JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES,

SCHOOL OF GRADUATE STUDIES DEPARTMENT OF BIOLOGY

ISOLATION AND CHARACTERIZATION OF SOME DOMINANT YEAST ISOLATES FOR PRODUCTION OF ETHANOL FROM WET ARABICA COFFEE (*COFFEA ARABICA* L.) PROCESSING EFFLUENT AND PULP

BY

BUZAYEHU DESISA

A Thesis Submitted to the School of Graduate Studies of Jimma University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

August, 2014

Jimma, Ethiopia

JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES,

SCHOOL OF GRADUATE STUDIES DEPARTMENT OF BIOLOGY

ISOLATION AND CHARACTERIZATION OF SOME DOMINANT YEAST ISOLATES FOR PRODUCTION OF ETHANOL FROM WET ARABICA COFFEE (*COFFEA ARABICA* L.) PROCESSING EFFLUENT AND PULP

BY

BUZAYEHU DESISA

A Thesis Submitted to the School of Graduate Studies of Jimma University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

Advisors: - Main advisor: Dawit Abate (Ph.D)

Co advisor: Diriba Muleta (Ph.D)

August, 2014

Jimma, Ethiopia

TABLE OF CONTENTS **PAGE**

LISTS OF TABLES

LIST OF FIGURES

LIST OF APPENDIXES

LISTS OF ABRIVATIONS

ACE	Arabica coffee effluent
ACP	Arabica coffee pulp
CFU	Colony forming unit
CH_3CH_2OH	Ethanol
$C_6H_{12}O_6$	Glucose
$C_6H_{12}O_5$	Xylose
g/l	Gram per litter
GHG	Green house gases
NOx	Oxides of nitrogen
rpm	Round per minute
w/v	Weight per volume
v/v	Volume per volume

ACKNOWLEDGMENTS

Above all, I thank Almighty God for always being with me in all my endeavors and giving me endurance to complete my study.

I express my deepest appreciation to my major advisor Dr. Dawit Abate and co-advisor Dr. Diriba Muleta for their very useful comments, guidance, and willingness to supervise my research, support and professional advice from the inception to the completion of this thesis.

I am sincerely thankful to the Department of Microbial, Cellular and Molecular Biology (DMCMB), Addis Ababa University (AAU) for allowing me to use the laboratory, and staff members of the Applied Microbiology Stream of DMCMB, AAU for their heartily cooperation and I would also like to thank Dr. Fassil Assefa for his generously supported me during my stay in the institute, and Jimma University, Department of Biology for giving me the chance and thought me effectively to reach on this progress.

I would like to express my gratitude to W/t Zenebech Aytenew for her encouragement and cooperation in provision of materials and technical support in laboratory activities.

It is my greatest pleasure to thank Ato Zerihun Belay, the owner and Managing Director of Balezaf Alcohol and liquors PLC for his kind permission to use facilities in the factory to measure alcoholic content of my samples. The remarkable hospitality of all the staff members of Sebata Balezaf Alcohol and Liquor Factory is also highly appreciated.

I would like to thank all my families, especially my brother Mr. Dereje Desisa, for their love, financial support, encouragement, moral support and patience that made this work possible. I also acknowledge Mr. Mulatu Workiye for his moral, technical support and editing my thesis and individuals who helped me in one way or another during my study.

Finally, I would like to express my deepest love, respect and gratitude to Mr. Dejene Gezaw for his financial support and unreserved encouragement that was always with me in my tedious and painful occasions.

ABSTRACT

Biofuels are alternatives to fossil fuels to ensure energy security and mitigate climate change. Currently, most biofuels are in the form of bioethanol that is generated from starch or sugar. Hence, the current study was initiated to isolate and characterize yeasts from wet Arabica coffee processing for ethanol production. Fifteen (15) yeast were isolated from wet Arabica Coffee processing effluent1, effluent 2, effluent 3, pulp 1 and pulp2. The yeast isolates were screened and characterized for ethanol production using yeast extract peptone dextrose agar medium (YEPDA) and characterized for ethanol production. All the isolates were first tested for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth using common fermentative carbohydrates. Two isolates (ACP12and ACE12) which showed relatively high fermentative ability in Durham tube fermentation method were selected for ethanol production from wet Arabica Coffee processing effluent and pulp. Ethanol producing isolates were tested for ethanol, sugar, and temperature and pH tolerance using yeast extract peptone dextrose broth. The yeasts isolated from pulps (ACP12) and effluents (ACE12) recorded maximum population at 20% glucose concentration with a maximum population of (216.0±1.00, 121±1.00 $x10^6$CFU/ml), respectively and the reference culture showed maximum growth at the same concentration (153.0±1.00 $x10^6$ CFU/ml) compared to the other two isolates. Yeast isolate (ACP12) showed higher population (97.0±1.00 $x10^6$CFU/ml) at 30ºC, followed by the yeast isolate from Arabica Coffee effluent (ACE12) that showed (67.7±1.54 $x10^6$ CFU/ml). However, the standard culture *(Saccharomyces cerevisiae*) showed the maximum population of (87.0±1.00 $x10^6$ CFU/ml) at 30ºC. The isolates (ACP12) and (ACE12) were recorded maximum population (98.0±1.00 $x10^6$ CFU/ml) and (78.0±1.00 $x10^6$ CFU/ml) compared to the standard strain (87.0±1.00 $x10^6$ CFU/ml) at pH 5.0. Based on morphological, physiological and biochemical characteristics the two isolates (ACE12 and ACP12) were grouped to genus *Saccharomyces*. The amount of total sugar concentration obtained from pulp1, pulp2, effluent1, effluent2 and effluent 3 were 90%, 85%, 51%, 43.71%, 40.26%, respectively. Isolate ACP12 showed the maximum ethanol production (6.2g/l) from pulp1 compared to the standard isolate (5.49 g/l). The ethanol produced from pulps 1 was satisfactory result compared to pulp 2, effulent1, effulent2 and effulent3. From this study, it can be concluded that isolate ACP12 has a potential of ethanol production from coffee pulps compared to the rest yeast isolates and needs further supplementary activities to qualify it for industrial application.

Keywords: Coffee wastewater, Arabica Coffee, fermentation, isolates, yeast, ethanol production

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). The continued use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentration of CO_2 in the atmosphere that poses global warming (Demirbas *et al*., 2004). The combustion of fossil fuel is responsible for 73 % of the CO_2 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 ((Demirbas *et al*., 2004) which embody (i) reduce energy consumption, (ii) increase the efficiency of energy conversion or utilization, (iii) switch to lower carbon content fuels, (iv) enhance natural sinks for CO_2, and (v) capture and store CO_2. Reducing use of fossil fuels would considerably lessen the amount of CO_2 produced and also potentially can reduce the levels of pollutants (Demirbas, 2006). As concern about global warming and dependence on fossil fuels grows, the search for renewable energy sources that reduce CO_2 emissions becomes a matter of widespread attention (Oliveria *et al*., 2005).

Coffee is one of the most important beverages in the world and its yearly production is about seven million tons in more than 60 countries (Mutua, 2000). Among different species of coffee plant, two alone dominate world trade the Coffee Arabica and Robusta. Substances to be found in coffee waste water Arabica coffee are toxic chemicals like tannins, alkaloids (caffeine) and polyphenolics (GTZ-PPP, 2002). These components make the environment for biological degradation of organic material in the waste water more difficult. The organic and acetic acids from the fermentation of the sugars in the mucilage and pulp make the wastewater very acid (pH down to 3.8). Under these acid conditions, higher plants and animals will hardly survive.

Thus creating anaerobic conditions in quickly causing bad smells and speed up the death of aquatic life due to the quick use up of oxygen dissolved in the water. Waste waters are normally discharged untreated into small waterways causing serious environmental problems. Bacteria living in anaerobic conditions can also cause health problems for humans when found in drinking water (GTZ-PPP, 2002).

Solid and liquid state fermentation has emerged as an appropriate technology for the management of agro-industrial residues like Arabica coffee waste water and for their value addition such as mushrooms and ethanol production (Fan *et al*., 2000). Among the bioconversion processes, bioethanol production is an appropriate technology for the management of agro-industrial residues (Demirbas, 2006). Ethanol is an important industrial chemical with emerging potential as a biofuel to replace fossil fuels. Ethanol can be produced by fermentation of sugars from agricultural products or waste plant materials. The most commonly used ethanol producer is strain of yeast such as *Saccharomyces cerevisiae*. Efficient ethanol production requires a rapid fermentation leading to high ethanol concentrations. Therefore, a yeast strain and isolates must have a good specific growth rate and good specific ethanol production rate at high osmotic activities and ethanol concentration.

Bioethanol used for production of gasoline and can reduce vehicle carbon dioxide emission by 90% (Demirbas, 2006). Wastewater from wet coffee processing firms is an important environmental pollution (Deepa *et al*., 2002). These investigators further noted that bioethanol produced from crop by-products would most likely be a part of any strategy designed to minimize food security problems. Organic waste products such as mucilage and pulp and other wastewaters from coffee processing represent a major source of environmental pollution and their disposal is usually done in the water sources close to the processing sites such as rivers and lakes. Pulp, mucilage and wastewater effluent from coffee processing site discharged to water, decrease oxygen content of water resulting in the death of plants and animals lived in the water due to the lack of oxygen or the increased acidity.

This fact can later result in a proliferation of undesirable microorganisms, bringing foul odors that attract flies and other insects rendering the water undrinkable and useless for other purposes large (De Matos *et al*., 2001). The idea of using these products came from the need to minimize their negative environmental impacts, to give them added value, and to satisfy the demand for suitable resources for ethanol production. Thus, the use of effluent and pulps from wet coffee processing would offer raw materials of a second generation liquid fuel wherever coffee is being processed.

In turn, this would diversify sources of energy production and promote sustainable development by directly benefiting the inhabitants around coffee producing areas. In order to reduce the cost and amount of petrol consumption, many countries use a mixture of ethanol and petrol's (Sree *et al*., 2000) and the method of ethanol production from various agro residues is of prime importance as the raw materials are easily available and cheap in cost along with *Saccharomyces cerevisiae*.

Currently in Ethiopia, there is only one sugar mill producing ethanol and few distilleries participating in downstream chemicals from ethanol. Among molasses driven products, ethanol takes the largest part, but its utilization must attract the attention of government policy makers in order to utilize as a bioethanol. With the present trend, sugar sector expansion and transformation of the country from agriculture-led to industry-led,, promotion of bioethanol production and utilization has to take place. At present, about 5.6 million liter of ethanol is annually produced, but there are projects towards increasing the products to cover 142,000 cubic meter (Ethiopian sugar develop agency (ESDA, 2005). Recently, the three Ethiopian sugar factories, Fincha, Wonji-Shoa and Metehara, produced more than 11 million liters of ethanol (Ethiopian Sugar Corporation (ESC, 2012).

Therefore, the current study has been initiated to isolates and characterize some potential yeast isolates for the production of ethanol from wet Arabica coffee processing effluent and pulp to ensure a cheap source of raw material for the production of bioethanol as new source of energy can be very helpful to meet the rising energy demand and reduce environmental pollution.

1.2. The statement of the problems

The increase in the price of petroleum and environmental pollutions due to combustion of fossil fuels has renewed the interest to search for alternative fuels. The use of food crops (like corn, maize) for biofuel production may cause inflation of cost of these crops leading to food insecurity. To alleviate such problems, alternative and non-edible agricultural products must be investigated.

The coffee plant, which is indigenous to Ethiopia, produces fruit once per year about six to nine months after flowering. The bean represents about 40 % of the fruit; the other 60 % is generally discarded as waste (pulp and mucilage and wastewater). Coffee pulp and effluent represent the most abundant and non edible agricultural waste obtained after pulping ripe fruit (Yishak Seboka *et al*., 2009) and availability of 30,275 tonnes per year (t/yr) of coffee residue has been estimated in Ethiopia that obtained from wet coffee milling (Kebede Dawit, 2001). Similarly, Alemayehu Teshome *et al*. (2007) have estimated 525,000 t/yr coffee residues from processing plants. The use of coffee pulp and other by-products has become a priority in coffee producing countries for economic, ecological and social reasons. According to Urbaneja *et al*. (1996), sugar contents in coffee pulp hydrolysates (g/l) are xylose (0.08-3.23), arabinose (0.23- 11.26), fructose (0.9-3), glucose (1.30-6.31), sucrose (0.08-3.96), and maltose (0.01-3.50). The above literature (Urbaneja *et al*. 1996) showed that a wet coffee residue will be desirable and very beneficial when it is used as raw material for bioethanol production. Therefore, the aim of this research was to produce ethanol by fermentation from Arabica coffee effluent and pulp using selected fermentative yeast isolates such as *Saccharomyces cereviciae* to reduce environmental pollution caused around wet Arabica coffee process and produce renewable energy ethanol. The sources of potential fermentative yeasts could be yeasts isolated from the same environment as they are already adapted to the environment.

1.3. OBJECTIVES

General Objectives

The general objective of this study was to isolate, identify, characterize and evaluate some dominant yeast isolates for production of ethanol from wet Arabica coffee *(coffea arabica* L.*)* processing wastes (effluent and pulp).

Specific Objectives

The specific objectives of the present work were:

1. To isolate some fermentative yeasts from wet Arabica coffee processing waste water (effluent) and pulp
2. To characterizes the yeast isolates by Durham tube fermentation test, and determine the tolerance best alcohol producers of yeast isolates to some physicochemical factors such as glucose, ethanol, temperature and pH.
3. To screen for high ethanol producing yeast isolates and characterize morphologically
4. To determine of moisture content (Dry rot of pulp)
5. To determine total sugar in liquid waste effluent and pulp of Arabica coffee waste water
6. To evaluate ethanol yield and yeast isolates biomass after fermentation of coffee wastes (effluent and pulp)

2. LITERATURE REVIEW

2.1. World coffee production

Coffee is one of the most important beverages of the world. Green coffee beans are deemed as a commodity ranking second only to petroleum in terms of currency and traded worldwide. The crop is cultivated in Latin American, Asian and African countries. The world annual coffee production is around 7 million tons, of which Brazil produces one-third (Gaur, 2006). Coffee is produced in more than 80 countries and three of them (Brazil, Vietnam and Columbia) account for more than half (52 per cent) of the world's production (Petit, 2007).

2.2. Processing of coffee cherries and wet processing method

Once the cherries are harvested, the beans have to be extracted by using either the dry or the Wet method. The wet method is more expensive than the dry method but the coffee it produces has better quality properties (Bertolini *et al*., 1991).

The wet method requires the use of specific equipment and substantial quantities of water. Properly done, the qualities of the coffee beans are better preserved, producing a green coffee which is homogeneous and has few defective beans. Hence, the coffee produced by this method is usually regarded as being of better quality and commands higher prices. As in the dry method, preliminary sorting and cleaning of the cherries is usually necessary and should be done as soon as possible after harvesting. This operation can be done by washing the cherries in tanks filled with flowing water. Screens may also be used to improve the separation between the ripe and unripe, large and small cherries (Bertolini *et al*., 1991). After sorting and cleaning, the pulp is removed from the cherry. This operation is the key difference between the dry and the wet method, since in the wet method the pulp of the fruit is separated from the beans before the drying stage.

The pulping is done by a machine which squeezes the cherries between fixed and moving surfaces. The flesh and the skin of the fruit are left on one side and the beans, enclosed in their mucilaginous parchment covering, on the other. The clearance between the surfaces is adjusted to avoid damage to the beans. The pulping operation should also be done as soon as possible after harvesting to avoid any deterioration of the fruit which might affect the quality of the beans (Mutua, 2000). The pulped beans go onto vibrating screens which separate them from any un pulped or imperfectly pulped cherries, as well as from any large pieces of pulp that might have passed through with them. From the screens, the separated pulped beans then pass through water washing channels where a further flotation separation takes place before they are sent to the next stage (Boccas *et al*., 1994).

Because the pulping is done by mechanical means it normally leaves some residual flesh as well as the sticky mucilage adhering to the parchment surrounding the beans. This has to be completely removed to avoid contamination of the coffee beans by products resulting from the degradation of the mucilage. The newly pulped beans are placed in large fermentation tanks in which the mucilage is broken down by natural enzymes and can easily be washed away.

Unless the fermentation is carefully monitored, the coffee can acquire undesirable, sour flavours (Mutua, 2000). When the fermentation is complete, the coffee is thoroughly washed with clean water in tanks or in special washing machines. The wet parchment coffee at this stage consists of approximately 57% moisture. To reduce the moisture to an optimum 11% the parchment coffee is dried either in the sun, in a mechanical dryer, or by a combination of the two. Sun drying should take from 8 to 10 days, depending upon ambient temperature and humidity (Daniels, 2009). The final stages of preparation of the coffee, known as "curing", usually take place at a special plant just before the coffee is sold for export. The coffee is hulled, to remove the parchment, then passes through a number of cleaning, screening, sorting and grading operations which are common to both wet- and dry-processed coffee. The wet method is generally used for all the Arabica coffee to produce coffee waste water.

2.3. Coffee wastewater

Coffee wastewater, also known as coffee effluent, is a byproduct of the coffee processing steps whose treatment and disposal is an important environmental consideration. Coffee wastewater is a form of industrial water pollution. The unpicked fruit of the coffee tree, known as the coffee cherry, must undergo a long process to make it ready for consumption. This process often entails the usage of massive amounts of water and the production of considerable amounts of both solid and liquid waste. To determine the type of waste stemming from coffee processing, it is important to know how the coffee cherries are processed.

2.3.1. Characteristics of coffee wastewater

The wastewaters from wet coffee processing can be basically divided into two parts. Firstly, the pulping water with a high content of quickly fermenting sugars using enzymes from the bacteria on the coffee cherries. Secondly, depending on the processing method applied the water from fermentation/washing or the thick effluents from the mechanical mucilage removers. The main pollution in coffee wastewater stems from the organic matter set free during pulping, particularly the difficult to degrade mucilage layer surrounding the beans.

During wet method of coffee cherry processing, coffee pulp contains 8.25% protein and 23-27% fermentable sugars on dry weight basis (Bressani, 1979). In spite of such high nutrient content, the coffee pulp cannot be an animal feed, mainly due to its toxic components such as caffeine, mineral salts, amino acids, tannins, phenols and other polyphenols(Table1) (Bressani, 1979). Coffee pulp poses many problems in the coffee producing tropical countries and areas.

Table 1 Composition of coffee pulp

Composition of coffee pulp (ether extract)	
Crude fibers	21.4%
Crude Protein	10.1%
Ash	1.5%
Nitrogen free extract	31.3%
Tannin	7.8%
Pectic Substances	6.5%
Non reducing sugars	2.0%
Reducing sugars	12.4%
Chlorogenic acid	2.6%
Caffeine	2.3%
Total caffeic acid	1.6%

Source: GTZ-PPP, 2002

Coffee pulp disposal in nature, without any treatment, causes severe environmental pollution, due to putrefaction of organic matter (Bressani, 1979). Consequently, the coffee pulp forms a major source of the pollution of rivers, lakes and environment in the vicinity of the coffee processing sites (Boccas *et al*., 1994). Other substances to be found in coffee wastewater are toxic chemicals like tannins, alkaloids (caffeine) and polyphenolics. These components make the environment for biological degradation of organic material in the wastewater more difficult.

