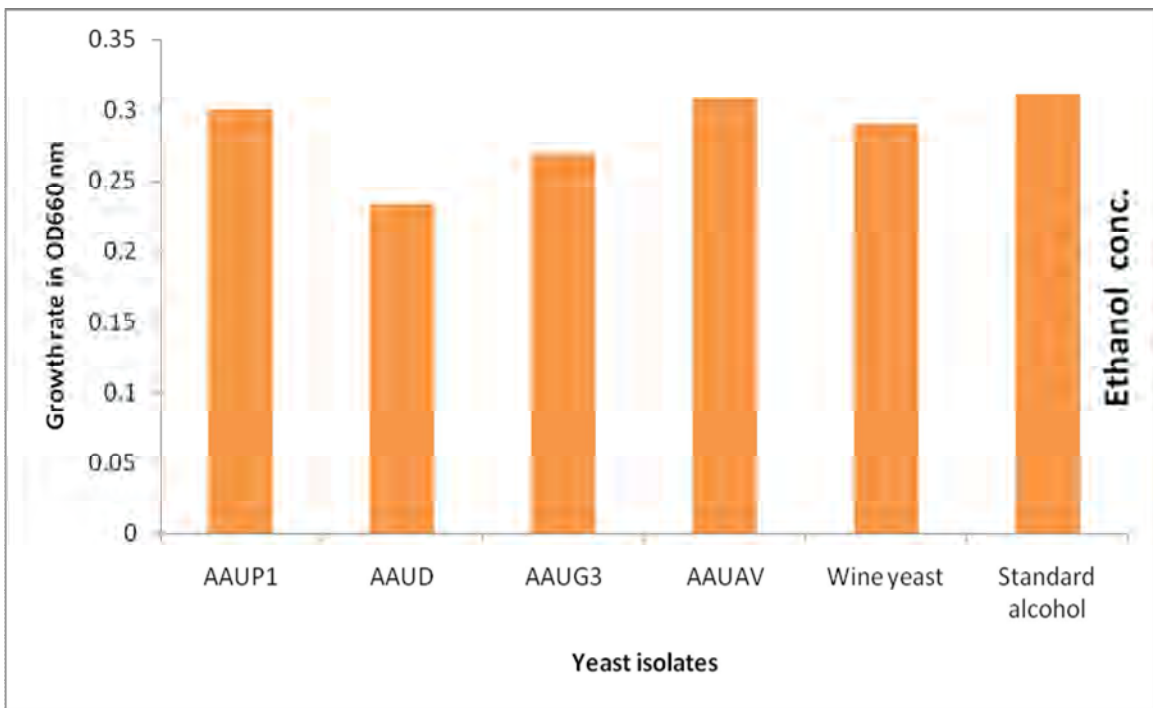


Figure 9. Determination of alcohol production by yeast isolates (Dry weight in OD₆₆₀ nm)

4.11. Effect of Acid Hydrolysis concentration on sugar content

The sacchrification of coffee husk was increased progressively when the concentration of H₂SO₄ increased from 0 – 3 M there after it was declined (Figure 10). The optimal free sugar obtained (85%) when the coffee husk was acid hydrolyzed at 3M.

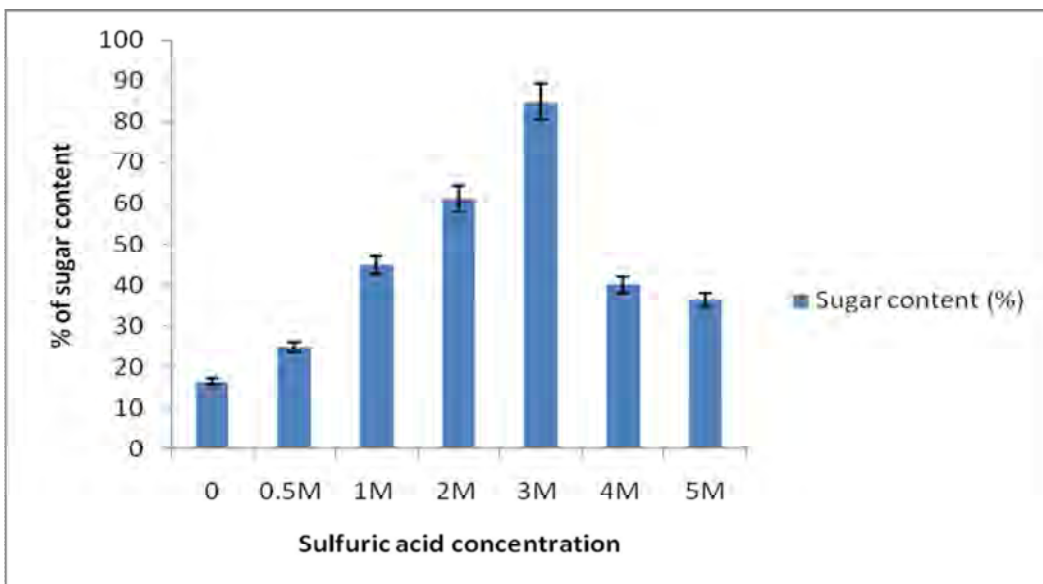


Figure 10. The effect of acid concentrations on sugar content (error bars with percentage, chart series with 5% value)

Figure 11 shows that, the ethanol yields obtained in distilled water, 0.25 M and 0.5 M H_2SO_4 concentrations were 0.66, 1.04 and 1.1% v/v respectively and the maximum ethanol yield 5.56 % was obtained in 3M H_2SO_4 . Further increase in acid concentration resulted in a decrease in ethanol yield (4 M H_2SO_4 concentrations the ethanol yields were 2.3%).

