KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
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EVALUATION OF ETHANOL PRODUCTION FROM PITO MASH USING *Zymomonas mobilis* AND *Saccharomyces cerevisiae*

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CHARLES OFOSU APPIAH

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DECLARATION AND CERTIFICATION

This is to certify that the thesis entitled "Evaluation of Ethanol Production from Pito mash using *Zymomonas mobilis* and *Saccharomyces cerevisiae*" submitted by Charles Ofosu-Appiah in partial fulfillment of the requirements for the award of Degree of Master of Science in Biotechnology to faculty of Bioscience, Kwame Nkrumah University of Science and Technology consists entirely of my own work produced from research undertaken towards the award of a Master of science in Biotechnology and that to the best of my knowledge, it contains no material previously published by another person; nor has it been presented for another degree elsewhere, except for the permissible excepts/references from other sources, which have been duly acknowledged.

Ofosu-Appiah Charles …….. ……

(Student) Signature Date

Certified by:

Dr. H. D. Zakpaa …… …….

(Supervisor) Signature Date

Mr. E. E Mak-Mensah …… ……..

(Supervisor) Signature Date

Prof Ibok Oduro …….. ……..

(Head of Department) Signature Date

DEDICATION

This work is dedicated to my mother-----Madam Elizabeth Asare and my wife Rhoda (Acquah) Ofosu, who, through unlettered, struggled to bring me this far.

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ABSTRACT

This study investigated bioethanol production by means of fermentation using *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from freshly tapped palm wine. *Pito* mash (waste from sorghum brewing with no appreciable value to industries or competitive use as food) was examined as alternative and cost-effective feed stock for the production of bioethanol. The parameters of ethanol fermentation, such as initial pH of the fermentation medium, temperature and period of fermentation were studied. *Saccharomyces cerevisiae* was tentatively identified using their morphological characteristics and by determining their pattern of fermentation and assimilation of glucose, sucrose, maltose, lactose. On the other hand, *Zymomonas mobilis* was identified using API^{TM} test kit. Analysis of reducing sugar residue was performed using Dinitrosalisilic acid (DNS) method, while analysis of ethanol content was performed using gas chromatography. *Pito* mash recorded total starch content of 6.69 %, reducing sugar content of 11.1 mg ml^{-1} and cellulose content of 0.41 mg g^{-1}. Saccharifficatio n by malting produced reducing sugar content of 19.75 mg ml^{-1}. The results revealed that an initial pH of 6.0, temperature of 30^{o}C and fermentation time of four days were the optimum conditions for *S. cerevisiae* while the optimum condition for *Z. mobilis* were pH of 5.5, temperature of 35^{o}C and fermentation time of 3days. The maximum ethanol yield of 3.03 g l^{-1} and efficiency of 62% was obtained for *S. cerevisiae* while yield of 3.63g l^{-1} and efficiency of 74.2% was obtained for *Z. mobilis*. *Z. mobilis* may be better organism for ethanol production from *pito* mash.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Energy consumption has increased steadily over the last century as the world's population increases, and more and more countries become industrialized. The traditional source of fuel, fossil fuel is continuously being depleted irrespective of the new geographical discoveries. Campbell and Laherrere (1998) estimated the current known oil reserve as well as the reserves yet to be discovered and concluded that the world crude oil production will begin to decline in 2010. There have also been concerns about the pollution and various health risks associated with the use of petroleum as fuel. In view of these, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of the limited fossil fuel stock but also for safe and better environment (Chandel *et al.,* 2007). The interest in biomass as the alternative source of energy is gaining momentum more and more over the last century. Production of bioethanol from biomass is one of the alternative sources of fuel that has gained a lot of attention over the past years. Ethanol produced from renewable energy source is the most promising future biofuel (Marszalek and Kaminski, 2008). To be a viable substitute for a fossil fuel, Hill *et al.*, (2006) contended that an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, and be producible in sufficient quantities to make a meaningful impact on energy demands, but it should also provide a net energy gain over the energy sources used to produce it. Bioethanol meets most of these criteria but the quantity of ethanol produced annually has not overtaken petroleum.

Crops such as maize have traditionally been utilized as the substrate for the production of ethanol. It has however been reported that the cost of raw material accounted for 70% of the

total cost of producing the ethanol (Ramesh *et al.*, 2004). Research into alternative substrates such as agricultural waste, municipal waste, molasses etc is on going to find low cost as well as efficient substrate for ethanol production (Ramesh *et al.*, 2004). The problem however is the technology for conversion of the lignocellulosic part of these materials to bioethanol. The choice of the best technology for the conversion of lignocelluloses to bioethanol should be decided on the basis of overall economics (lowest cost), environmental (pollutants), and energy (higher efficiencies). Many investigations have been performed on the appropriate technology for the conversion of the lignocellulosic to ethanol as well as substrate with little or no lignin such as molasses (Chandel *et al.*, 2007), but research work on the utilization of *pito* mash has been very limited.