During the fermentation process in the wastewater, the acidification of sugars will drop the pH to around 4 or less, and the digested mucilage will be precipitated out of solution and will build a thick crust on the surface of the wastewater, black on top and slimy orange/brown in colour underneath. If not separated from the wastewater, this crust will quickly clog up waterways and further contribute to anaerobic conditions in the waterways (Gathuo *et al*., 1991).

2.3.2. Effects of Arabica coffee waste water on the environment

The organic acids from the fermentation of the sugars in the mucilage and pulp make the wastewater highly acidic (pH down to 3.8). Under these acid conditions, higher plants and animals will hardly survive. After the first fermentation of sugars in the wastewater takes place, the organic substances that diluted in the wastewater breakdown only very slowly by aerobic microorganisms (Gathuo *et al*., 1991).

This process causes problems as the demand for oxygen to breakdown organic materials in the wastewater exceeds the supply, dissolved in the water, thus creating anaerobic conditions. Values for Biological Oxygen Demand (BOD) oxygen needed to breakdown organic matter in coffee wastewater are high (up to 150 g/l for effluents from demucilators) (Gathuo *et al*., 1991).

Consequently, the very slowly degrading compounds indicated by the Chemical Oxygen Demand (COD), make up around 80% of the pollution load and are reaching 40 g/l and more (GTZ-PPP, 2002). Luckily, most of these materials can be taken out of the water stream as precipitated mucilage solids and made into compost. As a result of the high values of COD and BOD, anaerobic conditions ("rotting") set in quickly causing bad smells and speed up the death of aquatic life due to the quick use up of oxygen dissolved in the water. Bacteria living in anaerobic conditions can also cause health problems to humans when found in drinking water. In addition to the bad smell, coffee wastewater will turn dark green to black after a while. This discoloration is caused by the chemical components of the red colour of the coffee cherry (flavanoids). Generally, wastewaters that are normally discharged untreated into small waterways pose serious environmental problems (GTZ-PPP, 2002).

2.4. Properties and uses of ethanol

Ethanol or ethyl alcohol, CH_3CH_2OH, 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 miscible with water in all proportions. Ethanol that is completely free of water is called absolute ethanol. Ethanol is a psychoactive agent and it produces a variety of physiological and behavioral effects (Gaur, 2006).

2.4.1. Bioethanol as a sustainable fuel

The main use of ethanol is as a motor fuel and fuel additive. Efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases (Altintas *et al*., 2002). In 2005, the world ethanol production was approximately 46 billion liters per year and reached, 76 billion liters per year in 2010 (Olfert and Weseen, 2007). (Table 2) shows world ethanol production by country wise.

Table 2. World annual ethanol production by country (2005)

Country	production (millions of liters)	Country	Production (millions of liters)
United States	16,139	South Africa	390
Brazil	999	Spain	352
China	3,800	United Kingdom	348
India	1,699	Thailand	299
France	908	Ukraine	246
Russia	749	Canada	231
Germany	431	Others	1,707

Source: (Olfert and Weseen, 2007).

Bioethanol is appropriate for the mixed fuel in the gasoline engine (Kim and Dale, 2004). In Brazil, bioethanol for fuel is derived from sugar cane and is used pure or blended with gasoline in a mixture called gasohol (24% bioethanol, 76% gasoline) (Oliveria *et al*., 2005). In several states of the United States, small amount of bioethanol (10 % by volume) is added to gasoline, known as gasohol or E10.

Blends having higher concentrations of bioethanol 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 biofuel program involving both forms of bioethanol–gasoline blend program, e.g. 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).

2.4.2. Other uses of ethanol

One of the major uses of ethanol is in alcoholic beverages. Alcoholic beverages vary considerably in their ethanol content and in the substrates from which they are produced. Most alcoholic beverages can be broadly classified as fermented beverages made by the action of yeast/other microbes on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. Fermented beverages can be broadly classified by the substrate from which they are fermented. Beers are made from cereal grains or other starchy materials, wines and ciders from fruit juices, and meads from honey. Fermented beverages may contain up to 15–20 % ethanol by volume, the upper limit being set by the yeast's tolerance for ethanol, or by the amount of sugar in the starting material (Gaur, 2006).

Absolute ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62 %.

2.4.3. Ethanol and the environment

Ethanol represents closed carbon dioxide cycle because during both fermentation of biomass to ethanol and combustion of ethanol, the released carbon dioxide is recycled back into plant material because plants use CO_2 to synthesize cellulose during photosynthesis cycle (Chandel *et al*., 2007).

Ethanol production process only uses energy from renewable energy sources; no net carbon dioxide is added to the atmosphere, making ethanol an environmentally beneficial energy source. In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Wyman and Parekh, 1990). Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the greenhouse gas effect (Foody, 1988). As energy demand increases, the global supply of fossil fuels cause harm to human health and contributes to the GHG emission (Hahn-Hagerdal *et al*., 2006).

The reduction of GHG pollution is the main advantage of utilizing biomass conversion into ethanol (Demirbas, 2007). Ethanol contains 35 % oxygen that helps complete combustion of fuel and thus reduces particulate emission that poses health hazard to living beings. A study conducted by Bang-Quan *et al.* (2003) on the ethanol blended diesel (E10 and E30) combustion at different loads found that addition of ethanol to diesel fuel simultaneously decreases cetane number, high heating value, aromatics fractions and kinematic viscosity of ethanol blended diesel fuels and changes distillation temperatures. These factors lead to the complete burning of ethanol, less emission and its ability to reduce ozone precursors by 20 – 30%, bioethanol can play a significant role in reducing the harmful gasses in metro cities worldwide (Wyman and Parekh, 1990). Ethanol blended diesel (E-15) causes 41% reduction in particulate matter and 5% NOx emission (Chandel *et al.*, 2007; Subramanian *et al.*, 2005). One of the disadvantage in using ethanol as fuel is that aldehyde predominantly acetaldehydes emissions are higher than those of gasoline. However, acetaldehydes emissions generate less adverse health effects in comparison to formaldehydes emitted from gasoline engines.

2.5. Feed stocks for bioethanol production

Production of bioethanol from renewable lignocellulosic sources such as wood and agricultural residues is a promising means to decrease the accumulation of GHG and alleviate pressure on fossil fuel shortage (Wyman and Parekh, 1990). Various raw materials like sugarcane juice and molasses (Morimura *et al.*, 1997), sugar beet, beet molasses (EI-Diwany *et al.*, 1992), sweet sorghum (Bulawayo *et al.*, 1996) and starchy materials like sweet potato (Sree *et al.*, 2000), *Prosopis juliflora* (Negusu Tefera, 2009), corn cobs and hulls (Beall *et al.*, 1992), cellulosic materials like cocoa, pineapples and sugarcane waste (Othman *et al.*, 1992), coffee husk (Franca *et al.*, 2008) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva *et al.*, 1995) have been studied. Of these, simple sugar bearing materials are the easiest to process, since yeasts ferment these directly, while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies (physiochemical/enzymatic preparation) before they can be fermented to yield ethanol.

Dabas *et al.* (1997) studied ethanol production from wheat starch. Hydrolyzed wheat starch was used as a substrate for ethanol production using the strains of *S. cerevisiae*. Wheat flour slurry (25% w/v) was gelatinized and conditions were standardized for saccharification and fermentation of wheat starch for ethanol production. Bioethanol feed stocks conveniently classified as sucrose-containing, starchy materials and lignocellulosic biomass (Malca and Freire, 2006).

2.5.1. Sucrose containing feed stocks

Feedstock for bioethanol is essentially comprised of sugar cane and sugar beet (UNCTAD, 2006). Two third of world sugar production is from sugar cane and one-third is from sugar beet (Linoj *et al*., 2006). These two are produced in geographically distinct regions. Sugar cane is grown in tropical and subtropical countries, while sugar beet is only grown in temperate climate (Kim and Dale, 2004).

The advantages with sugar beet are a lower cycle of crop production, higher yield, and high tolerance of a wide range of climatic variations, low water and fertilizer requirement. Sweet sorghum is one of the most drought resistant agricultural crops as it has the capability to remain dormant during the driest periods. Of the many crops being investigated for energy and industry, sweet sorghum is one of the most promising candidates, particularly for bioethanol production principally in developing countries (Linoj *et al*., 2006).

A recent EU funded (*LAMNET program*) research program investigated the possibility of combining waste products of several crops for use in the processing of bioethanol. One of the studies concluded that sweet sorghum is a very useful plant, where by the complete plant can be used without leaving any waste (Kim and Dale, 2004). It is concluded that bioethanol produced from sugar cane is an attractive proposition (DSD, 2005). The conversion of carbohydrates with 5 and 6 carbons into bioethanol is easier compared to starchy materials and lignocellulosic biomass because previous hydrolysis of the feedstock is not required since this disaccharide can be broken down by the yeast cells.

In addition, the conditioning of the cane juice or molasses favors the hydrolysis of sucrose (Cardona and Sanchez, 2007).

2.5.2. Starchy materials

Starch based materials can also be used in ethanol production (Yoosin and Sorapipatana, 2007). Starch is a biopolymer and defined as a homopolymer consisting only one monomer, D-glucose (Pongsawatmanit *et al.*, 2007). During bioethanol production from starch, it is necessary to break down the chains of this carbohydrate for obtaining glucose syrup, which can be converted into bioethanol by yeasts. This type of feedstock is the most utilized for bioethanol production in North America and Europe. Corn and wheat are mainly employed with these purposes (Cardona and Sanchez, 2007). Starch can be converted to fermentable sugar by a method called the hydrolysis technique. Hydrolysis is a reaction of starch with water, which is normally used to breakdown the starch into fermentable sugar (Yoosin and Sorapipatana, 2007).

2.5.3. Lignocellulosic biomass

Lignocellulosic biomass such as agricultural residues (corn stover, sugar cane bagasse, wheat or rice straw, forestry), paper mill residues and municipal wastes are abundant, domestic and renewable. Lignocellulosic biomass has long been recognized as a potential low-cost source that can be converted to bioethanol 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 (Wiselogel *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 (beta1, 4)-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, make 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 and the process is called saccharification.

Hemicelluloses (20-40 % of lignocellulose) are highly branched heteropolymers containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid). They also contain smaller amounts of non-sugars such as acetyl groups (Lynd *et al*., 2001). The composition of hemicellulose depends on the source of the raw material (Wiselogel *et al*., 1996). Hemicelluloses in hardwood contain mainly xylans (15-30 %) while in softwood galactoglucomannans (15-20%) and xylans (7-10%) predominant. There are various enzymes responsible for hydrolysis of hemicellulose. Because of their branched, amorphous nature, hemicelluloses are easier to hydrolyze than cellulose (Brigham *et al*., 1996).

Lignin (10-30%) is a complex, hydrophobic, cross-linked aromatic polymer in nature. Lignins are polymers of phenylpropane units such as guaiacyl (G) units from the precursor transconiferyl- alcohol, syringyl (S) from trans-sinapyl-alcohol, and p-hydroxyphenyl (H) units from the precursor trans-p-coumaryl alcohol (Kirk *et al*., 1977).

The exact composition of lignin varies widely with species. Softwood contains mainly guaiacyl units while hardwood contains both guaiacyl and syringyl units (Wiselogel *et al*., 1996). It has been suggested that guaiacyl lignin restricts fiber swelling and thus the enzymatic accessibility is more than syringyl lignin. 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. Numerous pretreatment methods including physical, physicochemical, chemical, and biological methods that have been developed for separation of lignocellulosic to cellulose, hemicellulose, and Lignin (Sun and Cheng, 2002).

2.5.4. Biomass to ethanol process

Ethanol is produced from biomass both as petrochemical through the hydration of ethylene, and biologically, by fermenting sugars with yeasts. Ethanol for use as industrial feedstock is most often made from petrochemical feedstock, typically by the acid-catalyzed hydration of ethylene, represented by the chemical equation (1):

$C_2H_4 + H_2O \rightarrow CH_3CH_2OH$ (1)

The catalyst is most commonly phosphoric acid, adsorbed onto a porous support such as diatomaceous earth or charcoal (Gaur, 2006). Ethanol for beverages, and fuel, is mainly produced by fermentation. The process of fermentation is carried out by certain species of yeast commonly, *S. cerevisiae*.

When *S. cerevisiae* metabolizes sugar anaerobically, ethanol and carbon dioxide produced. The overall chemical reaction conducted by the yeast may be represented by equation (2) below:

$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$ (2)

The process of culturing yeast under anaerobic conditions to produce alcohol is referred to as brewing. Brewing can only produce relatively dilute concentrations of ethanol in water since concentrated ethanol solutions are toxic to yeast. The most ethanol tolerant strains of yeast can survive up to about 25% ethanol (v/v) (Gaur, 2006). Typical lignocellulose-to-ethanol processes consist of at least four steps. These are pretreatment to enhance biomass digestibility, hydrolysis of cellulose to sugar monomers, fermentation of sugars to ethanol, and recovery of ethanol by distillation/evaporation from process stream.

2.6. Ethanol producing microorganisms

2.6 1. Yeasts

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with about 1,500 species currently described (Kurtzman and Fell, 2005). Yeasts do not form an exact taxonomic or phylogenetic grouping. At present, it is estimated that only 1% of all yeast species have been described (Kurtzman and Piswkur, 2006). The term "yeast" is often taken as a synonym for *S. cerevisiae* (Kurtzman and Fell, 2005) but the phylogenetic diversity of yeasts is shown by their placement in both divisions Ascomycota and Basidiomycota. The budding yeasts ("true yeasts") are classified in the order Saccharomycetales. Yeasts dominate fungal diversity in the oceans (Bass, *et al*., 2007). Most reproduce asexually by budding, although a few do so by binary fission. Yeasts are unicellular, although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as pseudohyphae, or false hyphae as seen in most molds (Kurtzman and Fell, 2005). Yeast size can vary greatly depending on the species, typically measuring 3–4 μm in diameter, although some yeast can reach over 40 μm (Wayman and Parekh, 1990). Scientific classification of yeast *S.cervisiae* (Kurtzman and Piskur, 2006)

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Saccharomycotina

Class: Saccharomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Saccharomyces*

Species: *cerevisiae*

The yeast species *Saccharomyces cerevisiae* has been used in baking and fermenting alcoholic beverages for thousands of years (Table 3). It is also extremely important as a model organism in modern cell biology research, and is one of the most thoroughly researched eukaryotic microorganisms. Researchers have used it to gather information about the biology of the eukaryotic cell and ultimately human biology (Ostergaard, *et al.*, 2000) and produce ethanol for the biofuel industry. Yeasts convert sugars (through a process known as fermentation) into alcohol and carbon dioxide. This trait is what endears yeasts to winemakers, 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's to rise. To multiply and grow, all yeasts need the right environment, which includes moisture, food (in the form of sugar or starch) and a warm, nurturing temperature (21.11°C to 29.44°C). Wild yeasts' spores are constantly floating in the air and landing on uncovered foods and liquids. No one's sure when these wild spores first interacted with foods but it's known that the Egyptians used yeast as a leavening agent more than 5,000 years ago.

Today, scientists have been able to isolate and identify the various yeasts that are best for winemaking, 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 leavened. It is 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. Because it is a rich source of B vitamins, brewer's yeast is also used as a food supplement (Janse and Pretorius, 1995). Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, fructose, maltose, sucrose, lactose, raffinose, trehalose, dextrin, starch and xylose. With regard to fermentation of these sugars, Scheffers, (1987) has argued that the anaerobic liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast species.

Ethanol production assays are deemed to be more appropriate determinants of sugar fermentation by yeasts (Walker *et al*., 2006). Yeasts are used in many industrial processes such as the production of alcoholic beverages, biomass (food and other purposes) and various metabolic products (Table 3). Some of these products are produced commercially while others are potentially valuable in biotechnology (Jacobson and Jolly, 1989; Kurtzman and Fell, 1997)

Table 3. Some potential uses of yeasts in the food, beverage and fermentation industries

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

Modifieified from Jacobson and Jolly (1989)

2.6.2 Classification and identification of yeasts

The chief characteristics used to classify yeasts are microscopically appearance of the cells, their mode of sexual reproduction, certain physiological (especially nutritional) activities, certain biochemical futures(Table 4), comparison of genomes in terms base sequences, by DNA hybridization or RNA/DNAsequence comparison (Barnett *et al.*, 2000).

Table 4. Criteria used in yeast species classification and identification

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

The physiological features that distinguish different yeasts include range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi anaerobic and aerobic condition.

The relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids are other physiological parameters to be used. 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 (Figure 1a) about 8 µm long and 5 µm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990).

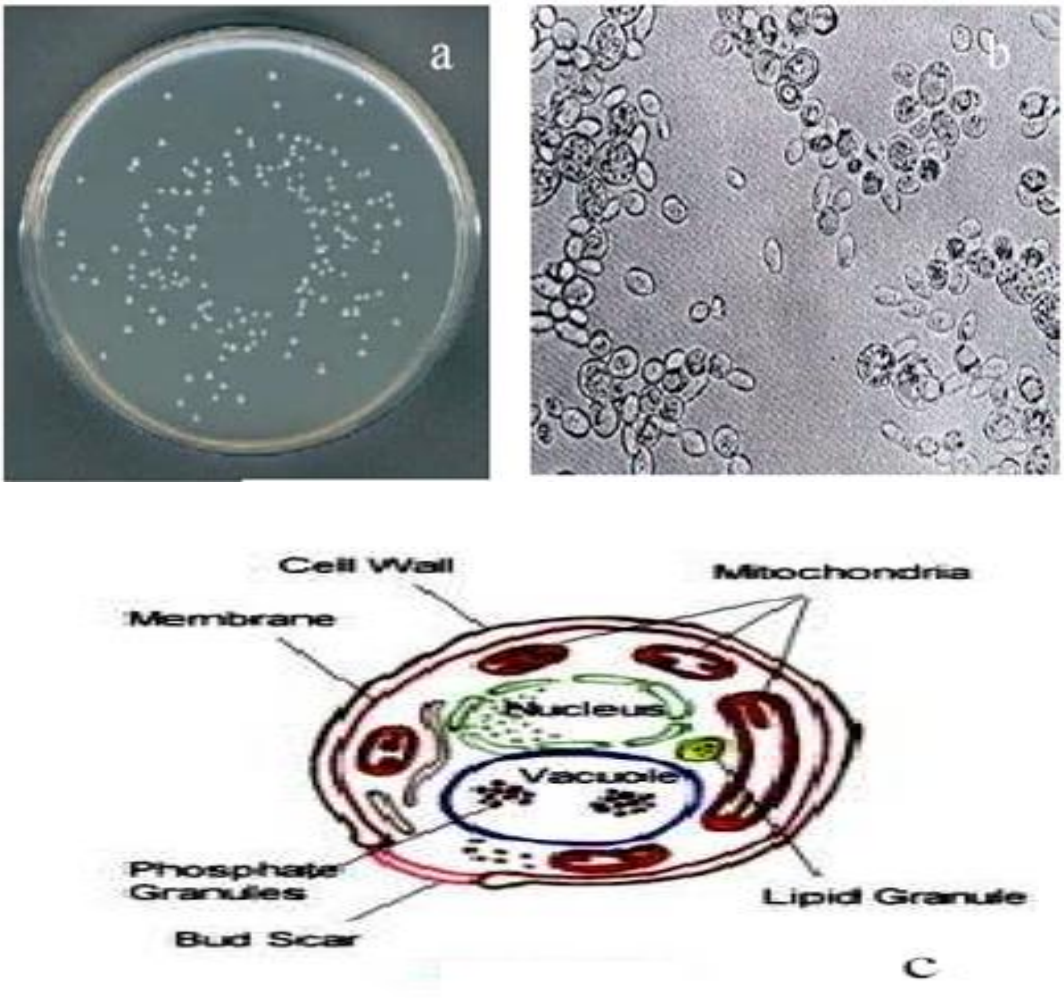


Figure 1. Yeast cell a) Colonies of *S. cerevisiae* on agar plate b), S. *cerevisiae* under microscope (400x) and c) Yeast cell composition(Barnett *et al*., 2000).