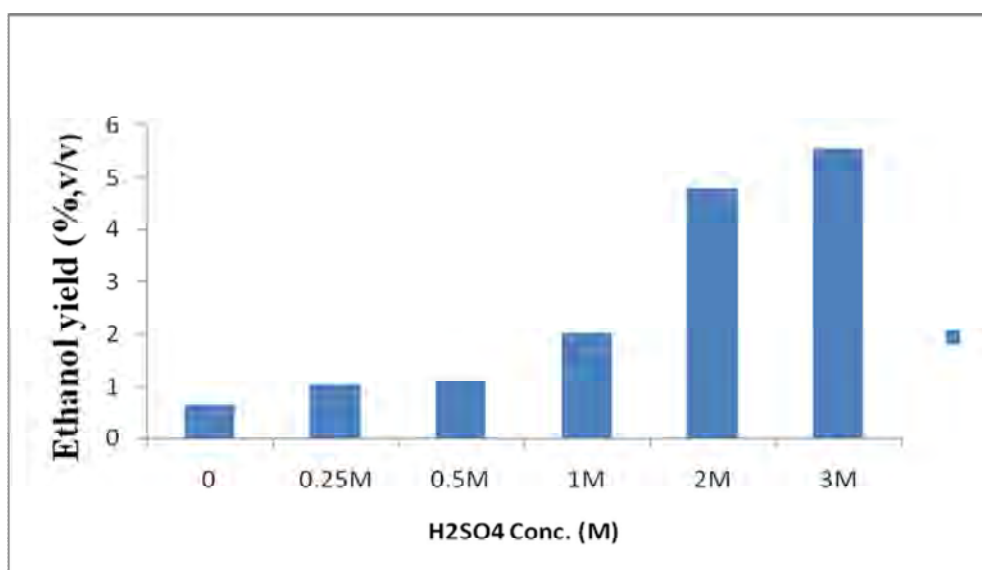


Figure 11: The effect of acid concentration on bio-ethanol production

4.11.1. Fermentation Time

At fermentation time of 72 hrs resulted in ethanol concentration of 1.73% v/v (Figure 12). The maximum ethanol yield of 5.56% v/v was obtained at 48 hrs fermentation time. The result revealed that the amount of ethanol increased with fermentation time and reached maximum at 48 hrs fermentation time and decreased at the end of fermentation time (Figure 12).

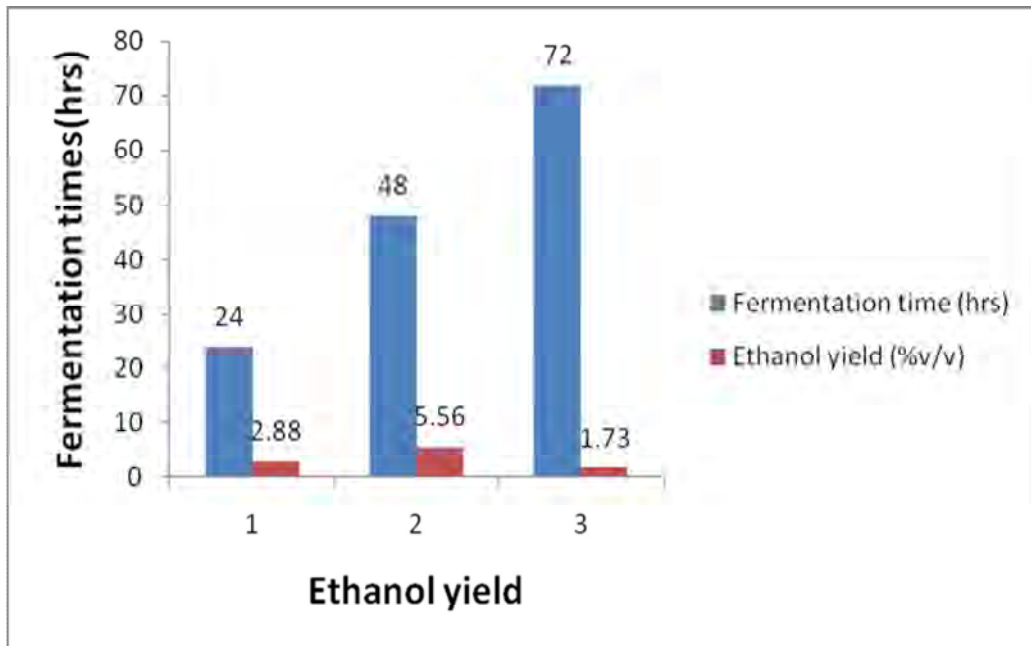


Figure 12. The effect of fermentation hours on bio-ethanol production

4.11.2. Hydrolysis Time

The result showed that ethanol yield in 5, 10 and 15 hydrolysis times gave 2.02, 5.56 and 3.41% v/v, respectively (Figure 13). The maximum ethanol yield (5.56% v/v) was obtained at 10 min hydrolysis time (Figure 13). The result showed that the ethanol yield increased with the hydrolysis time and reached optimum at 10 min hydrolysis duration and decreased after 15 minute hydrolysis time.