Even though several microorganisms, including *Clostridium* sp., have been considered as ethanologenic microbes, the yeast *Saccharomyces cerevisiae* and facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production (Gunasekaran and Raj 1999). Traditionally *Saccharomyces cerevisiae* has been used for the production of ethanol, however it has been associated with low alcohol tolerance and low productivity which for efficient ethanol production requires improvement. *Zymomonas mobilis*, a Gram negative bacterium possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance. *Z. mobilis* strain grown under anaerobic conditions can produce about 1.5 -1.9 mol of ethanol from each mol of glucose, which is much better than ethanol produced by *S. cerevisiae* (Swings and De Ley, 1977). *Zymomonas* grows and ferments glucose very fast, its preference for low pH prevents contamination and grows in high glucose and ethanol concentration (Swings and De-Ley, 1977).

1.2 PROBLEM STATEMENT

The rising cost of fuel has encouraged the search for products from biomass as alternatives to fossil fuel. Many countries have turned to bioethanol as the alternative and many researches are focused on finding efficient as well as cost-effective ways of producing bioethanol. Traditionally, the microorganism used in the production of bioethanol has been *Saccharomyces cerevisiae*. This organism is however associated with low productivity and low alcohol tolerance.

Pito is one of the alcoholic beverages common in Ghana. It is more common in the rural areas. After the production of the *pito* the marsh is disposed off, most of the time, indiscriminately for natural degradation. However, this waste can be utilized for the production of ethanol and other products such as fertilizers and animal feed.

1.3 JUSTIFICATION

Bioethanol is not only cheap and environmentally friendly, but can also help the country to improve its economic gains by producing her own fuel from renewable source. Even though many microorganisms, including *Saccharomyces cerevisiae*, *Clostridium* sp., have been considered as ethanologenic microbes, *Zymomonas mobilis* is considered the better candidate for industrial alcohol production. *Z. mobilis* possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance.

Traditional ethanol industries utilize crops such as maize and sugar cane as a substrate in the production of ethanol; however this raises the cost of production and renders the resulting bioethanol uncompetitive. The use of these crops will lead to competition with their use as food. Many investigations have been performed to find low cost as well as high efficient

substrate such as agro-wastes for bio-ethanol production but research into the use of waste product such as *pito* mash has not been investigated and documented.

1.4 MAIN OBJECTIVE

The main objective is to isolate and identify microorganism from locally produced palm wine that effectively ferment *pito* mash to ethanol.

1.5 SPECIFIC OBJECTIVES

1. Isolate and identify of bacteria and Yeast associated with palm wine.
2. Evaluate the production of ethanol by the isolates.
3. Examine some of the factors/condition favourable for production of ethanol with isolates.
4. Saccharification and fermentation of *pito* mash to ethanol.
5. Determination of percentage and concentration of ethanol produced.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is ethanol that is produced from biological materials (biomass). It is water-free alcohol produced from the fermentation of sugar or converted starch. It is colourless clear liquid with mild characteristic odour. It is volatile, miscible in both water and non-polar solvents at ordinary conditions and has density of 0.792 g/cm^3 at 15.5˚C (Purwadi, 2006; Thomsen *et al.,* 2008). Ethanol is less hygroscopic, contains a reasonable heat of combustion, and has lower evaporation heat. It is biodegradable, low in toxicity and causes little environmental pollution if spilled. Ethanol burns to produce carbon dioxide and water. Ethanol is a high octane fuel and has replaced lead as an octane enhancer in petrol (Bailey, 1996). It has boiling point of 78^{o}C and freezes at -112^{o}C. .

Ethanol as a solvent has many uses; potable ethanol in beer, saki, perry and variety of fermented fruit juices and in distilled beverages such as whisky, gin, brandy and other liquors. Ethanol is used in the pharmaceutical industry in preparations such as tonics and cough syrups, as solvent for hop constituents, and in cosmetics. Ethanol is also used as co-surfactant in oil-water microemulsions (Stewart *et al.*, 1983). Other uses of ethanol include antiseptic and sterillant. Ethanol is now used as fuel and fuel additive in automobiles.