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored (Figure1a) or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts. Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos *et al*., 1996**)** along with asexual or sexual reproduction (Wayman and Parekh, 1990).

A. Asexual reproduction

Alexopoulos *et al*., (1996) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. The budding yeasts reproduce by budding, in this process the protoplasm of the cell, covered by a thin membrane, pushes out of the cell wall in the form of a bud and forms daughter cells (Figure 2b). 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 (Figure 3d).

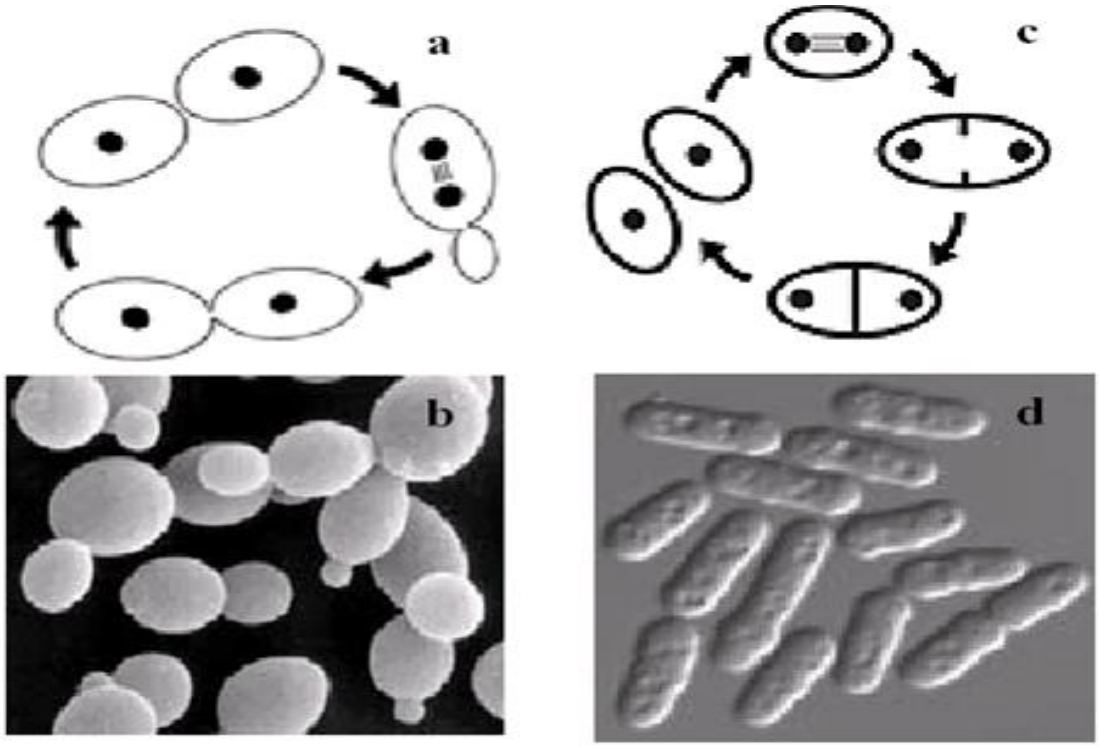


Figure 2. Sexual reproduction in yeasts

a. Budding yeast. Formation of new cell begins with blowing out of a new cell, at the pole of the cell. Mitosis follows with migration of one nucleus to the new cell. New wall material is then laid down in the passage between the two cells and separation of the cells will occur, b) shows S. cerevisiae cells reproducing asexually by budding (and c) fission yeast (Alexopoulos *et al*., 1996).

b. Mitosis of the nucleus occurs and, follows by elongation of the cell and formation of a cell wall that divides the cell in half, and separates the two nuclei, d) shows Schizosaccharomyces pombe cells reproducing asexually by fission (Alexopoulos *et al*., 1996).

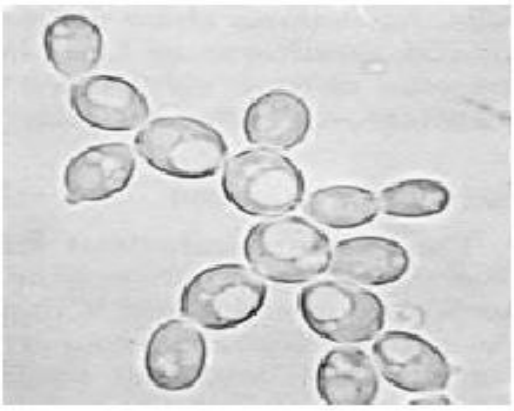


Figure 3. Chain of yeast cells (pseudo mycelium) produced by budding

During the process of budding, the nucleus divides, one daughter nucleus passing into the bud, the other remaining in the mother cell. Most known yeasts reproduce by budding such as S. cerevisiae. The fission yeasts reproduce by transverse division. The parent cell elongates, the nucleus divides, and a transverse wall (septum) in laid down 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 in ward. The new wall thickens before the daughter cells separation (Conti and Naylor, 1959).

B. Sexual reproduction

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

Figure 4 shows the reproduction of yeast, proceeding by the formation of buds on the cell surface, but sexual reproduction can be induced under special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and á. Each type can develop by budding into order haploid cells. The mating of an a haploid cell and an á haploid cell yields a normal á diploid cell. Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or á/ á) that can reproduce only asexually by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer and Nikaido, 1995).

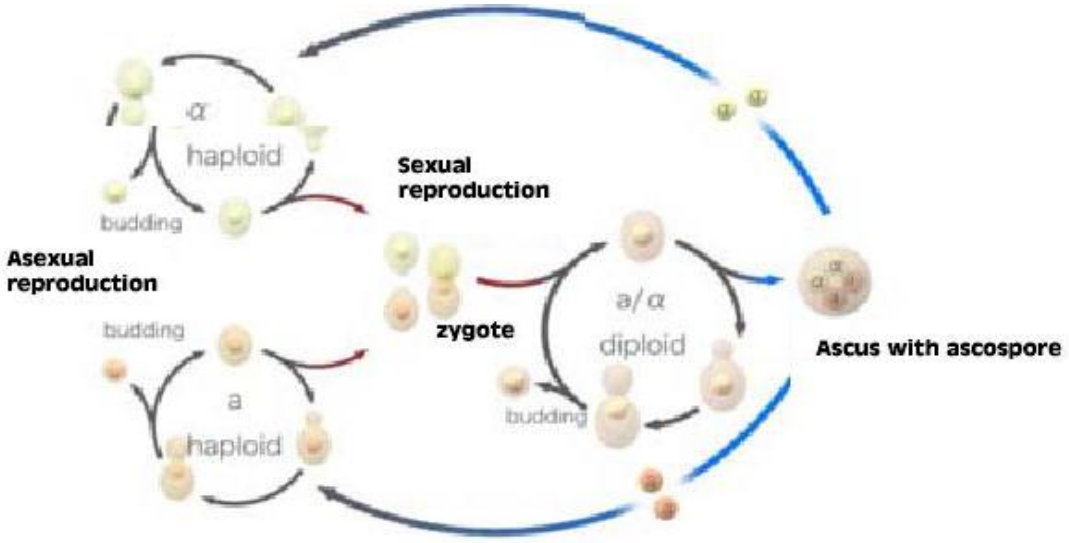


Figure 4.The reproduction of yeast by sexual and asexual (life cycle) (Kurtzman and Fell, 1998)

Ascospores formed by yeasts are often globose or ovoid, as in *Debaryomyces*, *Saccharomyces*, *Schizosaccharomyces*, and *Saccharomycodes* (Figure 5). Other yeasts form different types of ascospores. Thus, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped; in other species of *Hansenula* they may be hemispherical of shaped like the planet Saturn. Release of ascospores may occur when the ascus wall deliquesces; this is the usual method of release in species with hat- or Saturn-shaped spores. In other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopolos *et al*., 1996).

Miller (1989) pointed out that yeast ascospores are much more durable than somatic cells and have the ability to withstand even snail gut enzyme, a distinct advantage in their natural environment.

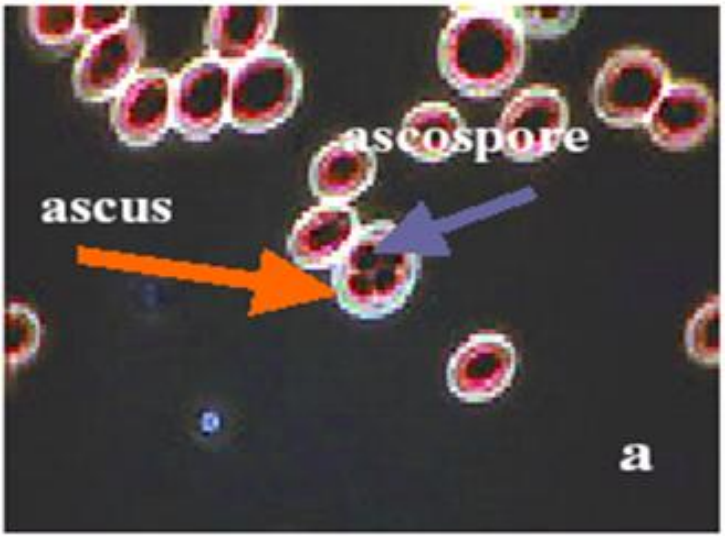


Figure 5. An ascus with four ascospores of S. cerevisiae

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

2.6.3 Fermentative yeasts

The genera of yeasts that are most frequently involved in ethanol production are *Saccharomyces, Brettanomyce* species, *Candida*, *Kluyveromyces, Debaryomyces, Trolopesis* and *Clavispora*. *Saccharomyces* does not have the genes for amylases, cellulases or B-galactosidases production or for degrading pentose (Ingledew, 1993).

Several fermenting yeasts including recombinant strain have been evaluated in converting lignocellulosic hydrolysates to ethanol **(**Table5). *Candida shehatae*, *Pichia stipitis kluyveromyes marxianus* were effective in degrading cellulose to ethanol.

Table 5. Yeasts capable of producing ethanol from different carbohydrates

Glucose and sucrose	**Lactose**	**Starch**	**Inulin**
Saccharomyces cerevisiae *S. ellipsoideus* *S.carlsbegensis* *S. ovifofmis* *Schizosaccharomyces pombe* **Xylose** *Kluyveromyces marxianus* *Candida tropicalis* *C. shehatae* *Clavispora spp.* *Pachysole tannophius* *Pichia stipitis* *Brattanomyces anomalus* *Brattanomyces clausenii*	*Brettanomyces spp.* *Brettanomyces lambricus* *Torulopsis spp.* *Torulopsis candida***.** **Cellobiose** *Brattanomyces anomalus* *Clavispora lustanae* *C.molischiana* *C. versalis*	*Saccharomyces diastaticus* *Kluyveromyces marxianus* *Candida tropicalis* *Candida shehatae* *Schwanniomyces occidentalis*	*Kluyveromyces marxianus* *Trolopesis spp.* *Torulopsis candida* *Schwanniomyces occidentalis* *Schwanniomyces castelli* *Saccharomycopsis fermentatans* *Saccharomycopsis cheresiensis* *Saccharomyces. kluyveri*

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

2.6. 4 Yeast isolates Selection for ethanol production

To obtain high quality and yield of ethanol in ethanol industry, selection of fermentative yeast is very essential. The most important factors to select microbial culture (Spencer and Spencer, 1997). Ability to utilize wide range of carbohydrates low pH optimum and high optimum temperature and resistance to several physicochemical stresses, rapid growth and fermentation rate and high osmo tolerance and ethanol tolerance.

In selecting yeasts for the efficient production of ethanol fuel (as opposed to potable ethanol), microbiologists have set out certain requirements of yeasts. The following are the most important one. An "ideal" yeast for fuel ethanol production should be ethanol tolerant, osmotolerant, acid tolerant, thermo tolerant, genetically stable, rapid and efficient fermentor, easy to propagate, able to utilize wide range of substrates, generate minimum heat during fermentation, possess flocculating or non flocculating characteristics depending upon the process requirements, possess "killer "activity, derepressed for di- or polysaccharide uptake in the presence glucose, resistant to certain toxic wastes . It is safe to assume that there is no singe yeast strain used in the industry today that posses the entire aforementioned characteristics and hence the research activity on this area needs further investigations (Panchal *et al*., 1981).

2.6.5. Ethanol Tolerant Yeasts

The conversion of carbohydrate to ethanol usually involve yeasts and optimal conversion requires cells that are tolerant to high concentration of both substrates and products and are able to efficiently produce ethanol (Waker, 1998). *Saccharomyces* yeasts are the most ethanol tolerant of the eukaryotic organisms and able to produce over 20% ethanol (Casey and Ingledew, 1986). There are different ways of improving ethanol production: increasing the range of substrate used as feed stock, improving the efficiency of substrate conversion to ethanol, raising fermentation temperature, or improving tolerance to ethanol and osmotic pressure. For this reason, attention has been given to yeasts other than *Saccharomyces* capable of fermenting substrates not accessible to the former such as inulin, starch, lactose, cellobiose, hemicelluloses/xylose(Ingledew, 1993; Waker, 1998).

Ethanol, the main end product of glycolysis in Saccharomyces, inhibits sugar fermentation and causes other unfavorable effects in yeast cells. For examples, it is a noncompetitive inhibitor of growth rate and inhibits the transport of sugar and amino acids, and other processes associated with membrane lipids (Ingram and Buttke, 1984; Waker, 1998). Lipid composition of plasma membrane is very important for ethanol tolerance, consistent with structural changes observed in the cell membrane of microorganisms tolerant to high concentration of ethanol (Ingram and Buttke, 1984).

Ethanol tolerance also depends on environmental and nutritional conditions (Ingledew, 1993). However, under fixed conditions, non-isogenic strains differ in their ability to tolerate ethanol, and tolerance is a reproducible characteristics implying that it is genetically controlled (Jimenez and Benitez, 1986, 1988). Genetic analysis confirmed that the characters are polygenic and that the genes responsible for ethanol tolerance are different in different strains (Osho, 2005; Jimenez and Benitez, 1986). For this reason hybridization has generated yeasts more tolerant to ethanol than their parental line.

2.6. 6. Ethanol fermentation

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

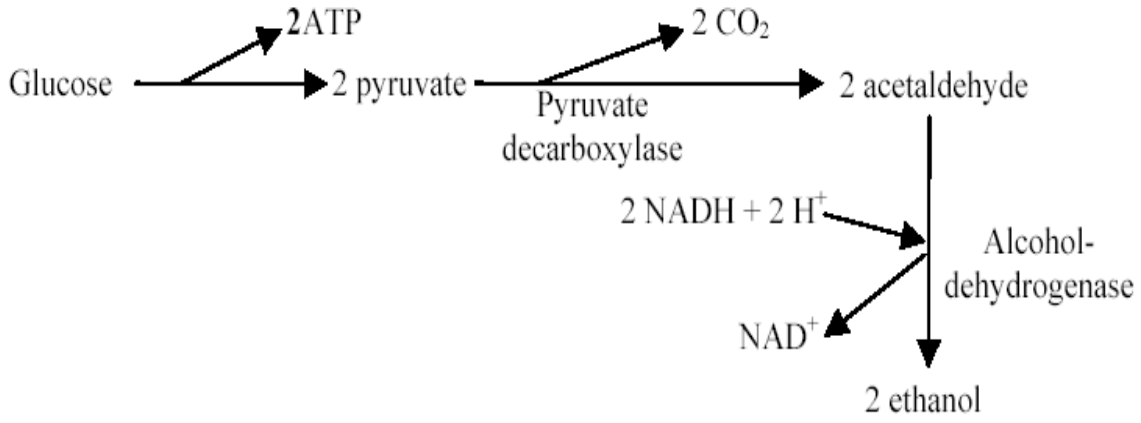


Figure 6. The ethanol fermentation pathway (Norr *et al*., 2003).

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

Lignocellulose is often hydrolyzed by acid treatment. The hydrolysate obtained is then used for bioethanol fermentation by microorganisms such as yeast. Because such lignocellulose 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 (3) below:

$(C_6H_{10}O_5)\ n + nH_2O \rightarrow nC_6H_{12}O_6 + \text{yeast} \rightarrow 2nC_2H_5OH + 2nCO_2$ (3)

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:

$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$, 4

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$, 5

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethyl alcohol and other byproducts. 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 bioethanol.

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, i*n-situ* detoxification and using less sensitive microorganisms to inhibitors (Taherzadeh, 1999). Microorganisms, termed ethanologens, presently convert an inadequate portion of the sugars from biomass to bioethanol (Demirbas, 2005). There are a number of microorganisms that produce significant quantities of bioethanol (Stewart and Russell, 1987). Xylose-fermenting microorganisms are found among bacteria, yeast and filamentous fungi (Hahn-Hagerdal *et al.*, 2006). Today, xylose fermenting bacteria include both native and genetically engineered organisms, and many have characteristics useful for simultaneous saccharification and fermentation (Jeffries and Jin, 2000).

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). 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 bioethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment.

2.7. Factors affecting Fermentation

A. 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. However, too high sugar concentrations can inhibit metabolism due to increased osmotic pressure. Very low levels of sugar may limit the rate of ethanol production (Jones *et al.*, 1981).