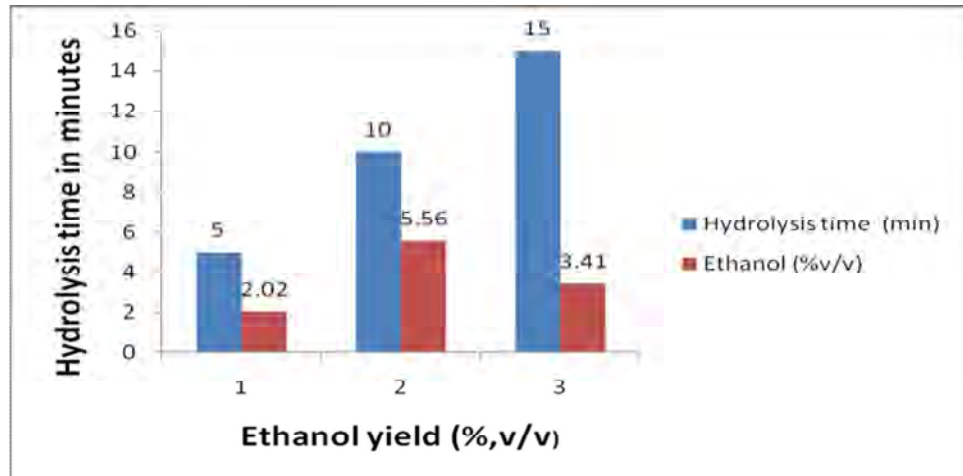


Figure 13. The effect of hydrolysis time on bio-ethanol production

4.11.3. Fermentation Temperature

The fermentation efficiency the yeast isolates with increasing temperature was measured (Figure 14). The ethanol yield increases with increasing temperature 25°C -30°C. Further increase in temperature resulted in yield reduction. The 30°C identified as the optimal temperature for maximal ethanol yield from acid hydrolyzed coffee husk.

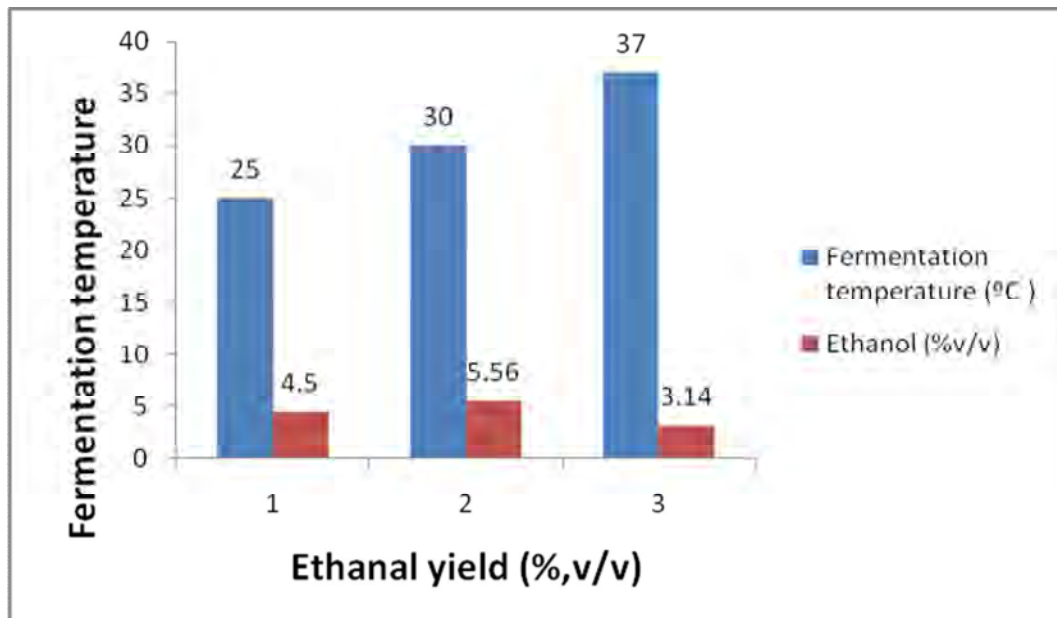


Figure 14. The effect of fermentation temperature on bio-ethanol production

4.11.4. The effect of pH

The result revealed that the ethanol yields obtained at pH 4, 5, and 6 were 4.14, 5.56 and 4.09% (v/v) respectively (Fig.15), and the maximum ethanol yield 5.56% (v/v) was obtained at pH 5.