2.2 Ethanol production technologies

Ethanol can be obtained by two main methods; chemical synthesis and biological production of ethanol (fermentation). The steps involved in biological production of ethanol depend on the starting materials. For simple sugars like glucose and fructose, the process involves fermentation by microorganisms or their enzyme to convert the sugar into ethanol. For

complex carbohydrates like starch, glycogen etc, production of ethanol requires saccharification of the carbohydrate to release the simple sugar before a microorganism can utilize the simple sugars and convert them to ethanol. Cellulosic and lignocellulosic material requires pretreatment of the raw material to release their components that can be utilized by microorganism (Sun and Cheng, 2002).

2.3 Raw materials

Ethanol can be produced from different kinds of raw materials. The raw materials are classified into three categories: simple sugars, starch and cellulose (Demiirba, 2005). Raw materials containing simple sugars, suitable for direct processing through fermentation include white beet, sugar cane, citrus fruits, tropical plants such as punk, and juice of trees like birches and maple and honey (Marszałek and Kamiński, 2008). Raw materials containing starch used for the production of ethanol include cereals such as rye, barley, corn, oat, wheat, sorghum etc. Most of the ethanol research has used raw material that contain starch, Hermann *et al.,* (1986) obtained maximum ethanol of 41-42 g l^{-1} from concentrated deproteinized whey having a lactose content of 23 percent using *Zymomonas mobilis,* immobilized with sodium alginate. Co-fermentation of sweet sorghum juice and grain was studied by Gibbons and Westby (1989) for production of fuel ethanol and obtained 3.5 percent (v/v) ethanol. Nimbkar *et al.,* (1989) successfully fermented unsterilized juice of sweet sorghum by using *Saccharomyces cerevisiae* strain 3319 and obtained maximum alcohol of 12.45 percent (v/v).

Cellulosic biomass materials can be classified into four groups based on type of resource: wood, municipal solid waste, waste-paper and crop residue resources (Marszałek and Kamiński, 2008). The lignocelluloses biomass comprises cellulose, hemicelluloses and lignin (Hayn *et al*., 1993). Cellulose is a linear, crystalline homopolymer with repeating units of glucose held together by beta-glucosidic linkages. The structure is rigid and harsh treatment

is required to break it down (Gray *et al*., 2006). Hemi-cellulose consists of short, linear and highly branched chains of sugars. In contrast to cellulose, which is a polymer of only glucose, a hemicellulose is a hetero-polymer of D-xylose, D-glucose, D-galactose, D-mannose and L-arabinose (Saha *et al*., 2005). Ethanol production from lignocellulosic biomass is not yet at commercial scale, even though many technologies are mooted. The total potential bioethanol production from crop residues and wasted crops is about 16 times higher than the current world ethanol production (Berg, 2004).

2.4 Production of Bioethanol

There are a number of advanced technologies of bioethanol production in the world presently, depending on the raw material subjected to fermentation. Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Bioconversion of biomass to ethanol requires four main stages, pretreatment, hydrolysis (saccharification), fermentation and product separation/ distillation.

2.4.1 Pretreatment

This refers to the solubilization and the separation of one or more of the four components of biomass: hemicelluloses, cellulose, lignin and extractives to make the remaining solid biomass more accessible to further chemical or biological treatment. Thus pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields (Sun and Cheng, 2002; Moiser *et al*., 2005; Chandel *et al*., 2007). Effects of pretreatment on the biomass structure include solubilizing hemicelluloses, reducing crystallinity and increasing the available surface area and pore volume of the substrate (Chandel *et al*., 2007). Generally

pretreatment techniques have been divided into three categories; physical, chemical and biological pretreatments. For pretreatment to be effective certain criteria have to be met; these include avoiding size reduction, preserving hemicelluloses fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and most of all cost effectiveness (Zheng *et al*., 2009). In addition to these, recovery of high value co-products (e.g. lignin and protein), pretreatment catalyst, catalyst recycling, and waste treatment are also considered (Zheng *et al*., 2009). Pretreatment is considered the most expensive processing step in the conversion of biomass to ethanol (Moiser *et al*., 2005). Therefore, in conversion of cellulosic material to bioethanol, pretreatment remains the major challenge (Chandel *et al*., 2007).