Hence, each fermentation process will have an optimal glucose or equivalent sugar concentration (Sofer and Zaborsky, 1981). A sugar concentration of 10-18% is usually satisfactory, although other concentrations are used (Dunn, 1959). Borzani *et al.* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar. Bertolini *et al.* (1991) isolated yeast strains from sample collected from Brazilian alcohol factories. These 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 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 osmotolerant *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 30ºC.

The maximum amount of ethanol produced by immobilization osmotolerant *S. cerevisiae* cells using 150, 200 and 250 g/L glucose was 72.5, 93 and 83 g ethanol per liter at 30 ºC after 48 hours. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%.

B. 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 recombinant strains are mesophilic organisms and function best between 30 to 38°C. Operating at greater temperatures is desirable for the following reasons (Hettenhaus, 1998).

- High fermentation temperature increases growth rate and productivity exponentially.
- Plant capital cost is less due to higher productivity per unit volume of fermentor 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 enzyme hydrolysis process for saccharification able to operate up to 55°C may be combined with fermentation, further reducing capital and glucose inhibition (Hettenhaus, 1998).

C. pH

A very important factor for cellular growth is external pH. Yeast and fungi tolerate a range of pH 3.5-5.0. The ability to lower pH below 4.0 offers as a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants. Yeast cultures can grow over a wide range of pH values from 3 to 8 with an optimum for growth generally in the slight acidic range 3.55.0. Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures. 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).

D. 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 (Osho 2005). At temperatures above 35°C, current strains lose viability at ethanol concentrations of 10% (*w*/*v*) (Hettenhaus, 1998).

E. 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).

F. Inhibitor tolerance

McMillan (1994) grouped the fermentation inhibitors into three classes. Compounds originating in the biomass by hydrolysis. These include organic acids such as acetic, glucuronic and galacturonic acids from the hemicellulose and phenolic compounds from the lignin. The inhibitoriest of these for both yeast and bacteria is acetic acid and solubilized lignin. Compounds formed by degradation of the products resulting from pretreatment and hydrolysis of the biomass. Furfural from xylose and hydroxymethylfurfural from Glucose leads to this group. It is completed by an assortment of aldehyde, acids and alcohols from lignin, sugar and protein degradation. And compounds from other sources. Metal ions resulting from equipment corrosion, sulfites, sulfur dioxide and lactic acid introduced with other streams containing nutrients, cleaning solutions.

2.8. Method of Ethanol determination

Determination of ethanol is perhaps the most important routine analysis in a modern n fermentation process. Frequent, fast, and accurate results are needed to control the quality of the alcohols from substrates to bottle, as well as for state and federal government tax and regulatory purposes (Jain and Cravey, 1972).

The alcoholic beverage industry and various regulatory agencies have devoted much effort in recent years to developing a faster, specific, more accurate, and automated method.

A. Gas-liquid chromatography

Gas-liquid chromatography is one of the most modern analytical tools, dating from 1952. Even more recently, with the utilization of electronic digital data-processing equipment, gas chromatography has become increasingly preferred for accurate quantitative as well as qualitative analyses of many substances. Gas chromatography is inherently specific in separating volatile compounds on the basis of compound specific partitioning properties between a gas phase and a liquid (or a solid) phase (Jain and Cravey, 1972).

B. Ebulliometer

For measuring the concentration of ethanol, Ebulliometer is equipment designed to evaluate the boiling point of different types of liquids. Its use in the alcohol industry is based on the fact that alcohol boils at (78.4°C) a lower temperature than water, so the boiling point of alcohol-water mixtures changes as a function of their concentration. A precision thermometer is involved to determine the boiling temperature of the ethanol (Samarajeewa and Tissera, 1975).

C. Alcoholmeter

An alcoholmeter is used to find the alcohol percentage of alcohol by volume (abv) in completely dry or distilled liquor. It does this by measuring the density of the liquid compared to that of alcohol; any residual sugar or other cause of change to the density will result in a false low reading. Because there is often a minute amount of unfermented sugar following standard fermentation, along with dissolved carbon dioxide and spent yeast or solids remaining after fermentation, it is very difficult to obtain an accurate reading of the abv in such a liquid. In addition, an alcoholmeter is increasingly difficult to read at lower alcohol percentages, compounding the problem. For this reason, it is suggested that you use an alcoholmeter only for the testing of distilled spirits. The use of an alcoholmeter is very straightforward. Pour a sample of the spirit to be tested into a tall glass or high chemical tolerant plastic cylinder, leaving enough space for the alcoholmeter to disperse the liquid (Lachenmeier *et al*., 2005).

3. MATERIALS AND METHODS

3.1 Study area description

The study was conducted in Agaro and Gomma Wereda coffee processing station Gomoli area. Gomma Wereda is located at about 390 km southwest of the capital, Addis Ababa. It is one of the administrative units of Jimma Zone of Oromia Regional State were the largest Wereda producing Arabica coffee. The altitude of Goma Woreda ranges from 1387 to 2870 meters above sea level (m.a.s.l). The mean annual rainfall was reported to be 1524 mm with bi-modal distribution. The mean monthly temperature varies between 12.67^0C and 29.10^0C (IPMS, 2007).

Gomoli is study areas where wet Arabica coffee processing is practiced. Different environmental characteristics of the study area, with polluted rivers around coffee processing, poor waste management systems, coffee processing waste water effluents and pulps discharged into traditional wastewater lagoon or pools, the environment has bad smell and contribution to pollution. This situation initiated me to undergo this study to examine the potentiality of Arabica coffee waste water and produce environmental friendly product bioethanol to reduce environmental pollution.

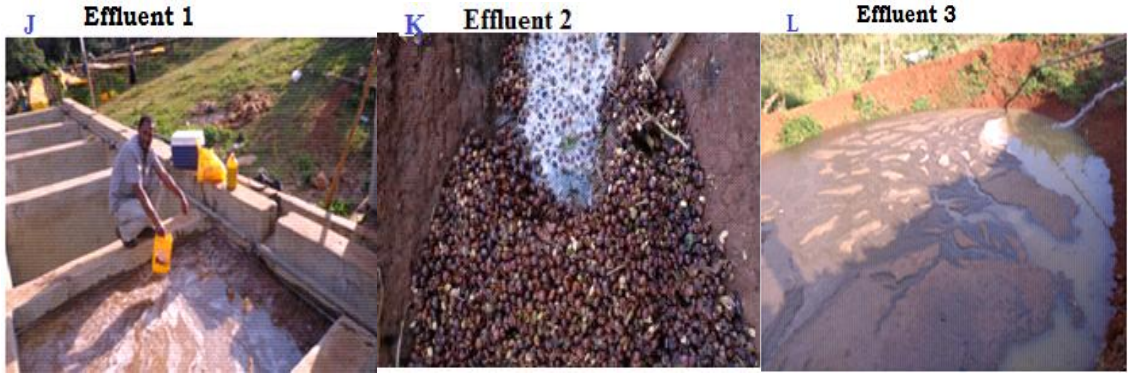
3.2. Samples collection

Wet Arabic coffee wastewater (effluents) and two samples pulps were collected in icebox from a pulping center that is located in Agaro and, Goma Wereda and Gomoli area, Jimma Zone. The samples were, taken to Addis Ababa University, Mycology Laboratory for analysis (Figure 7). The pulp was oven-dried at 60°C for 48 h (to moisture content of 15 %), grinded by coffee grinder and sieved (Urbaneja *et al*., 1996). The samples were stored in hermetically closed plastic containers at room temperature for further study.


A
B
C


D
E
F


G
H
I


J
Effluent 1
K
Effluent 2
L
Effluent 3

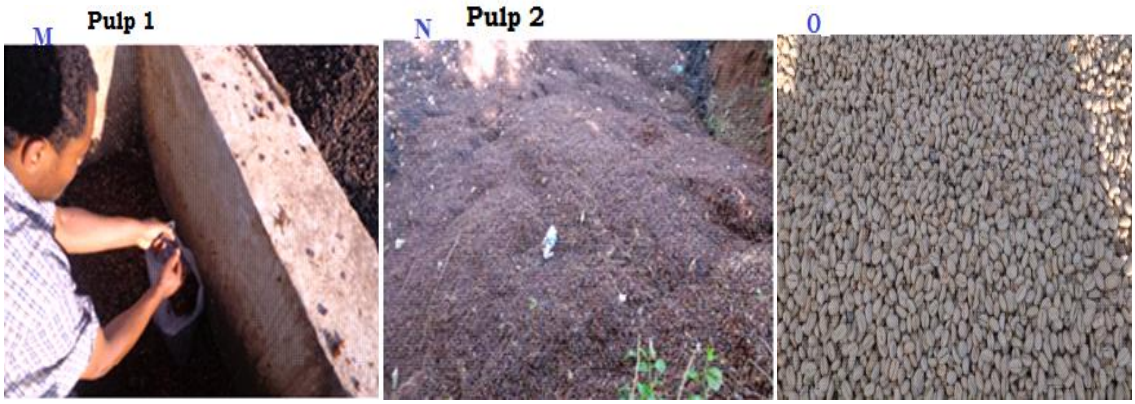


Figure 7. A) Fresh Arabic Coffee bean on the trees, B) Harvesting of the red coffee beans, C) Collecting the red cherries, D) Wet processing area (small industry),E) Ready for reception of cherries), F) Release of water to the opening for process, G,) Sorting by Floaters (removal of fruits of bad quality), H) Pulping (pulp removal), I) washing or mechanical demucilaging (mucilage removal), J) Washing coffee in channel, and, K)effluent mixed with pulp), L) effluent storage hole , M) Temporarily storage of pulp, N) Pulp stored area, O) Dried, Hulled (hull/parchment removal) and prepared for Bagging / shipping.

3.3. Isolation of dominant yeasts from Arabica coffee wastewater (effluents) and pulps

Fermentative yeasts were isolated from the samples on pre-solidified plates of yeast extract peptone glucose (YEPD) agar medium with the following ingredients (yeast extract, 10 g; peptone, 20 g; glucose, 20 g; agar 20 g; distilled water 1,000 ml) containing 50µg chloramphenicol/ml). Ten (10ml) of Arabica coffee effluent 1, 2, and effluent 3 and 10g of pulp1 and pulp2 were separately mixed with 90 ml sterile distilled water. One ml of the mixture was taken and serially diluted in test tubes each containing 9 ml sterile distilled water. This was followed by spread plating of the aliquots of 0.1 ml from appropriate dilutions (10^{-1}-10^{-6}) on YEPD agar medium. All the inoculated plates were incubated at 30^{o}C for 2 to 3 days. The yeast isolates were purified by subsequent streaking on YEPDA medium.

The colonies of pure culture were transferred to YEPDA slant. After growth, the slant cultures were preserved at 4^{o}C for further study.

3.4. Characterization of yeast isolates

3.4.1. Testing of isolates for carbohydrate fermentation using Durham tube

Yeast fermentation broth with Durham tube was used for testing of yeast isolates for carbohydrate fermentation. Yeast fermentation broth medium consists (g/l) of 4.5 yeast extract, 7.5 peptone, 20g respective carbohydrate, 17g of Bromcresolbule and 1000 ml distilled water) with Durham tube were used for identification yeasts based on fermentation of specific carbohydrates of fermentation pattern. At least the two isolates (ACP12 and ACE12) were selected for further characterization which were rapid and fastidious fermentative of the tested carbohydrates and screened for ethanol production. The color of the medium changed from blue to yellow due to the alcohol and gas production in the medium (Warren and shadomy, 1991).

The carbohydrates used were: glucose (dextrose), galactose, maltose, sucrose, lactose, fructose, trehalose, raffinose, starch and cellulose.

A 4.5 g of Yeast extract and 7.5 g of peptone were transferred to 1000 ml of deionized distilled water and thoroughly mixed with gent heating and brought to boiling and bromcresol blue was added to yeast extract peptone broth after heating. A 10 ml of the broth was distributed into the larger tube (with about 150 mm by 15 mm) size. A Durham tube of about 50 mm by 6 mm was inserted. The flasks were autoclaved for 15 min at 15 psi pressure and 121^{o}C. After autoclaving flasks were cooled to room temperature and 1ml of teste carbohydrate was transferred to test tube that contains 10ml of yeast extract peptone broth. Finally, one drop of 72 hrsr old yeast culture grown in YEPD broth was added to each flask and incubated at 25^{o}c for one week. Each day, the flasks were shaken to help sediment the yeast cells and examined for bubbles of gas (CO_2) in the inserted Durham tubes with color change of the medium (Barnett *et al.*, 2000). The fermentation broth with Durham tube that lacked yeast culture served as control.

3. 4.2. Tolerance of yeast isolates to some physicochemical factors

A. Tolerance of glucose concentration

Tolerance of isolates to different levels of glucose concentration was accessed and tested in comparison with the standard strain *S. cerevisiae.* Different concentrations of glucose (10%, 20%, 30%, 40%, 50%, and 60%) were added to 100 ml YM broth containing (g/ml) yeast extract, 3 g; peptone, 5 g; glucose, 10 g and 1000 ml distilled water) and autoclaved. The flasks were then cooled to room temperature and 1 ml of 24 h old yeast culture was inoculated aseptically. The flasks were incubated at 30ºC for 7 days. After incubation, the yeast cell was taken from 10^{-5} were counted by serial dilution and plating. One ml of the mixture was taken and serially diluted in test tubes each containing 9 ml sterile distilled water. This was followed by spread plating of the Aliquots of 0.1 ml from appropriate dilutions (10^{-1}-10^{-6}) and were spread plated on YEPD agar medium (Subashini *et al.,* 2014).

B. Tolerance of ethanol

Tolerance of yeast cultures to ethanol was tested in comparison with the standard strain *S. cerevisiae* and the isolated yeast from coffee wastewater (effluent) and pulp. One ml of 24 h old culture grown in YEPD broth was inoculated to 100 ml YM broth and subjected to different concentrations of ethanol 4%, 8%,12%,16% ,20%, 24% (v/v) to the YM broth and incubated at 30ºC for 7 days based on the standard procedure. After incubation, the yeast cell was taken from $10^{-}5$ (I am not sure and believe on this dilution factor for the estimation of the yeast population and the population was estimated by serial dilution and plating like that tolerance to glucose concentration procedure section (A) (Subashini *et al.,* 2011).

C. Tolerance of temperature

Tolerance of yeast isolates to different temperature level was tested in comparison with the standard *S. cerevisise.* One ml of 24 h old yeast culture grown in YEPD broth was inoculated aseptically into 100 ml YM broth and incubated at 10, 20, 25, 30, 40, 50, and 60ºC for 7 days. After incubation the population was estimated by serial dilution and plating followed the same procedure of glucose tolerance in section (A) (Subashini *et al.,* 2011).

D. Tolerance to pH

Tolerance of yeast isolates to different pH levels was tested in comparison with the standard *S. cerevisiae*. In 100 ml YM broth, the pH was adjusted to 2.5, 3.5, 4.5, 5.5, and 6.5 using 1N HCl and 0.1N sodium hydroxide. One ml of 24 h old yeast culture was inoculated into flasks containing the adjusted pH values and incubated for 7 days at 30 ºC. After incubation the population was estimated by serial dilution and plating (the detail procedure similar to yeasts tolerance to glucose concentration) in section (A) (Subashini *et al.,* 2011).

3. 4.3. Morphological characterization

3.4.3.1. Microscopic examination for vegetative cells

From YEPD Agar, (two days old) yeast culture was inoculated into 30 ml of sterile YEPD broth in a 100 ml conical flask. The culture was examined microscopically after incubation for 2 days at 30^{o}C (Barnett *et al*., 2000). The culture from the broth was taken and dropped on the center of sterilized slide then covered by cover slip, the culture examined microscopically.

3.4.3.2. Microscopic examination for filamentous growth

Filamentous growth was detected by using the method of Barnett *et al.* (2000). Slide cultures were prepared as indicated below. A sterile piece of filter paper, a sterile U-shaped glass rod support, two sterile microscope slides and sterile cover slips were put down in each sterile petridishes. Working aseptically, autoclaved corn meal agar was melted and poured into a boiling-tube which is wide and deep enough to hold a microscope slide. Each slide was dip into the agar, drained a little, and replaced on its glass rod support in the Petri dish.

The medium was lightly inoculated along the length of each slide with a straight wire (the inoculums from an actively growing culture) and a cover slip was placed over a part of inoculated agar. The filter paper was wet with sterile water to prevent drying. The cultures were incubated at 30^{o}C and examined microscopically every two days for about two weeks.

3.4.3.3 Growth in liquid medium

The yeast cells were cultured in YEPD liquid medium. Cells from a young actively growing culture were inoculated into test tube containing 7 ml of YEPD broth medium, incubated at 30°C for 72 hrs. The culture was examined for the growth of yeast visually on the surface of YEPD liquid medium.

3.4.3.4. Microscopically examination for ascospores

For production of ascospores by yeasts, the method of Prave *et al.*, (1987) was followed. Accordingly, two types of media were prepared, i.e. presporulation and sporulation media. The presporulation medium consists of 20 g of glucose, 2 g of ammonium sulfate ($(NH_4)_2SO_4$), 2 g of potassium dihydrogen phosphate (KH_2PO_4), 5 g of yeast extract and 1000 ml of distilled water, the medium was kept in sterile state for 7 days in flasks. The medium were inoculated with a loopful young culture of 48h old and incubated at 30^{o}C on shaker at 121rpm for 3 days.

The sporulation medium consists of 1 g of glucose, 8.2 g of potassium acetate, 2.5 g of yeast extract, 1.86 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and 1000 ml of distilled water. The flask contained the sporulation medium was autoclaved and prepared. The prepared medium was inoculated with one up to two drops of yeasts from the presporulation medium. In contrast to the presporulation medium, this medium was used immediately after sterilization and incubated at 30^{o}C for 72hrs and examined microscopically. Yeast samples were wet-mounted on a glass slide to observe types of ascospores. The yeast cells were also heat fixed and spore stained according to (Lodder, 1971).

Accordingly, the heat fixed samples were flood with 5% aqueous malachite green for 30-60 seconds and heated to steaming 3 to 4 times and washed with tap water for half a minute. The slides were counterstained with 0.5% safranin red for about 25-30 seconds. The excess stain was gently washed with running tap water for half a minute. The preparations were observed under high power (40X) and oil immersion objectives (100X).