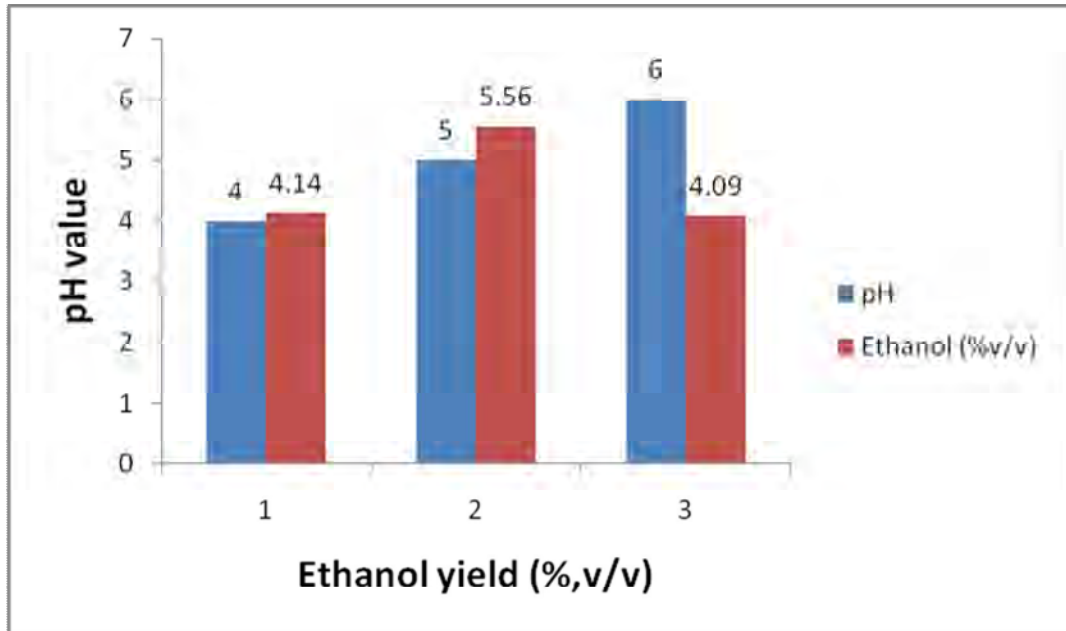


Figure 15. The effect of pH on bio-ethanol production

4.11.5. Ethanol yield

In present study, fermentation of the hydrolyzate samples showed that the maximum ethanol yield (5.56% v/v) using yeast isolates (AAUP₁) which was isolated from papaya (Figure 16). The concentration of bio-ethanol increased with increasing fermentation time and decreased at the end of fermentation time. Maximum ethanol concentration, 5.56 % v/v was obtained at 48 hrs and the result started to decrease after 48 hrs of fermentation time (Figure 12). From the optimization experiment, the highest concentration of ethanol was achieved at 48 hrs of fermentation and started to level off (Figure 12).

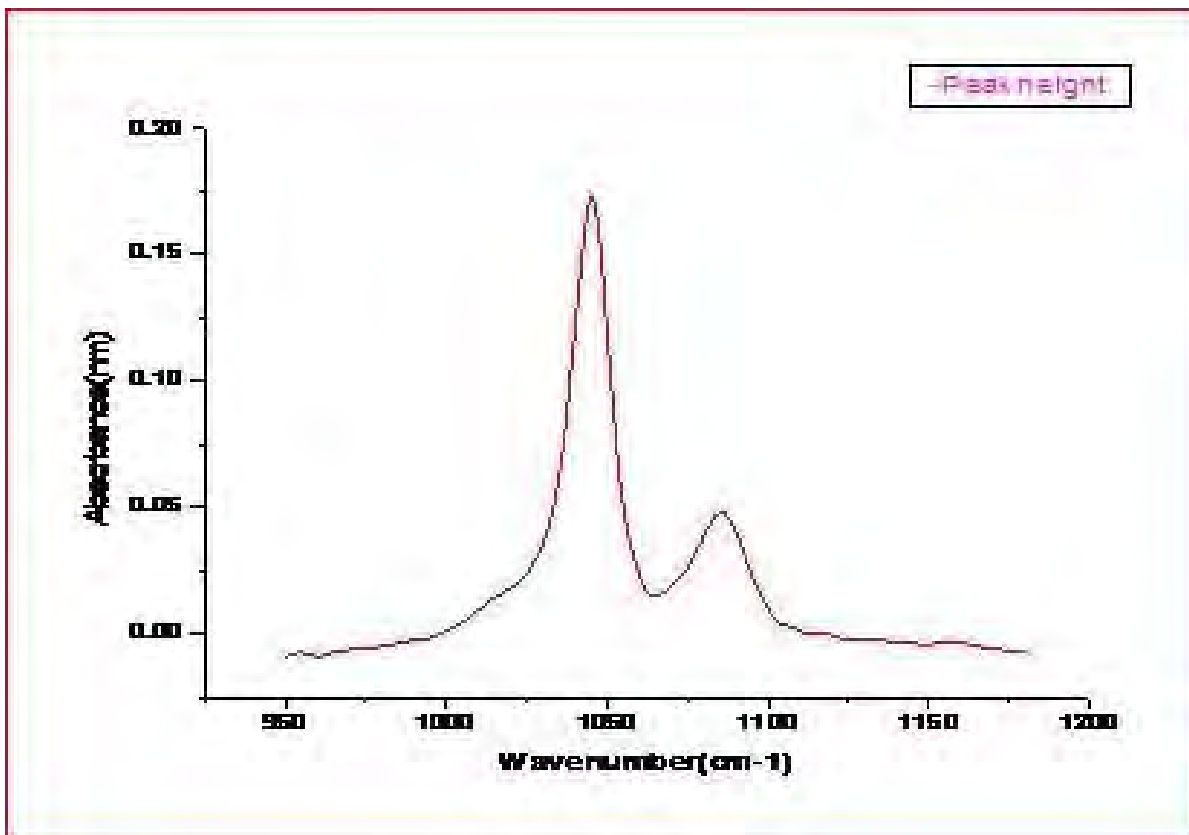


Figure 16: FTIR spectra of ethanol formed by hydrolysis of coffee husk in 3M H₂SO₄ concentrations, 10 minute hydrolysis time, 30⁰c temperature, and pH 5 after 48 hour fermentation using AAUP₁ yeast isolate.

5. DISCUSSION

Yeast isolates formed smooth white raised colonies on YPDA medium. The budding stage of the yeast isolates was observed under (40X) microscope and confirmed them to be yeast. Filamentous pseudomycelium is characteristic of *Saccharomyces cerevisiae*, which is dimorphic, existing either in a spherical, unicellular yeast like morphology or in a filamentous form (Gimeno *et al.*, 2013).

In this study, eight different types of sugars were used, glucose, galactose, maltose, trehalose, xylose sucrose fructose and lactose .The results of sugar utilization were presented in Table 4. The data indicated that almost all the isolates utilized glucose, galactose, maltose, trehalose, sucrose and fructose extensively and weak utilization of xylose and lactose was observed in AAUP₁, AAUD, AAUG₃ and AAUAV.

Among the isolates AAUP₁, AAUD and AAUG₃ were found good in utilization of all the types of sugars, whereas, AAUP₁ performed better than AAUD and AAUG₃.This variation is due to their preference towards the carbon sources. Optimum yeast growth was obtained when carbon source were glucose, galactose, fructose, sucrose, maltose and trehalose (Table 4). This was similar with results reported by Sathees Kumar *et al.* (2011).