2.4.1.1 Physical pretreatment:

Physical pretreatment includes chipping, grinding and milling and can be applied to reduce the size of substrate and increase the surface area for enzyme activity. Corn ethanol producers use grinding and milling; these reduce the size of the corn kernels and open them for enzymatic hydrolysis. Cellulosic and lignocellulosic raw materials however require much more intense physical pretreatment. However chipping, grinding and milling have been applied to reduce cellulose crystallinity (Sun and Cheng, 2002). Vibratory ball milling was found to be more effective in reducing cellulose crystallinity compared to ordinary milling (Millet *et al.,* 1976). Pyrolysis is another physical method of pretreatment used to treat lignocellulose materials. Treating cellulose at temperature greater than 300^{o}C will decompose the cellulose to produce gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). Lignocellulose materials require the combination of both physical and chemical methods. Commonly used physiochemical method is the steam explosion. The lignocellulose material is chipped and the chips are treated with high-pressure saturated steam and the pressure is swiftly reduced. This makes the materials to undergo an

explosive decompression (Sun and Cheng, 2002). This process causes degradation of hemicelluloses and lignin transformation due to the high temperature; this increases the potential of cellulose hydrolysis. Grous *et al.,* (1986) achieved 90% efficiency of enzymatic hydrolysis in 24h for poplar chips pretreated by steam explosion, compared to 15% hydrolysis of untreated chips. Addition of H_2SO_4 or CO_2 in steam explosion has been found to improve enzymatic hydrolysis, decrease the production of inhibitory compounds and can lead to complete removal of hemicelluloses (Morjanoff and Gray, 1987).

2.4.1.2 Chemical pretreatment

Chemicals used for pretreatment of cellulosic materials include dilute acid, alkaline, organic solvent, Ammonia, Sulphur dioxide, Carbon dioxide etc. Both concentrated and dilute acids such as HCl and H_2SO_4 have been used in the pretreatment of lignocellulose materials. However both acids are toxic, corrosive and hazardous and therefore require reactors that are resistant to corrosion, thus their use as agent of pretreatment has not been successful (Sivers and Zacchi, 1995; Sun and Cheng, 2002). Dilute acid pretreatment however has been successfully developed and many researchers have been done with different substrate such as wheat straw (Saha *et al*., 2005), wheat bran (Palmarola-Adrado*set al*., 2005), spruce (Taherijadeh, 1999). Bases such as NaOH have also been used in the pretreatment of lignocelluloses materials. Krishna *et al*., (1999) used alkaline to treat sugarcane leaves. Dilute NaOH was also found to be effective in treating straw with low lignin content (Bjerre *et al*., 1996), Chosdu *et al*., (1993) used irradiation and NaOH for the pretreatment of corn stalk, peanut husk and cassava barks. Organic solvents such as methanol, acetone, ethylene, glycol, triethylene and tetrahydrofurfural alcohol (Chum *et al*., 1988) have also been used in pretreatment of lignocelluloses. These are used with inorganic acids such as HCl or H_2SO_4 as a catalyst or organic acids such as oxalic, acetylsalicylic or salicylic acid as a catalyst (Sarkanen, 1980). Ozone has also been used in the pretreatment of wheat straw (Ben-

Ghedalia and Miron, 1981), bagasse and peanut (Neely, 1984) and poplar sawdust (Vidal and Molinier, 1988).

2.4.1.3 Biological pretreatment

Most of the pretreatments described above require expensive instruments or equipment and high energy. Also waste produced by chemical pretreatment can be hazardous to the environment. Microorganisms and their enzymes offer pretreatment method which is cheap and environmentally friendly. Brown rots, white rot and soft rot fungi are known to attack different components of cellulose and lignocellulose materials. Brown rot fungi attack only cellulose while imparting minor modifications to lignin, while white and soft rot fungi attack both cellulose and lignin (Schurz, 1978). The ligninolytic system is an extracellular enzymatic complex that includes peroxidases, laccases and oxidases responsible for the production of extracellular hydrogen peroxide (H_2O_2) (Ruiz-Dueñas and Martinez, 2009). These enzyme systems exhibit differential characteristics depending on the species, strains and culture conditions (Kirk and Farrell, 1987). The enzymes responsible for lignin degradation are mainly: lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper containing phenoloxidase, known as laccase (Maciel *et al*, 2010). Hwang *et al.*, (2008), studied the biological pretreatment of wood chips using four different white-rot fungi for 30 days and found that the glucose yield of pretreated wood by *Trametes versicolor* MrP 1 reached 45% by enzymatic hydrolysis while 35% solid was converted to glucose during fungi incubation. A Japanese red pine *Pinus densiflora* (softwood) was pretreated biologically by white-rot fungi of *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis*, and it was found that *S. hirsutum* was the most effective to degrade lignin and improved the enzymatic digestibility of wood (Lee *et al*, 2007). Keller *et al*. (2003), reported a 3- to 5-fold improvement in enzymatic digestibility of corn stover after pretreatment with *Cyathus*

stercoreus; and a 10- to 100-fold reduction in shear force needed to obtain the same shear rate of 3.2 to 7.0 rev/s, respectively, after pretreatment with *Phanerochaete chrysosporium*.