3.5. Determination of moisture content (Dry rot of pulp)

The moisture content of the pulps was determined by oven drying method. The sample was weighed with glass crucible and placed in the air drying oven for 48 hrs at 60 ℃ 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 (Bilanski and Ghate,1978.). The moisture content was then calculated as follows:

$$\%\text{Moisture contents} = \frac{W1-W2}{W1} \times 100$$

Where:

W1= Weight of the sample before drying in g

W2= Weight of the sample after drying in g

3.6. Total sugar determination

3.6.1. Coffee processing effluent (liquid).

The amount of sugar in the samples of coffee effluent was determined by Fehling method (Periyasamy *et al*., 2009). A 50 ml of sample from the coffee wastewater (effluent) was taken for determination of total sugar. A 50ml of sample was filtered through filter paper and dissolved in 10 ml of distilled water and 2 ml of concentrated HCl was added and boiled for 5mins. The mixture was allowed to stand at room temperature for 24hours. The acidified sample was neutralized with concentrated 0.1N NaOH and the solution was made up to a volume of 300 ml and transferred to the burette.

A 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. Two drops of methylene blue indicator was added (Fig 8I). The solution was titrated with burette solution in boiling conditions until disappearance of blue color. Finally, the volume at which a brick red color observed and recorded (Fig 8II, B, C and D). For each sample of both coffee effluents, the sugar content was calculated by using the formula given below

$$\text{Sugar Contents (\%)} = \frac{300\ \text{ml} * f}{V} * 100$$ (Periyasamy *et al*., 2009).

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

3.6.2. Determination of sugar content of pulps

The pulp was oven-dried at 60 °C for 48 hrs, ground by mortal and pistil and sieved (Urbaneja *et al*., 1996). The sample of 20g was diluted with1000ml of distilled water and filtered by filter paper. The amount of sugar in the sample was determined by Fehling method as detailed in section (3.6.1) of coffee processing effluent. The solution in the flask is titrated with burette solution under boiling condition until the blue color disappeared. Finally, the volume at which a brick red color observed was recorded (Fig 8II E and F).

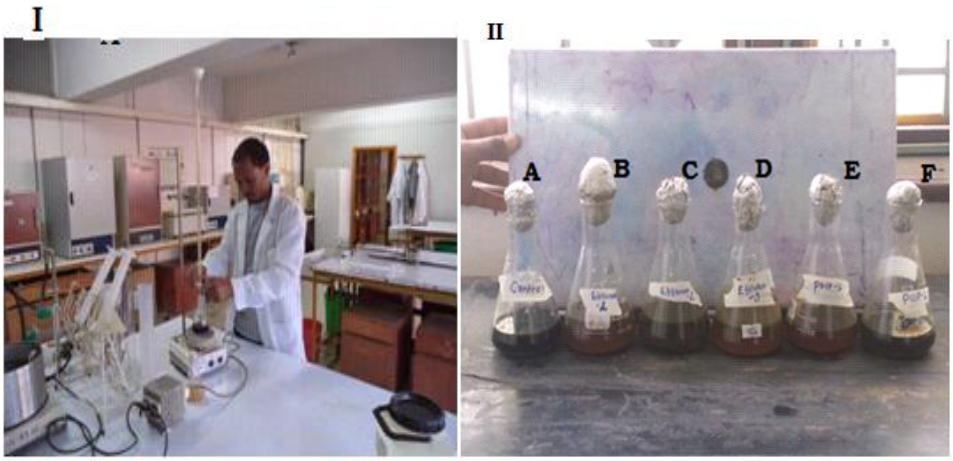


Figure 8. Total sugar determination of Arabica coffee effluent and pulp process (I) and were the color change observed (II).

3.7. Fermentation process

3.7.1. Fermentation of coffee effluent

The flasks containing the coffee effluent 750ml of effluent were diluted with 250ml of distilled water (v/v) and covered with cotton wool, wrapped in aluminum foil, autoclaved for 15 minutes at 121°C and allowed to cool at room temperature. Fermentation was carried out in 1000 ml capacity Erlenmeyer flask with optimum inoculum 3 g/l of yeast isolates used by Turhan *et al*., (2010), and standard yeast *S. cereviciae* at incubation temperature of 30°C and fermented for 72hrs. The standard yeast strain was maintained in the Addis Ababa University, Mycology Laboratory and obtained for the experiment.

3.7.2. Fermentation of coffee pulp

The powdered pulp (20 g) was hydrolyzed with 1000ml of distilled water contained in the flask for 4h and the samples were covered with cotton wool, wrapped in aluminum foil, autoclaved for 15 minutes at 121°C and allowed to cool at room temperature. Fermentation was carried out in 1000 ml capacity Erlenmeyer flask with 3 g/l of yeast isolates and standard *S. cereviciae* and incubated at temperature of 30°C (Franca *et al.*, 2008; Thuesombat *et al*., 1990) fermentation was carried out for 72hrs

3.7.3. Cell biomass determination

After 72hrs, the fermentation broth composition of coffee effluents and pulp in each flask was filtered and centrifuged at 10,000 rpm for 5 minutes. Each yeast biomass (pellet) was measured using watch glass (Campelo and Belo, 2004).

3.7.4. Determination of ethanol amount in the fermentation broth

After centrifugation at 10,000 rpm for 5 minutes, the supernatant was filtered. The filtered ethanol was measured and carried from Addis Ababa University, Mycology Laboratory to Sebata town where Balezaf Alcohol and Liquors Factory found. Ethanol concentration was measured using Ebulliometer at Balezaf Alcohol and Liquors Factory. Ebulliometer is equipment designed to evaluate the boiling point of different types of liquids (water and alcohol). Its use in the alcohol industry is based on the fact that alcohol boils at (78.4°C) a lower temperature than water and the amount of ethanol was determined by calculating between the difference of the boiling of the two solution based on the standard manual. A precision thermometer is involved, to determine the boiling temperature of the ethanol.

3.7.5. Method of data analysis

Statistical analyses were performed using SPSS version 16 in order to setup mean, standard deviation of the laboratory result. Tolerance of Yeast isolates to some physicochemical factors comparisons with standard *S.cerevisiae* were done by one-way ANOVA to determine significant difference of the isolates. Finally the results were displayed using tables.

4. RESULTS

4.1. Isolation of fermentative yeasts

A total of fifteen (15) yeast isolates were isolated from five samples of Arabic coffee effluent 1, effluent 2, effluent 3, and Arabic coffee pulp 1 and pulp 2 and standard yeast strain of *Saccharomyces cerevisiae* was taken from Addis Ababa University Mycology Laboratory, most of the isolated colonies exhibited smooth surfaces with circular margins (Appendix 1). The colour of the pure colonies of effluents and pulps showed creamy white but some colonies of pulps slightly red and pinkish (Appendix1 and 2). The cells were found to be of various shapes such as round; oval, spherical and ellipsoidal (Appendix 1). The isolates from effluent 1 were designated as ACE1 (ACE11, ACE12 and ACE13). The yeast isolates from effluent 2 were designated as ACE2 (ACE21, ACE22 and ACE23). Similar designation (ACE3) was given to the isolates from effluent 3 (i.e., ACE31, ACE32 and ACE33). The yeast isolates from pulp1and pulp2 were designated by ACP1 (ACP11, ACP12and ACP13) and ACP2 (ACP21, ACP22 and ACP23), respectively (Appendix 1). The standard yeast (*S.cerevisiae*) was taken from Addis Ababa University Mycology Laboratory for this study.

4.2. Screening of fermentative yeast isolates

4.2.1. Testing of isolates for carbohydrate fermentation by Durham tube

The yeast isolates were capable of utilizing 6-10 different carbon sources with variation in utilization of different sugars (Table 6). Almost all isolates utilized glucose, galactose, fructose and maltose. All the isolates were failed to grow on starch except ACE12, ACE22, ACE23, ACE32 andACP12 (Table 7). ACE12, ACP12 and ACP13 were the only isolates capable of fermenting cellulose. Likewise, isolates ACE12, ACE13, ACE31, ACE32, ACP12 and ACP21 were the most fermentative ones in xylose broth. Almost all isolates fermented raffinose and trehalose except ACE13, ACE23, ACE32, ACP22 and ACP13 and ACP22. Isolates ACE11, ACE12, ACE13, ACE22, ACE23, ACE31, ACE32, ACE33, ACP11, ACP12 and ACP13 were highly fermentative in

several of the test carbohydrates (Table 6), but the rate of fermentation varied in most of the isolates. The most potent fermenter were ACE12 and ACP12 and taken for further morphological and physiological characterization for ethanol production (Table 6). The fermentation process was indicated by the color change from red to yellow (appendix 2).

Table 6.Comparison and selection of isolates by Durham tube carbohydrates fermentation method

Isolates	Fermentation											
	Glucose	Galactose	Fructose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Starch	Cellulose	xylose	Total carbohydrate fermented
ACE11	+++	++	++	+	+	+	+	+	-	-	-	8
ACE12	+++	++	+	++	+	+	++	+	-	+	+	10
ACE13	+	+	+	+	+	-	-	+	-	-	+	8
ACE21	++	++	+	-	+	+	+	+	-	-	-	7
ACE22	++	+	+	+	+	++	+	+	+	-	-	9
ACE23	+	+	+	+	+	++	_	+	+	-	-	8
ACE31	++	+	++	++	++	+	+	+	-	-	+	9
ACE32	++	++	+	-	+	+	_	+	+	-	+	8
ACE33	+++	++	++	++	++	+	-	+	-	-	-	7
ACP11	++	+	++	+	+	+	+	+	-	-	-	8
ACP12	+++	+	+	+	+	++	+	+	+	-	+	10
ACP13	+	+	+	+	+	-	+	-	-	+	-	7
ACP21	++	+	+	-	+	-	+	+	-	-	+	7
ACP22	+	+	++	-	+	+	+	-	-	-	-	6
ACP23	++	+	+	+	+	_	_	+	-	-	-	6
S.cerevisiae	++	+	+	++	+	_	+	+	_	_	_	7

+ = Fermentative, ++ =moderately fermentative, +++ = Highly fermentative (Durham tube empty), - = No carbohydrate utilization

4.2.2. Physiological characterization of yeast isolates ACP12 and ACE12

The two isolates ACP12and ACE12 were screened for ethanol production since these isolates utilized most of the test carbohydrates and based on colony characteristics (Table 6 and Appendix 1).

A. Sugar tolerance (Osmotolerance)

Table- 7 summarizes the percentage of sugars tolerated by the isolates and standard yeast *S.cerevisiae* growth in different glucose concentrations in YM medium. The growth of ACP12 and ACE12 was gradually increased with concentrations of sugar. The results indicated that the two isolates had maximum population at 20% glucose concentration (Table 7). The yeasts isolated from pulps (ACP12) and effluents (ACE12) recorded maximum population count at 20% glucose concentration with the mean value of (216.0±1.00 and 121±1.00x10^6 CFU/ml), respectively (Table 7). However, as the sugar concentration increased from 20% to 60%, the growth of both isolates and standard yeast *S.cerevisiae* was decreased gradually. The reference culture showed maximum growth at 20% glucose concentration (153.0±1.00 x10^6CFU/ml) compared to other two isolates. Among the three strains, isolate ACP12 showed the highest sugar tolerance of up to 20% with maximum mean count of (216.0±1.00x10^6 CFU/ml) compared to the two isolates and there was significantly different ($P<0.05$) (Table 7).

Table 7. Growth of yeast isolates at different glucose concentrations

Glucose (%)	Mean count (CFUx10^6/mL) of yeast isolates		
	ACP12	ACE12	*S.cerevisiae*
10	77.0±1.00^{g}	48.5±0.50^{j}	56.0±1.00^{i}
20	216.0±1.00^{a}	121±1.00^{c}	153.0±1.00^{b}
30	112.0±1.00^{d}	91.0±1.00^{f}	94.7±5.86^{e}
40	65.0±1.00^{h}	43. 0±1.00^{k}	48.0±1.00^{j}
50	49.3±1.52^{j}	23.0±1.00^{l}	36.0±1.00^{l}
60	37.0±1.00^{l}	13.0±1.00^{m}	21.0±1.00^{m}

The isolates have different growth pattern from the above data expressed as % glucose concentration (Table 7). Mean ± SD in the same column and row the letters are different, this indicate the growth of the isolates were significantly different at ($P< 0.05$).

B. Temperature tolerance

The effect of temperature on growth of yeast isolates is shown in Table 8. Yeast isolated from Arabic Coffee pulp (ACP12) showed higher mean count (97.0±1.00x10^6CFU/mL) at 30ºC followed by the standard culture *S. cerevisiae* with maximum population of (87.0±1.00 x10^6CFU/ml). However, the yeast isolated from Arabica Coffee effluent (ACE12) showed (67.7±1.54 x10^6 CFU/mL) at the same temperature. The growth of selected yeast isolates increased from 15ºC to 30ºC. Beyond 30ºC, the growth of test yeasts and the standard strain was declined and there was significantly different ($P<0.05$) among the yeast isolates (Table 8)

Table 8. Growth of yeast isolates at different temperature in YM medium

Temperature(°C)	Mean count (CFUx10^6/ml) of yeast isolates		
	ACP12	ACE12	*S.cerevisiae*
15	22.0±1.00^{k}	12.0±1.0^{m}	17.0±1.00^{l}
20	26.0±1.00^{i}	15.0±1.00^{m}	23.0±1.00^{jk}
25	71.7±.58^{c}	56.7±1.53^{fg}	59.0±1.00^{f}
30	97.0±1.00^{a}	67.7±1.54^{d}	87.0±1.00^{b}
40	63.0±1.00^{e}	54.0±1.00^{g}	55.0±1.00^{g}
50	36.3±1.53^{h}	21.0±1.00^{k}	32.3±1.53^{i}

The growth of yeast isolates in different temperature. Mean ± SD in the same column and row letters are different. This indicate the growth of the isolates were significantly different at ($P< 0.05$).

C. pH Tolerance

The growth of yeast isolates at different pH is given in Table 9. The isolates from Arabica Coffee pulp (ACP12) and effluent (ACE12) recorded maximum mean counts of (98.0±1.00 x10^6CFU/ml), (78.0±1.00 x10^6CFU/ml) and the standard strain (87.0±1.00 x10^6 CFU/mL) at pH 5.0, respectively. Above pH 5.0 yeast cells declined (Table9). There were maximum mean count at pH 5.5 by the test isolates (ACP12, ACE12) and the standard yeast strain; there was statistically significant difference ($P<0.05$) (Table 9).

Table 9. Growth of yeast isolates at different pH in YM medium

pH	Mean count (CFUx10^6/mL) of yeast isolates		
	ACP12	ACE12	*S.cerevisiae*
2.5	14.0±1.00^{j}	12.0±1.00^{j}	12.0±1.00^{j}
3.5	26.0±1.00^{i}	15.0±1.00^{j}	23.0±1.00^{i}
4.5	47.7±.58^{f}	35.0±1.00^{h}	43.0±1.00^{g}
5	98.0±1.00^{a}	78.0±1.00^{c}	87.0±1.00^{b}
5.5	95.0±1.00^{a}	76.0±2.65^{c}	87.3±1.53^{b}
6.5	58.3 ±.58^{d}	45.0±1.00^{fg}	53.0±1.00^{e}

The mean count of yeast isolates at different pH, the growth of the isolates varied. Mean ± SD in the same column and row and letters are different and this indicate their growth were significantly different at $P < 0.05$.

D. Ethanol tolerance

As concentration of ethanol increased in medium, a reduction in growth was generally observed (Table 10). Difference in ethanol tolerance was observed among yeast isolates and the standard *S.cervisiae (*Table 10). Yeast isolated from Arabica Coffee pulp (ACP12) showed maximum population (96.0±1.00 x10^6CFU/mL) followed by isolate from Arabica Coffee effluent (ACE12) with maximum mean count of (77.7±1.53 x10^6CFU/ml). At 4% concentration and gradually decreased at higher concentrations. The isolate from coffee pulp exhibited maximum tolerance up to 16% ethanol with a maximum population of (78.0±1.00 x10^5 CFU/ml) similar to that of standard strain *S. cerevisiae (*68.0±1.00 x10^5 CFU/ml), whereas the one which was isolated from coffee effluent (ACE12) showed poor tolerance and there was significant ($P<0.05$) among the

three yeast strain (Table 10). As the concentration of ethanol increased from 4% to 24%, cell number gradually drastically decreased.

Table 10. Growth of yeast isolates at different ethanol concentration in YM medium

Ethanol (%)	Mean count ($CFUx10^6$/mL) of yeast isolates		
	ACP12	ACE12	*S.cerevisiae*
4	96.0 ± 1.00^{a}	77.7 ± 1.53^{de}	89.0 ± 1.00^{b}
8	$87.0\pm1.00b^{c}$	64.0 ± 1.00^{gh}	77.0 ± 1.00^{de}
12	81.0 ± 1.00^{cd}	57.7 ± 1.53^{h}	72.0 ± 1.00^{ef}
16	78.0 ± 1.00^{de}	45.0 ± 2.00^{i}	68.0 ± 1.00^{fg}
20	24.0 ± 2.00^{j}	19.7 ± 6.03^{j}	24.0 ± 4.00^{j}
24	12.0 ± 2.00^{k}	9.0 ± 1.00^{k}	10.0 ± 1.00^{k}

The mean count of yeast isolates at different alcohol concentration, the growth of the isolates varied. Mean ± SD in the same column and row and letters are different and this indicate their growth were significantly different at $P < 0.05$.

4.2.3. Morphological, microscopic observations and identification of the yeast isolate ACP12 and ACE12

Appendix 4 shows the features of the appearance of cultures when cells grown in YEPD broth and on YEPD agar. After 72hrs of incubation at 30^0C, heavy, dry climbing pellicles were formed on the surface of YPED broth medium. The growth was smooth and white cream color on YPD agar (Table-11). The cell morphology of the ethanol tolerant, temperature, sugar and pH of ACP12 and ACE12 under compound microscope (appendix 4), are ovoidal circular to elongate have single, pairs, or triple budding cells

were present, filamentous form showing aberrant and elongated morphology distinct from pseudohyphae morphology were formed (Appendix 6). Besides of, ascospores were formed in ascospore forming medium after incubating at 30°C for 3 weeks also developed (Table 11). All the dominant yeasts isolated from the Arabica Coffee effluents and pulps did have round or oval shape or spherical or ellipsoidal (Table11). The screened isolates (ACP12, ACE12) and standard *S.cerevisiae* were reproduced asexually by budding (Appendix 5). The yeast isolates reproduced sexually by forming round ascospores in which their asci contained four ascospores (Table11). The isolated yeasts also showed a filamentous growth when they were inoculated to corn meal agar, nitrogen deficient medium (Appendix 6). The Arabica coffee effluent isolate (ACE12) and the pulp isolate yeast (ACP12) were compared with the standard yeast *S.cerevisiae*. Morphological characteristic of the isolates were summarized in (Table 11).