The effect of temperature on the growth of yeast isolates were studied at different range of temperature (30-45 °C) (Figure 3). All most all the isolates produced good (OD_{660nm}) growth best at 37°C but the wine yeast was 30°C. Among the yeast AAUD isolates thermo tolerance at (OD_{660nm}) growth, followed by AAUG₃ 45°C. These results are in line with the observations made by Apiradee (2006) the optimum growth rate of thermo tolerant yeast isolate was at 37 °C concisely all isolates were thermo tolerant. As the temperature increased higher than 40 °C the growth of the yeast isolates decreased.

Ethanol tolerance by isolates and standard yeast are presented in figure 4. The results showed that all the isolates showed good growth at 5 per cent ethanol concentration. Whereas, at 15.0 per cent, higher (OD_{660nm}) value was recorded by AAUG₃ followed by 10.0 per cent OD value was recorded by AAUD. The yeast isolates AAUP₁ and AAUAV tolerated ethanol up to 10% and 5% (v/v), respectively (Figure 4). Accordingly, AAUP₁, AAUD, AAUG₃ and AAUAV were rapidly fermentative under high ethanol concentration. Similarly, Fakruddin *et al.* (2013) showed that thermo tolerant yeast C, T and DB2 were grown at 0-20% (v/v) ethanol concentration. Certain strains P and C can grow well up to 18% (v/v) but T and DB2 were up to 15% (v/v). *Saccharomyces cerevisiae* tolerate up to 15% of ethanol in the medium (Sathees Kumar *et al.*, 2011). Also Khaing *et al.* (2008) have reported that (KY1 and KY3) yeast isolates have tolerated up to 15% of ethanol in the medium and the yeast isolate (KY2) tolerated up to 20% of ethanol and maintained maximum ethanol production over a long incubation period.

In this study on yeast isolates AAUP₁, AAUD, AAUG₃ and AAUAV decreased in growth when the concentration of sugar reached 45% (Figure 5). The optimum sugar concentration for all yeast isolates and reference yeast was 30% (w/v). Increasing the sugar concentration for the isolates prolonged their lag phase for certain times when their concentration was beyond 30% (w/v) concentration and decreased the growth of yeast isolates. Similarly, Osho (2005) showed that growth rate remain lowest in 25% (w/v) of sugar concentrations in all the yeast isolates. The highest growth rate was shown by the isolate BSOSU 0271. Only slight differences were observed in the growth rates with increasing sugar concentrations, the differences being most obvious between 10 and 25% sugar concentrations. Dechasa Tolasa (2010) reported that the yeast isolates, TBY1, TGY2 and reference yeast were ability to grow best at the concentration from 24% to 32% of the medium containing sucrose. Their optical density were decreased as the sucrose concentration increased and their optical density at the concentration of 32% of sucrose decreased the growth of all yeast isolates in the first twelve hours and less than the initial optical density of the inoculums and increased gradually after twelve hours of incubation at 30⁰C.

The effect of different nitrogen source on the growth of yeast isolates was tested and the relatively low concentration of glucose (1%, v/v) in M9 minimal medium was used. The result showed that $(\text{NH}_4)_2\text{HPO}_4$ was the best nitrogen source for growth, which slightly decreased from $\text{NH}_4\text{H}_2\text{PO}_4$, NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, and NaNO_3 , respectively (Figure 6).

The growth rate of yeast isolates increased when the pH level increase to 3 (Figure 7). The pH of the medium had no more different effect on growth rate at pH 4.5 to 6.5, but beyond that the growth of the isolates decreased. The yeast isolate AAUG₃ the highest optical density at pH 3 to 5 than the rest isolate and the AAUD isolate the lowest optical density. The highest optical density was observed in the medium pH equal to 5.0 for all isolates. Similarly, Apiradee (2006) showed that, the pH of the medium did not affect the growth rate at pH 2.5 to 6.8, but beyond that the growth rates decreased. Fakruddin *et al.* (2013) showed that the yeast isolates P, C, T and DB2 were able to grow at wide range of pH (2 to 8). At pH 2, growth was decreased by highly acidic condition but the isolates grew up to pH 8.

The growth of yeast isolates in glucose concentration was increased with the 15% (v/v) glucose but decreased beyond that (Figure 8). The final glucose utilization in the fermentation broth was found to be used up at the glucose concentration equal to or below 15% (v/v), but above the glucose concentration of 15% (v/v), the final glucose utilization became quite appreciable.

From this study the optimal free sugar obtained (85%), when the coffee husks acid hydrolyzed at 3M concentration of H_2SO_4 . Similarly, Ayantu Girma (2013) have found 95% free sugar when brewery grain spent acid hydrolyzed at 1M concentration of H_2SO_4 . The concentration of ethanol obtained by the hydrolysis of the coffee husks using 3M H_2SO_4 , which is about (5.56%v/v) is comparable to the maximum amount of ethanol obtained from the enzymatic fermentation of barley straw (10 g/l) (Belkacemi *et al.*, 2002), maximum ethanol concentration obtained by the batch fermentation of acid hydrolyzate of coffee husk using *S. cerevisiae* (13.6 g/l) (Franca *et al.*, 2008) and coffee pulp (7.4 g/l) (Ayele Kefale *et al.*, 2012). This result is much higher than the maximum amount of ethanol from corn stalks (5 g/l) (Belkacemi *et al.*, 2002). Furthermore, there are many possibilities for improving ethanol production from agricultural residues,

including the addition of pre-treatment steps (enzymatic saccharification), use of fungal and simultaneous saccharification and fermentation.