Table 11. Morphological characteristics of the two selected yeast isolates

Character	ACP12	ACE12	Standard *S.cerevisiae*
Surface	Smooth	Smooth	Smooth
Margin	Circular	Circular	Circular
Colour	Creamy, white	Creamy, white	Cream, white
Cells	Ellipsoidal/oval	Ellipsoidal/oval	spheroidal, ellipsoidal
	Single/Multilaterial budding	Single/Multilaterial budding	Single/Multilaterial budding
Ascospores	+	+	+
Filamentous	+	+	+

+= Formation of ascospores and filamentous growth

4. 3. Total sugar determination of coffee effluent and pulp

In this section, the results of the experiment carried out on coffee effluent and pulp for bioethanol production through distilled water hydrolysis of pulp and the amount of sugar formed was investigated. The sugar content of the coffee effluent and pulp hydrolysates is presented in (Figure 9) bellow.

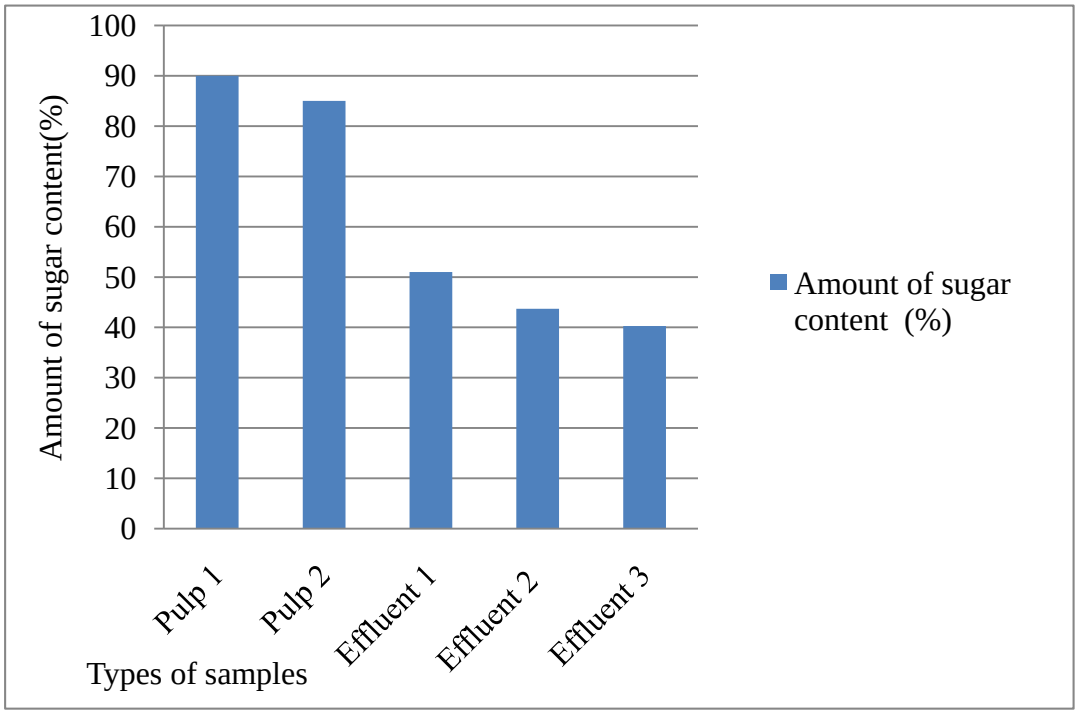


Figure 9. Total sugar content of Arabica coffee waste water effluents and pulps

The maximum reducing sugar concentration of 90% was produced from distilled water hydrolysate of coffee pulp1 followed by pulp 2 (85%), effluent1 (51%), effluent2 (43.71%) and effluent3 (40.26%).

4.4. Fermentation and bioethanol concentration

Ethanol production was laboratory scaled by batch fermentation system (appendix 7). After fermentation, bioethanol was filtered (appendix 8) and centrifuged for ethanol determination. Among all the strains, isolates ACP12, ACE12 and standard *S. cerevisiae* were found to be potential ethanol producers as they produced the highest amount(6.2%) of ethanol yield (Table12). The isolates showed different pattern of ethanol production of 4.5% (g/l) for standard *S.cerevisiae*, 6.20% (g/l) for isolate ACP12 and 5.01% (g/l) for isolate ACE12 which were produced from pulp1. (Table 12) showed that ethanol production from all substrate and standard sucrose with the yeast isolates and amount of ethanol presented.

Table 12. Comparison of ethanol production from Arabica coffee effluents, pulps and standard sucrose

Types of sample	Yeast isolates	Alcohol contents(g/l)
Standard sucrose	ACE12	4.0
	ACP12	5.8
	Saccharomyces cerevisiae	4.83
Pulp 1	ACE12	5.01
	ACP12	6.20
	Saccharomyces cerevisiae	5.49
Pulp 2	ACE12	4.14
	ACP12	5
	Saccharomyces cerevisiae	4.34

Types of sample	Yeast isolates	Alcohol contents(v/v)
	ACE12	2.1
Effluent 1	ACP12	2.5
	Saccharomyces cerevisiae	2.3
Effluent 2	ACE12	1.86
	ACP12	2.01
	Saccharomyces cerevisiae	1.98
Effluent 3	ACE12	0.96
	ACP12	1.23
	Saccharomyces cerevisiae	1.01

4.5. Biomass yield at the end of fermentation

The maximum cell density was recorded for ACP12 and ACE12 compared to standard *S. cerevisiae* in batch fermentation with initial sugar concentration of 20% standard sucrose, pulp 1 and pulp 2 as well as coffee effluents as indicated in Table 13.

Table13. Biomass of isolates after fermentation

Types of Samples	Isolates and standard isolates		
	Net weight (g/l)		
	ACP12	ACE12	Standard *S.cerevisiae*
Standard sucrose	2.43	1.25	2.30
Pulp 1	2.45	1.26	2.32
Pulp 2	2.23	1.18	2.21
Effluent 1	2.24	1.01	2.15
Effluent 2	1.98	1.01	1.76
Effluent 3	1.8	0.05	1.65

4.6. SUMMARY OF THE RESULTS

Microbial production of ethanol is a very popular concept in respect of alleviating energy demand nowadays. In this regard, two potential fermenting isolates of *Saccharomyces spss* were isolated for the production of ethanol from Arabica coffee effluent and pulp. They are characterized for alcoholic fermentation using biochemical, physiological and morphological with respect to different concentration and method for estimating percentage of ethanol was employed. The summary of optimum results obtained during this study is discussed here under (Table 14).

Table 14. Summary of the results

The findings and parameters of the results		Fermentative yeasts		
		ACP12	ACE12	Standard *S.cervisiae*
1.	Fermentation using Durham tube	Fermenting 10 sugars	Fermenting 10 sugars	Fermenting 7 sugars
2	Sugar Tolerance (20%)	216.0 ± 1.00^{a}	121 ± 1.00^{c}	153.0 ± 1.00^{b}
3	Temperature tolerance(30ºC)	97.0 ± 1.00^{a}	67.7 ± 1.54^{d}	87.0 ± 1.00^{b}
4	pH Tolerance (5)	98.0 ± 1.00^{a}	78.0 ± 1.00^{c}	87.0 ± 1.00^{b}
5	Ethanol tolerance (4%-16%)	78.0 ± 1.00^{de}	45.0 ± 2.00^{i}	68.0 ± 1.00^{fg}
6	% of Bioethanol production from pulp 1	6.20%	5.01	5.49
7	Biomass yield from pulp 1	1.26	2.45	2.32

5. DISCUSSION

A total of fifteen yeast isolates were isolated from five samples of Arabica coffee effluent 1, effluent 2, effluent 3, pulp 1 and pulp 2. Based on the colony characteristics (creamy and white texture), some colonies of pulps slightly red and pinkish, smooth surfaces with circular margins and ovoid microscopic shape. Based on the colony characteristics (white and creamy texture) ovoid microscope shape, the presence of ascospore, and budding pattern (multipolar), the selected isolate (ACP12 and ACE12) were found to belong *sacharomyces* type unicellular ascomycete according to (Lodder, 1971).

All the test isolates were capable of utilizing 6-10 sugars indicating their potential in utilization of diverse sugars. It is important to produce more ethanol. The isolates were tested for fermentation of carbohydrates and ACP12 and ACE12 were capable to ferment ten (10) sugars out of the eleven (11) sugars tested. Whereas, standard *S.cerevisiae* was successfully fermented 7 (seven) out eleven sugars. But the rate of fermentation varied in most of isolates because of the types of isolates, their potential difference, may be their species difference. Among the isolates, two of them (ACP12and ACE12) were relatively highly fermentative selected for further physiological and morphological characterization and to be used for ethanol production.

Keeping in view data obtained when glucose tolerance was compared among yeast isolates, it was observed that potential yeast isolates ACP12 and ACE12 compared to standard yeast *S.cerevisiae* could tolerate a maximum sugar concentration of 20% and decrease when the glucose concentrations were increased. Similarly, Osho (2005) has reported that the sugar tolerance of wine yeast (*S. cerevisiae)* were tolerated maximum of 20%.

The effect of temperature on growth of the selected yeast strain was showed that the maximum population was displayed at 30^0C whereas, beyond 30ºC the growth of isolated population and the standard strain were declined and at higher temperatures growth was inhibited (Subashini *et al*., 2011).

Similar results were obtained in this study. Yeast isolated from Arabica Coffee pulp (ACP12) showed the highest population at 30ºC, followed by the standard culture *S. cerevisiae* and the yeast isolate from Arabic Coffee effluent (ACE12) showed a minimum population at 30ºC.

The isolates and the standard strain recorded maximum population at pH5 and pH 5.5, while pH 5.5 yeast populations declined. Similarly Linden *et al*., (1992), have carried out fermentations, with *S. cerevisiae* at pH 4.5, 5.0, 5.5, 6, 7 and 8 and found that the optimal pH for ethanol production and maximum population of yeast were around 5 and 5.5 and correlate with this result that the maximum growth and population of the isolates between pH 5.0 and pH 5.5.

Sacchromyces yeasts are the most ethanol tolerant of the eukaryotic organisms, and able to tolerate over 20% ethanol. In a previous study by (Casey and Ingledew, 1986), yeast strain TGY2 could tolerate up to 16% (v/v) ethanol. Almost Similar ethanol tolerance of 16.5% (v/v) has been observed for *saccharomyces cerevisiae* by (Teramoto, *et al*., 2005). In this study, yeast isolated from Arabica Coffee pulp (ACP12) showed the highest population compared to (ACE12) and standard strain *S. cerevisiae* at 4% concentration and gradually decreased at higher concentrations. The isolate from coffee pulp recorded maximum tolerance up to 16% ethanol than the standard strain *S. cerevisiae*. However, the one which isolated from coffee effluent (ACE12) showed poor tolerance. From this finding, as the concentration of ethanol increase from 4% to 24% there were decline in population of the yeast isolates. The yeast strain isolated from coffee pulp (ACP12) recorded $78.0\pm1.00x10^{6}$CFU/mL at 16% of ethanol concentration. Similar result reported by, Subashini *et al*., (2011) have observed that, the yeast *S. cerevisiae* tolerated ethanol concentration of 15% and effectively utilized 97.5% available glucose in the medium with a population of $62x10^{6}$CFU/ml. There fore, from this study observed that, isolate ACP12 has a potential to tolerate ethanol.

Morphological and microscopic observations of identification and screening of the yeast isolates ACP12 and ACE12 were, based on their colony characteristics (white and creamy texture), ovoid microscope shape, filamentous growth pattern, the presence of four ascospore in ascus, and budding pattern (multipolar). All isolates were found to belong to *Saccharomyces* type unicellular ascomycete according to Lodder (1971); Boekhout and Kurtzman, 1996).

These results were consistent with the previous findings that yeast from teff dough and tella are saccharomyces type (BerhanuAbegazGashe *et al.*, 1982; Samuel Sahle and Birhanu Abegaz Gashe, 1991 and Tamene Milkessa, 2009). The isolates were grouped under the genus *Saccharomyces* depending up on their morphological and physiological characteristics.

The maximum reducing sugar concentration of 90% was produced from distilled water hydrolysate of coffee pulp1 when compared to the other substrate of coffee waste water effluent and pulp. The result showed that the amount of sugar obtained decreases from the fresh sample to the storage area pond. The decrease of sugar content does to the formation of organic and acetic acids from the fermentation of the sugars in the coffee effluent and pulp after the first fermentation of sugars by microbiological processes using oxygen from the water. This process causes problems as the demand for oxygen to break down organic material in the waste water exceeds the supply, dissolved in the water, thus creating anaerobic conditions resulted the death of anaerobic organisms and cause the environmental pollution (Nutawan *et al*., 2010).

Inoculum sizes, 3% was chosen to be the optimum inoculum by comparing production rate, maximum growth rate and produced ethanol. The highest production rate, growth rate, and ethanol produced were obtained for 3% inoculum size. The results demonstrated that there is an increase of ethanol yield up to 3%, however 5% inoculum causes a decrease the growth yeasts for ethanol fermentation by *S. cerevisiae*. Fadel (2000) has reported that ethanol production increases by inoculum up to 4%.

Furthermore, it was reported that 3% inoculum size was the optimum for ethanol production (Turhan *et al*., 2010). Therefore, 3% inoculum size was also suggested to be the optimum level for ethanol by this study.

The isolates showed different pattern of ethanol production of 6.20% (g/l) for isolate ACP12, 4.5% (g/l) for standard *S.cerevisiae*, and 5.01g/l for isolate ACE12 were produced from pulp1. There were different amount of ethanol produced from pulp 2, effluent 1, effluent 2, effluent 3 and standard sucrose by the three yeast isolates , and the maximum amount of ethanol produced by ACP12 isolate from pulp 1 substrate compared to the two isolates with the other samples. Similarly, Ayele Kefale (2011) confirmed the maximum bioethanol concentration of 7.4 g/l from Arabica Coffee pulp was at 4hrs hydrolysis time. However, as hydrolysis time increased from 4 h it resulted in decreasing concentration of bioethanol (Ayele Kefale, 2011). From this finding maximum amount of ethanol was produced from pulp 1 than pulp 2,effluent , effluent 2, effluent 3 and sucrose. This shows that, the substrate pulp1 the highest and easily available substrate used for ethanol production to reduce environmental pollution from around where wet Arabica coffee processing. From this finding, ACP12 isolate was found to be produced more ethanol and were selected for bioethanol production being and more tolerant to pH, temperature, glucose and ethanol yeast isolates ACP12 than the isolate ACE12 and standard *S.cerevisiae*. Mir Naiman Ali and Mohammed Mazharuddin Khan, (2014) have studied that the maximum ethanol was produced from 20% of glucose concentration at 72hrs is high but, the yield was not increased when the concentration of sugar increased.

Bekatorou *et al*. (2006) and Verstrepen *et al.* (2004) showed that high substrate concentration would lead to catabolic repression by glucose and sucrose, may leads to several problems, such as incomplete fermentation, development of off flavors and undesirable by products as well as decreased biomass and yeast vitality. Ekunsanmi and Odunfa, (1990) asserted that the combination of sugar ,temperature, alcohol and pH tolerance is an advantage when a yeast is being considered for industrial use especially when ethanol is being produced. From this study, ACP12 and ACE12 have fit the above criteria.

The produced ethanol, together with oil extracted from low quality (defective) coffee beans, could be employed as reactants for biodiesel production (Franca *et al*., 2008). At this point it is worthwhile to mention that the concentration of ethanol obtained by the hydrolysis of the coffee pulp using distilled water, which is about 6.2g/l of pulp1 by ACP12 was the maximum ethanol, was produced as compared to the other. The ethanol produced from pulp1 (6.2g/l) was satisfactory product compared to the maximum amount of ethanol obtained from the enzymatic fermentation of barley straw (10 g/L) (Belkacemi *et al*., 2002).

The maximum ethanol concentration obtained by the batch fermentation was 13.6 g/L from of acid hydrolysate of coffee husk using *S. cerevisiae* (Franca *et al*.,2008), 11 g/L formed from wheat stillage hydrolysate (Davis *et al*., 2005), 59 g/l from cassava starch hydrolysate, 16.8 g/L from Corn stover (Ohgrem *et al*., 2007) and 18.1 g/L from wheat straw and 16.2 g/L from sweet sorghum bagasse reported by (Ballesteros *et al*. 2004). The result is much higher than the maximum amount of ethanol from Corn stalks (5 g/L) (Belkacemi *et al.*, 2002).

The biomass of the isolates were determined at the end of fermentation. Accordingly, they showed differences in biomass accumulation. The biomass produced by the isolates were 2.45g/l (ACP12), 1.26g/l (ACE12) and 2.32g/l (standard *S.cerevisiae*), from pulp 1 substrate and the biomass accumulation of isolate ACP12 was the highest when compared to the two isolates were applied on the other samples. This shows that biomass accumulation was directly proportional to the ethanol yield. In the conversion of carbohydrates to ethanol were yeasts are involved, optimal conversion requires cells that are tolerant to high concentration of both substrate and product and are able to efficiently produce ethanol (Walker *et al.*,2006).

7. CONCLUSION

- The colour of the pure isolates colonies of effluents and pulps were showed creamy white and some colonies of pulps slightly red and pinkish
- The cells were found to be of various shapes such as round; oval, spherical and ellipsoidal.
- The candidate yeast strain, labled ACP12 and ACE12 was found tolerant to at different level of sugar and ethanol concentration, temperature and pH revealed that, yeast isolated from pulp (ACP12) recorded maximum population than effluent(ACE12) and less population compared with standard *S. cerevisiae*.
- Potentially fermentative isolates of yeasts were found capable of fermenting up to ten(10) types of carbohydrates
- Yeast isolates of various morphological and physiological properties were identified , and they resemble more of *Saccharomyces spps*
- Coffee pulp and effluent are potential candidate and are promising lignocellulosic feed stocks for bioethanol production.
- The result of this study indicated that being available in plentiful amounts and non-edible material, coffee pulp and effluent will be potential feedstock for bioethanol production in Ethiopia.
- Based on these facts, data compared to food crops, coffee pulp which is an agricultural waste is a promising alternative feedstock for bioethanol production.

7. RECOMMENDATIONS

Based on the current investigation the following recommendations are forwarded:

- Evaluation of the chemical composition of coffee pulp and effluent extensive research should be undertaken.
- The reduction of the environmental impacts arising from dumping of the Arabica coffee waste water directly to the nearby rivers more research should be done to contribute the solution of fossil fuel replacement in Ethiopia.
- More work should be undertaken to optimize the production of bioethanol using coffee pulp and effluent at appropriate pH, temperature and substrate concentration.
- Further investigation should be done to analyze the potential of bioethanol production from coffee pulp and effluent using genetically modified yeasts isolated from different sources.
- An economic feasibility analysis of the overall conversion process from coffee pulp to ethanol is necessary for the purpose of commercialization.
- Considering the remarkable potential of ethanol that can be produced from coffee effluent and pulp further improvement is still needed for maximum results especially in the fermentation processes and by yeast specified species

REFERENCES

Alemayehu Teshome, Esayas Kebede and Kassu Kebede .2007. *Coffee development and marketing improvement plan in Ethiopia.* Proceeding of national workshop four decades of coffee research and development in Ethiopia, Addis Ababa. 375-387.

Alexopoulos, C.J, Mims, C.W and Blackwell, M. 1996. *Introductory Mycology*. 4th ed. United States of America. 280.

Altintas, M., Ulgen, K., Kirdar, B., Onsan, Z. and Oliver, S. 2002. Improvement of ethanol production from starch by recombinant yeast through manipulation of environmental factors. *Enzyme Microb. Technol.* **31**: 640-647.

Ayele Kefale. 2011. Bioethanol Production and Optimization test from Agricultural Waste: The case of wet coffee processing waste (pulp). M.Sc. Thesis. AAU.

Ballesteros, M., Oliva, J., Negro, M. and Manzanares, P. 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromycesmarxianus CECT 10875.*Process Biochem*. **39**:843-848.

Bang-Quan, H., Jian-Xin, W., Shi-Jin, S., and Hong, H. 2003. The effect of ethanol blended diesel fuels on emissions from a diesel engine. *Atmosph. Environ.* **37**: 4965-4971.

Bansal, R. and Singh, R. 2003. A comparative study on ethanol production from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis. Indian J. Microbiol.* **43**: 261-64.

Barnett, J.A, Payne, R.W, Yarrow, D. 2000. *Yeasts: Characteristics and identification*. 3rd edn, Cambridge University Press, UK.

Bass, D., Howe, A., Brown, N., Barton, H., Demidova, M., Michelle, H., Li, L., Sanders, H., Watkinson, SC., Willcock, S., Richards, TA. 2007. Yeast forms dominate fungal diversity in the deep oceans. Proceedings. *Biological Sciences*/The Royal Society. **274** (1629**)**: 3069–3077.

Beall, D., Bassat, A., Doran, J., Fowler, D., Hall, R. and Wood, B.1992. Conversion of hydrolysate of corn cobs and hulls into ethanol by recombinant *E.coli* B containing integrated genes for ethanol production. *Biotechnol.* **14**: 857.

Bekatorou, A., Psarianos, C. and Koutinas, A.A. 2006. Production of food grade yeasts. *Food Technol. Biotechnol*. **44**: 407- 415.

Belkacemi, K., Turcotte, G. and Savoite, P. 2002. Aqueous/steam fractionated agricultural residues as substrates for ethanol production. *Res. Ind. Eng. Chem*. **41**: 173–179.

Bertolini, M., Ernandes, J. and Laluce, C. 1991. New yeast strains for alcoholic fermentation at higher sugar concentration. *Biotechnol. Lett.* **13**: 197-202.

Bilanski ,W. K. and S. R. Ghate. 1978. Determining moisture content of alfalfa using a microwave Oven. School of Engineering, University of Guelph.21.

Birhanu Abegaz Gashe, Meaza Girma and Abrham Bisrat .1982. The role of microorganisms in fermentation and their effect on the nitrogen content of teff. SINET:*Ethiop.J. Sci*. **5**:69-76.

Boekhout, T. and Kurtzman, C.P. 1996. Principles and methods used in Yeast classification, and over view of currently accepted yeast genera. **In**: *Non conventional yeasts in bio technology*. A Hand book; SpringerVelag, Berlin, Heidelberg (Wolf). 1- 99.

Bocass, F., Roussos, S., Cutierrez M., Serrano, L. and Viniegra-Godez, G. 1994. Production of pectinase from coffee pulp in solid state fermentation system: Selection of wild fungal isolate of high potency by a three-step screening technique. *J. Food Sci. Technol.* **1**: 22-26.

Borzani, W., Garab, A., Pires, M., Piplovic, R. and De Ia Higuera, G. 1993. Batch ethanol fermentation of molasses: a correlation between the time necessary to complete the fermentation and the initial concentrations of sugar and yeast cells. *World J. Microbiol. Biotechnol.* **9**: 265-68.

Bulawayo, B., Brochora, J., Munzondo, M. and Zvauya, R.1996. Ethanol production by fermentation of sweet sorghum juice using various yeast strains. *World J. Microbiol. Biotechnol.* **12**: 357-60.

Bjerre, A., Olesen, A., Fernqvist, T., Ploger, A., and Schmidt, A. 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Bioengineer.* **49**: 568-577.

Bressani, R. 1979. By-products of coffee berries. **In**: *Coffee Pulp: Composition, Technology and Utilization*. 5-24

Brigham, J., Adney, W. and Himmel, M. 1996. Hemicelluloses: Diversity and applications. **In**: *Handbook on bioethanol: production and utilization*. Taylor and Francis, Washington. 119- 142.

Campelo, A.F. and Belo, I. 2004. Fermentative capacity of baker's yeast exposed to hyperbaric Stress. *Biotechnol. Lett*. 1237-1240.

Cardona, C. and Sanchez, O. 2007. Fuel ethanol production: process design trends and integration opportunities. *Biores. Technol*. **98**: 2415–2457.

Casey, G.P. and Ingledew, W.M. 1986 .Ethanol tolerance in yeasts. CRC .*Crit. Rev. Microbiol*. **13**: 219-280.

Coughlan, M. 1992. Enzymatic hydrolysis of cellulose. *Biores. Technol.* **39**: 107-115.

Conti, S.F. and Naylor, H.B.1959. Electron microscopy of ultrathin section of Schizosaccharomyces octosporus. A cell division. *J. Bacteriol*. **78:** 868-87.

Chandel, A., Rudravaram, R., Narasu, M., Rao, L. and Ravindra, P. 2007. Economics and environmental impact of bioethanol production technologies: an appraisal. *Biotechnol. Molec. Biol. Rev*. **2**: 14–32

Dabas, R., Verma, V. and Chauhary, K. 1997. Ethanol production from wheat starch. *Indian J. Microbiol.* **37***:* 49-50.

Daniels, N. 2009. *Variations in Coffee Processing and Their Impact on Quality and Consistency.* M.sc. Thesis, Michigan Technological University. 10-12.

Davis, L., Jeon, Y. and Svenson, C. 2005. Evaluation of wheat stillage for ethanol production by recombinant Zymomonas mobilis. *Biomass Bioenerg.* **29**: 49–59.

Deepa, G.B., Chanakya, H.N., de Alwis, A.A.P., Manjunath, G.R., Devi, V. 2002. "Overcoming Pollution of Lakes and Water Bodies Due to Coffee Pulping Activities with Appropriate Technology Solutions". *Proceedings "Symposium on Conservation, Restoration and Management of Aquatic Ecosystems"*. Canada: Centre for Ecological Sciences, Indian Institute of Science (IIS) and the Karnataka Environment Research Foundation (KERF), Bangalore and Common wealth of Learning.

De Matos, T, A., Lo Monaco, P.A., Pinto, A.B., Fia, R., Fukunaga, D.C. 2001. *Pollutant Potential of Wastewater of the Coffee Fruits Processing*. Viçosa-MG, Brazil: Federal University of Viçosa, Department of Agricultural Engineering.

Demirbas, A.2004.Ethanol from cellulosic biomass resources. *Int.J.Green Ener*.**1**: 79-87.

Demirbas, F., Bozbas, K. and Balat, M. 2004. Carbon dioxide emission trends and environmental problems in Turkey. *Energy Explor. Exploit*. **22**: 355–365.

Demirbas, A. 2005. Bioethanol from Cellulosic Materials: A Renewable Motor Fuel from Biomass. *Energy Sources.* **27**: 327-337.

Demirbas ,M.F .2006. Global renewable Energy Resources. Energy Sources.**28:** 779-792.

Demirbas, A. 2007. Progress and recent trends in biofuels. *Prog. Energy Combus. Sci*. **33**:1-18

Dunn, C. 1959. *Industrial Microbiology*. Third edition, London. 763.

Dutch Sustainable Development Group (DSD). 2005. Feasibility study on an effective and sustainable bio-ethanol production program by Least Developed Countries as alternative to cane sugar export, DSD group; study report on bio-ethanol production in LDC0s, the Netherlands.

European Biomass Industry Association (EUBIA) .2007. Biofuels for transportation, European biomass industry association, renewable energy house.

Ekunsanmi, T.J. and Odunfa, S.A. 1990. Ethanol tolerance, sugar tolerance and Invertase activities of some yeasts strains isolated from steep water of fermenting Cassava tubers. *J. Appl. Bacteriol*. **69:** 672-675.

El- Diwany, A., El-Abyad, M., El- Rafai, A., Sallam, L. and Allam, R. 1992. Effect of some fermentation parameters on ethanol production from beet molasses by *S. cerevisiae* Y-7. *Biores. Techno.* **42**: 191.

Ethiopian Sugar Corporation (ESC). 2012. Ethiopian sugar factories produced more than 11 million liters of ethanol. Ethiopia, Addis Ababa.

Fadel, M. 2000. Alcohol production from potato industry starchy waste. *J. Microbiol*. Egypt. **35**: 273–287.

Fan, L., Pandey, A., Mohan, R. and Soccol, C .R. 2000. Use of various coffee industry residues for the cultivation of *Pleurotus ostreatus* in solid state fermentation. *Biotechnol.* **20:** 41-52

Foody, B. 1988. *Ethanol from Biomass: The Factors Affecting Its Commercial Feasibility*.Iogen Corporation, Ottawa, Ontario.

Franca, A., Gouvea, B., Torres, C., Oliveira, L. and Oliveira, E. 2008. Feasibility of ethanol production from coffee husks. *J. Biotechnol.* **136**: 269–275.

Gaur, K. 2006. *Process optimization for the production of ethanol via fermentation*. Master thesis, Thapar Institute of Engineering and Technology, Patiala.

Gathuo, B., Rantala, P. and R. Maatta. 1991. Coffee Industry Wastes. *Water Science Technology*. **24(**1): 53-60.

Glazer, A.N. and Nikido, H.1995. Microbial diversity. **In***: Microbial Biotechnology:* Fundamental of Applied Microbiology. New York: Freeman and Company.76-87.

Gray, W.D. 1941. Studies on the alcohol tolerance of yeasts. *J. Bacteriol*. **42**: 561-574.

GTZ-PPP. 2002. Post harvest processing: Limit environmental damage by basic knowledge of coffee wastewater.

Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M., Liden, G. and Zacchi, G. 2006. Bioethanol the fuel of tomorrow from the residues of today. *Tre. Biotechnol.* **24**: 549-556.

Hamelinck, C., van Hooijdonk, G. and Faaij, A. (2003). *Prospects for ethanol from lignocellulosic biomass: techno-economic performance as development progresses*, Scientific report- NWS-E-2003-55. Utrecht University, Utrecht, the Netherlands: Copernicus Institute, Department of Science, Technology and Society.35.

Hamelinck, C., van Hooijdonk, G. and Faaij, A. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass Bioenergy*. **28**: 384– 410.

Harris, J., Baker, A. and Zerbe, J. 1984. Two stages dilute sulfuric acid hydrolysis of hardwood for ethanol production. *Energy Biomass Wastes.* **8**: 1151-1170.

Hettenhaus, J. 1998. Ethanol fermentation strains: present and future requirements for biomass to ethanol commercialization. USA, United States Department of Energy and National Renewable Enery Laboratory.

Ingledew,W.M.1993. Yeasts for the production of fuel ethanol. **In**: *The Yeasts*. (Rose, A. M. and Harrison, J.S, eds). London: Academic Press.**5**: 245-291.

Ingram, L.O. and Buttke, T.M. 1984. Effects of alcohol on microorganisms. *Adv. Microb. physiol*. **25**: 253-300.

IPMS, 2007. Goma pilot learning Wereda diagnosis and programming design. Improvement productivity and market Success. 85

Jain, N. C. and R. H. Cravey.1972. Analysis of alcohol. A review of gas chromatographic Methods. *Chrom. Sci*. 10:253-67.

Jacobson, G.K. and Jolly, S.O. 1989. Yeasts, Molds and Algae. *Biotechnol*.**7**: 279-314.

Janse, B. J. and I. S. Pretorius. 1995. One-step Enzymatic Hydrolysis of Starch Using a Recombinant Strain of Saccharomyces Cerevisiae Producing Alpha-Amylase, Glucoamylases and Pullulanase. *Appl. Microbiol. Biotechnol*. **42**:878–883.

Jeffries, T. and Jin, Y. 2000. Ethanol and thermo tolerance in the bioconversion of xylose by yeasts. *Adv. Appl. Microbiol*. **47**:221–268.

Jimenez, J. and Benitez, T. 1986. Characterization of wine ethanol for ethanol production. *Appl. Microbiol. Biotechnol*. **25**: 150-154.

Jime'nez, J and Beni'tez, T. 1988. Yeast cell viability under conditions of high temperature and ethanol concentration depends on mitochondria genome. *Curr. Genet*. **13**: 461-469.

Jones, R., Pamment, N. and Greenfield, P. 1981. Alcohol fermentation by yeasts- the effect of environmental and other variables. *Proc. Biochem.* **16**: 42-49.

Kadiman, O. 2005. *Crops: beyond foods*. In proceedings of the 1st international conference of crop security, Malang.

Katahira, S., Mizuike, A., Fukuda, H. and Kondo, A. 2006. Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose and cellooligosaccharide assimilating yeast strain. *Appl. Microbiol. Biotechnol*. **72**: 1136–1143.

Kebede Dawit. 2001. *Charcoal Production Efficiency Improvement and Supply Enhancement Options*. Ethiopian Rural Energy Development and Promotion Centre, Ethiopia.

Kim, S. and Dale, B. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioenergy.* **26**: 361–375.

Kirk, T., Connors, W. and Zeikus, J. 1977. Advances in understanding the microbiological degradation of lignin. **In**: *The structure, biosynthesis and degradation of wood.* 369–394. New York.

Kosaric, B. and Torget, R. 1993. An optimal temperature policy of percolation process as applied to dilute acid hydrolysis of biphasic hemicelluloses. *Appl. Biochem. Biotechnol*. **39**: 119- 129.

Kurtzman, C.P and Fell, J.W.1998. The Yeasts, a Taxonomic Study, 4th edn. Amsterdam: Elsevier.

Kurtzman, C.P. and Fell, J.W. 2005. Biodiversity and Ecophysiology of Yeasts. The Yeast Handbook. 11–30.

Kurtzman, C.P. and Piskur, J. 2006. Taxonomy and phylogenetic diversity among the yeasts. In: Comparative Genomics Using Fungi as Models. 29–46.

Kwiatkowski, J., McAloon, A., Taylor, F. and Johnston, D. 2006. Modeling the process and costs of fuel ethanol production by the corn dry grind process. *Ind. Crops Products.* **23**: 288–296.

Lachenmeier, D.W, Burri, P.A, Fauser, T, Frank, W, Walch, S.G. 2005. Rapid determination of alcoholic strength of egg liqueur using steam distillation and oscillation-type densimetry with peristaltic pumping. *Anal Chim Acta*. **537**:377-384.

Linoj, N., Kumar, V., Dhavala, P., Goswami, A. and Maithel, S. 2006. Liquid biofuels in South Asia: resources and technologies. *Asian Biotechnol. Develop. Rev*. **8**: 31–49.

Linden, T., J. Peetre and B.H. Hagerdal. 1992. Isolation and characterization of acetic acid tolerant galactose fermenting strains of Saccharomyces cerevisiae from a spent liquor fermentation plant. *Appl. Environ. Microbiol*. **58**: 1661-1669.

Lodder, J. 1971. The Yeasts. A taxonomic Study. North Holland Pub. Co. Amsterdam.

Lombardi, L.2003. Life cycle assessment comparison of technical solutions for CO2 emissions reduction in power generation. *Energy Convers. Manag*. **4**: 93–108.

Lynd, L., Lyford, K., South, C., van Walsum, P. and Levenson, K. 2001. Evaluation of paper sludge for amenability to enzymatic hydrolysis and conversion to ethanol. *TAPPI J*. **84:** 50-55.

McMillan, J. 1994. Pre-Treatment of Lignocelluloses Biomass. *Conversion of hemicellulose hydrolizates to ethanol*. American Chemical Society Symposium. Washington, DC. 292-324.

Malca, J. and Freire, F. 2006. Renewability and life-cycle energy efficiency of bioethanol and bio-ethyl tertiary butyl ether (bio ETBE): assessing the implications of allocation. *Energy.* **31**: 3362–3380 .

Miller, J.J. 1989. Sporulation in Saccharomyces cerevisiae. **In**: *The Yeast*. (Rose, A.H. and Harrison, J.S). London: Academic Press. **3**: 491-550.

Ministry of Finance and Economic Development (MoFED). 2010. *Ethiopia: Country Report on the Implementation of the Brussels Program of Action (BPOA)*. Addis Ababa.

Mir Naiman Ali and Mohammed Mazharuddin Khan. 2014. Screening, identification and characterization of alcohol tolerant potential bioethanol producing yeasts.

Morimura, S., Ling, Z. and Kida, K. 1997. Ethanol production by repeated batch fermentation at high temperature in a molasses medium containing a high concentration of total sugar by thermo tolerant flocculating yeast with improved salt tolerance. *J. Ferment. Bioeng.* **83**: 271-274.

Mutua, J. 2000. Post harvest handling and processing of coffee in African countries. Rome, Italy

Negusu Tefera .2009. *Potential of Prosopis Juliflora for Bioethanol Production*. M. SC Thesis, AAU, Center of environmental science, Ethiopia.

Norr, A.A., Hameed, A., Bhatti, K.P., and Tunio, S.A. 2003. Bio-ethanol fermentation by the bioconversion of sugar from dates by *Saccharomyces cerevisiae* strain ASN-3 and HA-*Biotechnol.* **2(1):** 8-17.

Nutawan, Y., Phattayawadee, P., Pattranit, T. and Mohammad, N. 2010. Bioethanol Production from Rice Straw. *Energy Research J.* **1**: 26-31.

Nwachukwu, I .Ewelike, C. Ibekwe, V. 2006. Isolation and screening of local yeast strains for attributes important for ethanol production. Journal of Raw Materials Research; **3(1):** 55 – 62.

Ohgrem, K. Bura, R. Lesnicki, G. 2007. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam pretreated corn stover. Process *Biochem*. **42**:834–839.

Olfert, R. and Weseen, S. 2007. Assessing the Viability of an Ethanol Industry in Saskatchewan. *The Saskatchewan Institute of Public Policy*: Paper 48.

Oliveria, M., Vaughan, B. and Rykiel, J. 2005. Ethanol as fuel: energy, carbon dioxide balances and ecological foot print. *Bio. Science.* **55**: 593–602.

Onuki, S. 2005. Bioethanol Industrial production process and recent studies. USA.

Osho, A. 2005. Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. African Journal of Biotechnology. **4 (7)**: 660-662.

Ostergaard, S., Olsson, L., Nielsen, J. 2000. Metabolic engineering of *S. cerevisiae*. *Microbiology and Molecular Biology Reviews*. 64: 34–50.