Highest ethanol yields (5.56 % v/v) from coffee husks was obtained at 48 hrs fermentation time and 10 minute hydrolysis time at pH 5, using yeast isolate (AAUP1). Similarly Ayantu Girma (2013) has found that, at 48hrs fermentation time, high ethanol yield obtained (6.13 % v/v). The result was lower than the maximum amount of ethanol obtained from cassava effluent (8.3%) (Akponah, and Akpomie 2012) and cashew apple juice (7.62 %) (Neelakandan *et al.*, 2009).

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

- Based on morphological and physiological characteristics, the four yeast isolates were grouped under the genus *saccharomyces* which were closely related to *S. cerevisiae*.
- The yeast isolates, AAUG₃ that was isolated from *gishita* (*Annona senegalensis pers.*) showed higher ethanol tolerance (15% v/v) than other yeast isolates.
- The yeast isolate, AAUD that was isolated from grapefruit was more thermo tolerant (45⁰C) than the other yeast isolates.
- Four yeast isolates showed best growth, at 37°C.
- In the M9 minimal medium, the optimization of yeast isolates using diammonium hydrogen phosphate ((NH₄)₂HPO₄), at pH 5 and 15 % glucose concentration.
- The bio-ethanol production from coffee husks and optimization test showed that 3M H₂S0₄ was preferable than distilled water and other dilute acid hydrolysis.
- The optimization study showed that the highest bio-ethanol concentration of 5.56 % (v/v) was observed by AAUP1 under the optimum conditions of with 3M H₂S0₄ hydrolysis at 48 hrs fermentation time and 30°C.

6.2. Recommendation

- More efforts should be directed toward discovering more ethanol, sugar and thermo tolerant yeasts especially from different fruit varieties and other sugar sources.
- Further studies on identification of the yeast like species, and molecular characterization.
- Collaborate research and pilot-scale production should be encouraged by involving different stake holders so as to determine the feasibility of bio-ethanol production from coffee husks.
- An economic feasibility analysis of the overall conversion process from coffee husk to ethanol is necessary for the purpose of commercialization.

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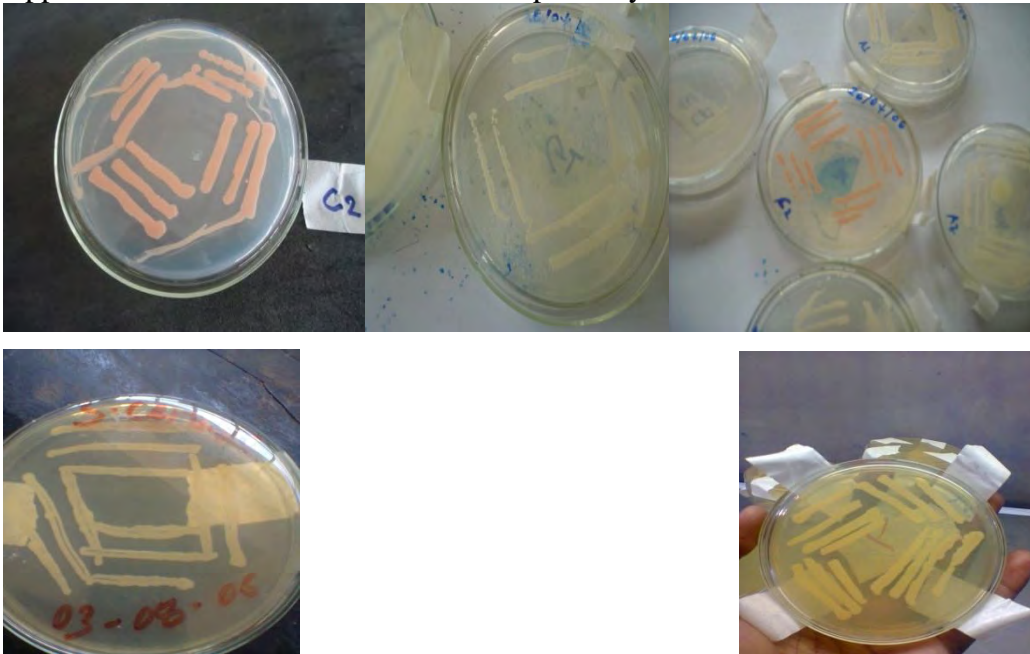
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8. APPENDIXES

Appendix 1. Different fruit samples fermentation for yeast isolation



Appendix 2. Pure isolates of the four samples of yeast isolates



A) *Saccharomyces cerevisiae*

B) AAUG₃

Appendix 3. Durham tube carbohydrate fermentation set up



A) Before fermentation

B) After fermentation

Appendix 4. Optimization of pH, nitrogen and carbon source for yeast isolates

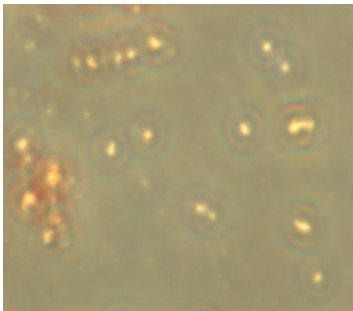


A) Carbon source

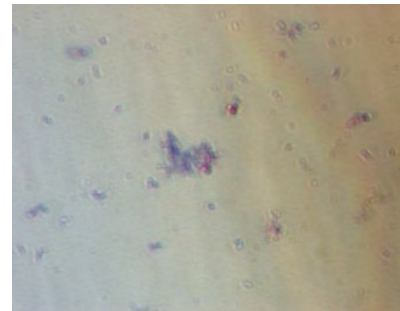
B) Nitrogen source

C) pH optimization

Appendix 5. Ascospores of the ethanol, sugar and thermo tolerant yeast isolates and baker yeast