Othman, A., Othaman, M., Abdulrahim, A. and Bapar, S. 1992. Cocoa, Pineapples, Sugarcane Waste for ethanol production. *Planter*. ***68***: 125.

Panchal, C.J., Harbison, A.Russell, I., and Stewart, G.G.1981. Ethanol production by Yeast strain. Dev. *Indust.Microbiol*. London: Academic Press.**5**. 245-291.

Periyasamy, S., Venkatachalams, S., Ramasamy, S. and Srinivasan, V. 2009. Production of Bioethanol from Sugar Molasses Using *S. cerevisiae. Modern Appl.Science.* **3**: 32-36.

Petit, N. 2007. Ethiopia's Coffee Sector: A Bitter or Better Future. *J. of Agrarian.* **7:** 225–263.

Pongsawatmanit, R., Temsiripong, T. and Suwonsichon, T. 2007. Thermal and rheological properties of tapioca starch and xyloglucan mixtures in the presence of sucrose. *Food Res. Int*. **40**: 239–248.

Prave, P., Faust, U., Sittig, W. and Sukatsch, D.A. 1987. Fundamentals of biotechnology.

Recek, M., Cadez, N., and Raspor, P. 2002. Identification and characterization of yeast isolates from pharmaceutical waste water. Food *Technol. Biotechnol*. **40(1):** 79-84.

Ribereau-Gayon, P., Dubordieu, D., Doneche, B., and Lonvaud, A. 2000. Handbook of Enology: The Microbiology of Wine and Vinifications. JohnWiley and Sons, Chichester. **1**.454.

Roehr, M. 2001. The Biotechnology of Ethanol: Classical and Future Applications. Chichester: Wiley-VCH. 232.

Samarajeewa, U. and Tissera, M.P. 1975. Use of Ebulliometer for alcohol determination in coconut toddy. *Ceylon Cocon*. **26**, 86-88.

Samuel Sahle and Birhanu Abegaz Gashe. 1991. Microbiology of tella fermentation SINET: Ethiop. *J.sci*. **14**: 81- 92.

Silva, C., Castro-Gomez, R., Abercio-da-Silva, C. and Gomez, R.1995. Study of the fermentation process using milk whey and the yeast *Kluyveromyces fragilis. Semina Londrina*. **16***:* 17-21.

Sofer, S. and Zaborsky, O. 1981. *Biomass conversion process for Energy and Fuels.* Third Eddition, Washington DC. 637.

Subashini, D., J. Ejilane, A. Radha, M.A. Jayasri and K. 2011. Suthindhiran School of Biosciences and Technology, VIT University, Tamil Nadu, India

Subramanian, K., Singal, S., Saxena, M. and Singhal, S. 2005. Utilization of liquid biofuels in automotive diesel engines: An Indian perspective. *Biomass Bioenergy.* **29**: 65-72.

Sun, Y. and Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Biores. Technol.* **83**: 1-11.

Scheffers, W.A. 1987. Alcoholic fermentation, Studies in Mycology. **30**: 321-332.

Spencer, G.F.T.and Spencer, D .M.1997. Yeast in natural and Artificial Habitats. Berlin: Springer-Verlang.

Sree, N., Sridhar, M., Suresh, K., Bharat, I. and Rao, L. 2000. High alcohol production by repeated batch fermentation using immobilized osmotolerant *S. cerevisiae. J. Indust. Microbiol. Biotechnol.* **24**: 222-226.

Taherzadeh, M. and Karimi, K. 2007. Acid- based hydrolysis processes for ethanol from lignocellulosic materials: A Review. *Bio Res.* **2**: 472-499.

Tamene Milkessa. 2009. Evaluation of yeast biomass production using molasses and supplements. M.Sc. Thesis. AAU.

Teramoto, Y. Sato, R. and Ueda, S .2005. Characteristics of fermentation yeast isolated from traditional Ethiopian honey wine, *ogol. Afr .J. Biotechnol*. **4 (2)**:160-163.

Turhan, I., Demirci, A.; Karhan, M. 2010. Ethanol production from carob extract by Saccharomyces Cerevisiae. Bioresource *Technol*. **101**: 5290–5296.

Thuesombat, P., Thanonkeo, P., Laopaiboon, L., Laopaiboon, P., Yunchalard, S., Kaewkannetra, Uma, V. and Polasa, H. 1990. *S. cerevisiae* of palm wine enhanced ethanol production by using mutagens. *J. Indust. Microbiol. Biotechnol.* **5**: 1-4.

United Nations Conference on Trade and Development (UNCTAD) .2006. Challenges and opportunities for developing countries in producing biofuels. UNCTAD publication, UNCTAD/DITC/COM/2006/15, Geneva.

Urbaneja, G., Ferrere, J., Paeza, G., Arenas, L. and Colina, G. 1996. Acid hydrolysis and carbohydrates characterization of coffee pulp. *Renew. Energy*. **9**: 1041-1044.

Van der Walt, J. P.1971. Criteria and methods used in classification. In: The Yeasts. A Taxonomic Study. North Holland *Pub. Co. Amsterdam*. 34-113

Verstrepen,K.J.,Iserentant,D.,Malcops,P.,Derdelinckkx,G.,Dijk,P.V.,Winderickx,J.,Pretorius, I.S., Johan , M.,Thevelein,J.T. and Delvaux,F.R. 2004. Glucose and sucrose: hazardous fast-food for industrial yeast. *Review: Trends in Biotechnol*. **22**: 531-537

Waker,G.M.1998 . *Yeast Physiology and Biochemistry*. John Wiley and Sons Ltd, London

Walker, L.P, Hii, H. Wilson, D.B.2006. Enzymatic hydrolysis of cellulose: An Overview. *Biores. Technol.* **36**:3-14.

Wayman, M. and Parekh, S.R. 1990 .Microbiology of fermentation catalysts. **In**: *Biotechnology of Biomass Conversion*. Open University press.75-100.

Warren, P. and shadomy, L.1991. Yeast fermentation broth base with carbohydrate and Durham Tube. **In:** *Manual of Clinical Microbiology*.5th ed. Washington D.C

Wildenborg, T. and Lokhorst, A. 2005. Introduction on CO2 Geological storage-classification of storage options. *Oil Gas Sci. Technol. Rev.* **60**: 513–515.

Wiselogel, A., Tyson, S. and Johnson, D. 1996. Biomass feedstock resources and composition. *Handbook on bioethanol: production and utilization.*105-118.

Yishak Seboka, Mulugeta Adamu and Yared Hailemeskel. 2009. *Biomass Energy for cement production: Opportunities in Ethiopia*. United Nations Development Program, New York, New York, 10017, USA.

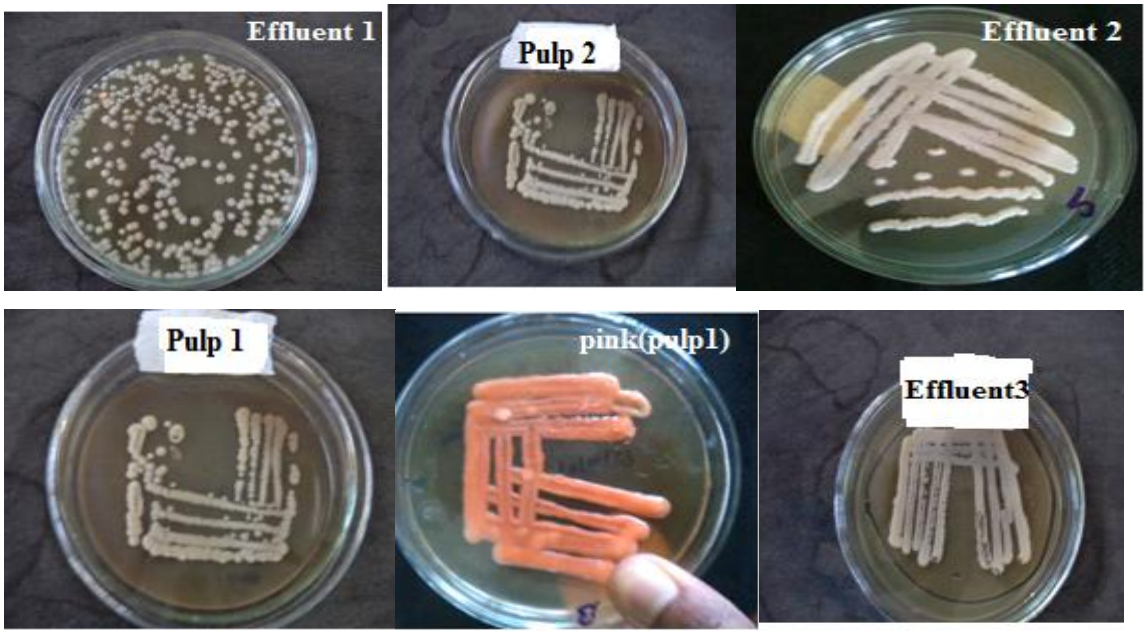
Yoosin, S. and Sorapipatana, C. 2007. A Study of ethanol production cost for gasoline substitution in Thailand and its competitiveness. *Thammasat. Int. J. Sci. Technol*. **12**: 69–80.

APPENDIXS

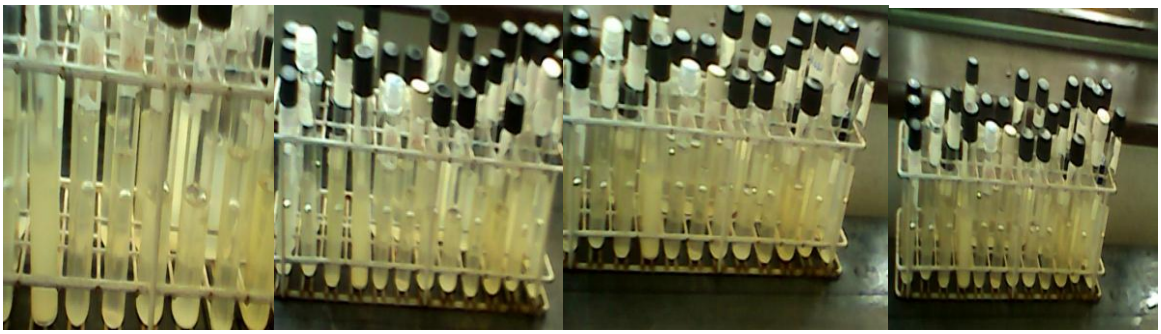
Appendix 1 Colony characteristics of yeast isolates

	Characteristics			
Isolates/strain	Surface	Margin	Colour	Cells
ACE11	Smooth	Irregular	Creamy white	Round/Oval
ACE12	Smooth	Circular	Creamy white	Round/Oval
ACE13	Rough	Irregular	Creamy white	Ellipsoidal
ACE21	Smooth	Circular	Creamy white	Spherical/Oval
ACE22	Smooth	Circular	Creamy white	Ellipsoidal
ACE23	Smooth	Irregular	Creamy white	Spherical/Oval
ACE31	Smooth	Irregular	Creamy white	Round/Oval
ACE32	Smooth	Circular	Creamy white	Round
ACE33	Smooth	Circular	white	Oval
ACP11	Smooth	Irregular	white	Round/Oval
ACP12	Smooth	Circular	Creamy white	Round/Oval
ACP13	Rough	Circular	Slightly red /Pinkish	Round/Oval
ACP21	Smooth	Circular	Creamy white	Round/Oval
ACP22	Rough	Circular	Creamy white	Ellipsoidal
ACP23	Smooth	Circular	Pinkish	Round/Oval
Saccharomyces cerevisiae	Smooth	Circular	Cream, white	spheroidal, ellipsoidal

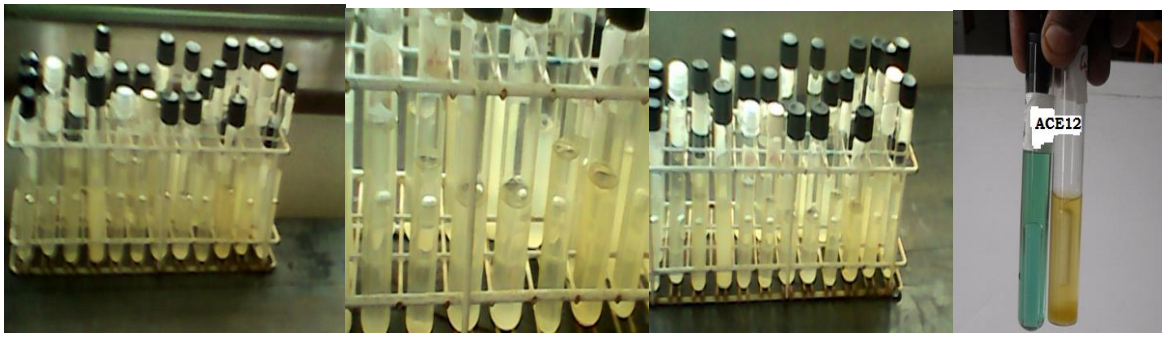
Appendix 2. Pure isolates of the five samples



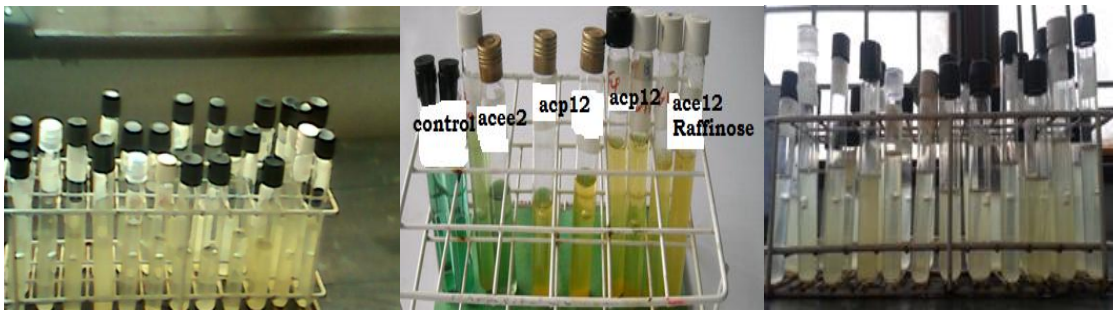
Appendix3. Carbohydrate fermentation test for characterizing of ACE12,ACP12 and *S.cerevisiae* *S.cerevisiae*



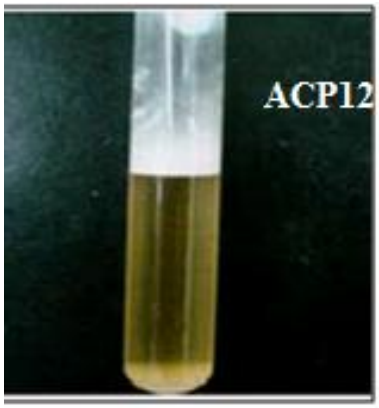
ACE12



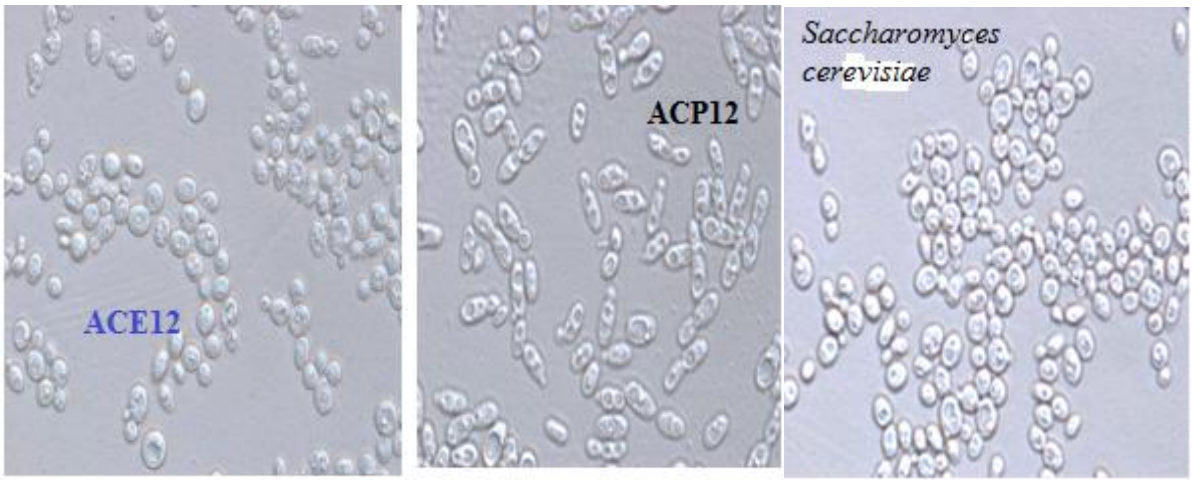
ACP12 and ACE12



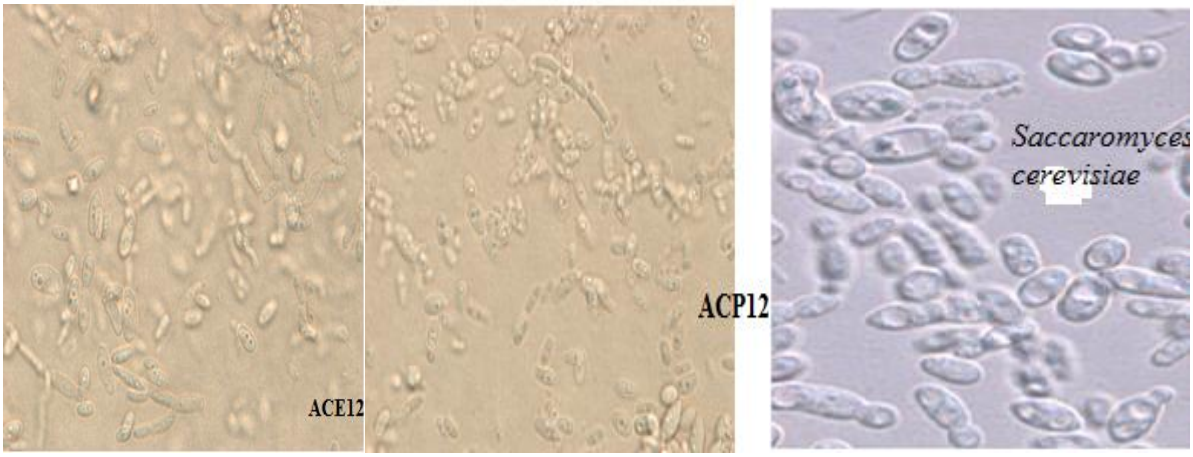
Appendix 4. Growth of isolates in YEPD broth



Appendix 5. Asexual reproduction of yeast isolated and standard *S.cerevisiae*



Appendix 6. Filamentous growth of yeast isolates and *S. cerevisiae*



Appendix 7 Laboratory scale ethanol production by batch fermentation system

Appendix 8. Filtration of ethanol after fermentation