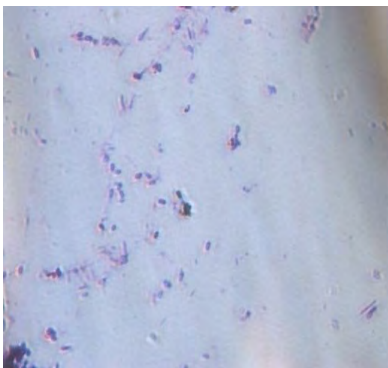


A) Ascospore of AAUP₁



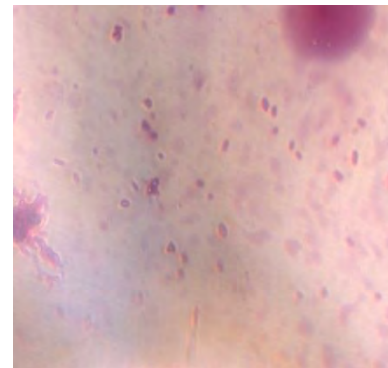
B) Ascospore of *Saccharomyces cerevisiae*

Appendix 6. Morphological characteristics of the ethanol, sugar and thermo tolerant yeast isolates



Mycelium and budding forming yeast isolates

A) AAUD



B) *Saccharomyces cerevisiae*

Appendix 7. The distilled of the samples collected from distillation process



Appendix 8: Sugar content formed after different acid concentration hydrolysis, at 90⁰c for 10 minute hydrolysis time.

Types of hydrolysis	Sugar content (%)
Distilled water	16.63
0.5M H ₂ SO ₄	25.08
1M H ₂ SO ₄	45
2M H ₂ SO ₄	61.20
3M H ₂ SO ₄	85
4M H ₂ SO ₄	40.26
5M H ₂ SO ₄	36.43

Appendix 9: Ethanol formed after different acid concentration hydrolysis at 48hrs fermentation time and for 10 min hydrolysis.

Isolates	Treatment types	Ethanol				
		concentration of ethanol	weight of solution collected after distillation (g)	Weight of ethanol in collected solution (g)	(% w/w)	(% v/v)
AAUP ₁	Distilled water	0.55	23.734	0.13	0.52	0.66
AAUP ₁	0.25MH ₂ SO ₄	0.87	23.596	0.21	0.82	1.04
AAUP ₁	0.5MH ₂ SO ₄	0.93	23.363	0.22	0.87	1.1
AAUP ₁	1MH ₂ SO ₄	1.69	23.565	0.40	1.60	2.02
AAUP ₁	2MH ₂ SO ₄	4.36	22.807	0.99	3.98	4.8
AAUP ₁	3MH ₂ SO ₄	4.63	23.761	1.1	4.40	5.56
AAUP ₁	4MH ₂ SO ₄	1.96	23.238	0.46	1.82	2.3
Baker yeast	Distilled water	0.43	22.297	0.096	0.38	0.48
Baker yeast	0.25MH ₂ SO ₄	0.46	23.604	0.11	0.43	0.54
Baker yeast	0.5MH ₂ SO ₄	0.97	23.435	0.23	0.91	1.15
Baker yeast	1MH ₂ SO ₄	1.40	23.119	0.32	1.3	1.64
Baker yeast	2MH ₂ SO ₄	3.8	22.873	0.87	3.48	4.40
Baker yeast	3MH ₂ SO ₄	1.89	23.788	0.45	1.80	2.27

Appendix 10: Ethanol formed after different acid concentration hydrolysis at 48 hrs fermentation time and for 15 min hydrolysis.

Isolates	Treatment types	Ethanol				
		concentration of ethanol	weight of solution collected after distillation (g)	Weight of ethanol in collected solution (g)	(%w/w)	(%v/v)
AAUP ₁	Distilled water	0.49	23.630	0.12	0.46	0.58
AAUP ₁	0.25MH ₂ SO ₄	0.77	23.560	0.18	0.73	0.92
AAUP ₁	0.5MH ₂ SO ₄	0.84	23.456	0.20	0.79	0.99
AAUP ₁	1MH ₂ SO ₄	1.34	23.465	0.31	1.26	1.60
AAUP ₁	2MH ₂ SO ₄	3.11	23.530	0.73	2.93	3.70
AAUP ₁	3MH ₂ SO ₄	2.86	23.661	0.68	2.70	3.41
AAUP ₁	4MH ₂ SO ₄	1.49	23.219	0.35	1.38	1.74
Baker yeast	Distilled water	0.39	23.285	0.09	0.36	0.45
Baker yeast	0.25MH ₂ SO ₄	0.44	23.660	0.10	0.42	0.53
Baker yeast	0.5MH ₂ SO ₄	0.89	23.635	0.21	0.84	1.06
Baker yeast	1MH ₂ SO ₄	1.27	23.123	0.29	1.17	1.48
Baker yeast	2MH ₂ SO ₄	2.88	23.801	0.69	2.74	3.46
Baker yeast	3MH ₂ SO ₄	1.56	23.600	0.37	1.47	1.86

Appendix 11: Ethanol formed after different minute's of hydrolysis with 3M H₂SO₄ conc. 48hrs, 30°C and pH 5 were keeping constant.

Isolates	Hydrolysis time (min)	Ethanol				
		concentration of ethanol	weight of solution collected after distillation (g)	Weight of ethanol in collected solution (g)	(%w/w)	(%v/v)
AAUAP1	5	1.69	23.565	0.40	1.60	2.02
	10	4.63	23.761	1.1	4.40	5.56
	15	2.86	23.661	0.68	2.70	3.41
Baker yeast	5	0.97	23.435	0.23	0.91	1.15
	10	1.89	23.788	0.45	1.80	2.27
	15	1.56	23.600	0.37	1.47	1.86

Hint:

Weight of ethanol in collected solution (g)

concentration of ethanol x weight of solution collected after distillation

100

Ethanol yield = $\frac{\text{weight of ethanol in collected solution (g)}}{\text{weight of initial sample (25g)}}$

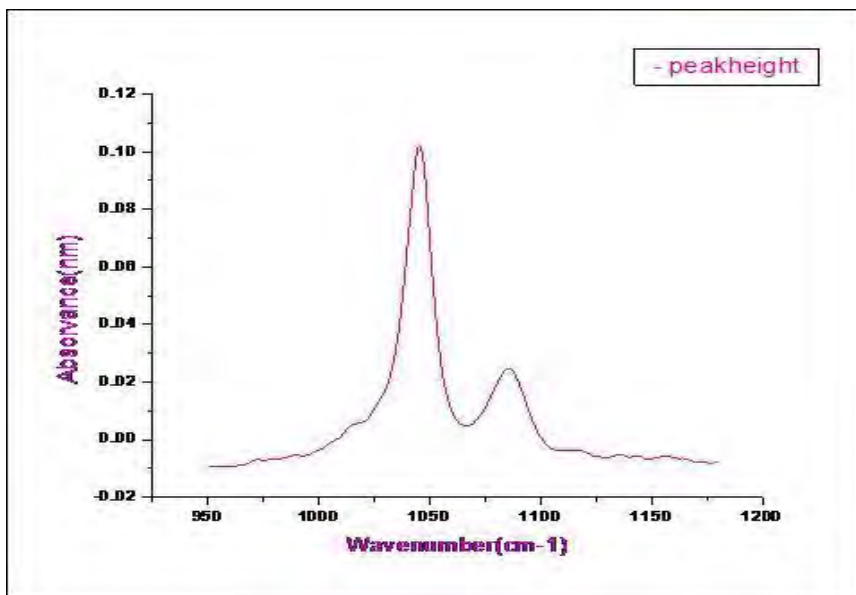
Appendix 12: Ethanol formed after 24, 48 and 72 hrs fermentation time and 10 minute's of hydrolysis.

Isolates	Fermentation time (hrs)	Hydrolysis time (min)	Ethanol yield	
			(% w/w)	(% v/v)
AAUAP1	24	10	2.28	2.88
	48	10	4.40	5.56
	72	10	1.37	1.73
Baker yeast	24	10	2.16	2.73
	48	10	3.48	4.40
	72	10	1.23	1.55

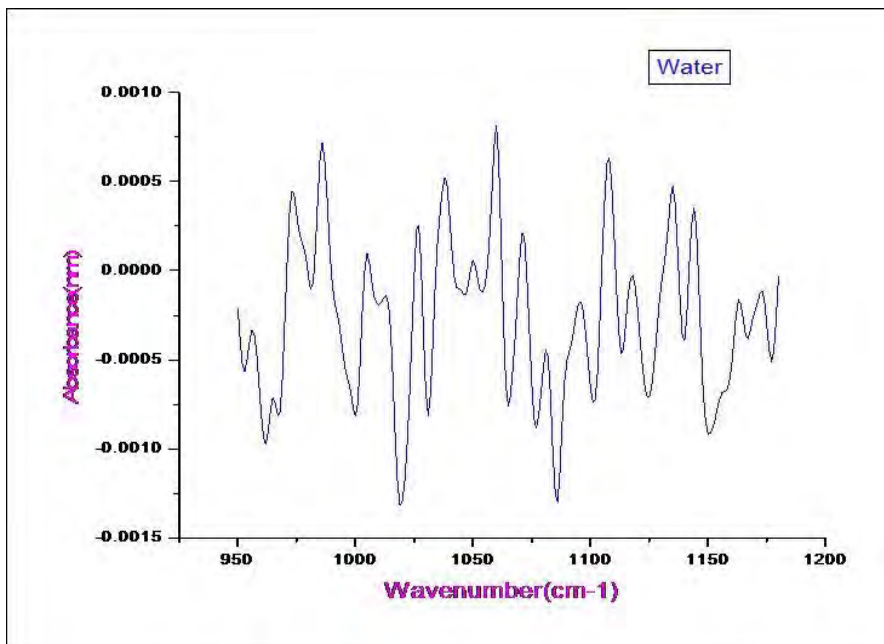
Appendix 13: Ethanol formed after 48 hours fermentation time, 10 minute's of hydrolysis and at different pH values.

Isolates	pH	Hydrolysis time(min)	Ethanol yield	
			(% w/w)	(% v/v)
AAUAP1	4	10	3.28	4.14
	5	10	4.40	5.56
	6	10	3.24	4.09
Baker yeast	4	10	2.87	3.63
	5	10	3.48	4.40
	6	10	2.64	3.33

Appendix 14: FTIR spectra of ethanol formed by hydrolysis of coffee husk in 3M H₂SO₄ concentrations, 15 minute hydrolysis time, 30⁰c temperature, and pH 5 after 72 hrs fermentation using AAUP1 yeast isolate.



Appendix 15: FTIR spectra of background with water



DECLARATION

I hereby declare that this thesis is the result of my original research work carried out at Addis Ababa University, Ethiopia, natural science department of Microbial, Cellular and Molecular Biology.

I hereby admit that this thesis has never been presented for the award of a degree in any university and all the sources of materials used for this thesis have been duly acknowledged.

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Signature	Date

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