2.4.2 Hydrolysis

Hydrolysis of the cellulose and hemicelluloses component to monomeric sugar is next after pretreatment. There are two types of hydrolysis commonly used: acids hydrolysis (dilute and concentrated) and enzyme hydrolysis (Chandel *et al*., 2007).

2.4.2.1 Acid hydrolysis:

There are two types of acid hydrolysis; dilute and concentrated acid hydrolysis. Dilute acid is conducted at high temperatures and pressure and has short reaction time. The hemicellulose component is depolymerized at lower temperatures than the cellulosic fraction (Chandel *et al*., 2007). The biomass is mixed with dilute sulphric acid and held at temperatures ranging from 120-220^{o}C for short period of time to hydrolyse the hemicellulose to xylose and other sugars. Depolymerization of the hemicellulose fraction of the cell wall enhances cellulose digestibility in the residual solids (Nigam, 2002; Sun and Cheng, 2002; Dien *et al*., 2006; Saha *et al*., 2005). The disadvantage with dilute acid hydrolysis is that at high temperature or long residence time the monosaccharides produced degrades and gives rise to fermentation inhibitors like furan compounds, weak carboxylic acids and phenolic compounds (Olsson and Hahn-Hagerdal, 1996; Klinke *et al*., 2004; Larsson *et al*., 1999). These inhibitors affect the performance of the ethanol producing microorganism (Chandel *et al*., 2007). To remove these inhibitors and increase the hydrolysate fermentability, several chemicals and biological methods have been used. These methods include overliming (Martinez *et al*., 2000), charcoal adsorption (Chandel *et al*., 2007), ion exchange (Nilvebrant *et al*., 2001), detoxification with laccase (Martin *et al*., 2002; Chandel *et al*., 2007), and biological detoxification (Lopez *et al*.,

2004). The cost is however higher than the benefits achieved (Palmqvist and Hahn-Hagerdal, 2000).

Concentrated acid hydrolysis uses concentrated acid followed by dilution with water to dissolve the hydrolysed sugar. The process allows for complete and rapid conversion of cellulose to glucose and hemicelluloses with a little degradation (Chandel *et al*., 2007). The concentrated acid process uses 70% sulphuric acid at between 40 – 50^{o}C for 2 to 4 hrs. The low temperature and pressure leads to minimize the sugar degradation.

2.4.2.1 Enzymatic hydrolysis

Lignocellulosic materials can be saccharified enzymatically to get fermentable sugars (Itoh *et al*., 2003; Tucker *et al*., 2003). Bacteria and fungi are a good source of cellulases and hemicellulase that could be used for the hydrolysis of pretreated lignocelluloses. This usually is enzymatic cocktail consisting of different hydrolytic enzymes such as cellulases, xylanases and mannanases (Chandel *et al*., 2007). Large numbers of microorganisms are capable of degrading cellulose (Miyamoto, 1997). For fungi, members of the genera that have received considerable attention under aerobic conditions are *Chaetomium,* and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium,* and *Trichoderma* (Deuteromycetes) and *Mucor* (Zygomycetes). The genera with prominent cellulolytic activity within anaerobic fungal division Chytridiomycetes are *Neocallimastix, Piromyces, Caecomyces, Orpimomyces and Anaeromyces* (Lynd *et al*., 2002; Miyamoto, 1997). However, only a few of these microorganisms are known to produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose *in vitro.* Fungi of the genera *Trichoderma* and *Aspergillus* are thought to be prominent cellulase producers, and crude enzymes produced by

these microorganisms are commercially available for agricultural use (Bon and Ferrara, 2007; Rajesh *et al.*, 2008).

2.4.3 Fermentation

An element common to essentially all proposed processes for producing ethanol from cellulosic biomass is microbial fermentation. A variety of microorganisms, generally bacteria, yeast, or fungi ferment carbohydrates to ethanol under oxygen-free conditions. Cells carrying out such fermentations do so to obtain energy (in the form of adenosine triphosphate) and are thus dependent upon ethanol production for growth and long-term survival (Stewart *et al.*, 1983).

2.4.3.1 Microorganism for ethanol production

A wide variety of microorganisms are known to produce ethanol as a product of carbohydrate fermentation. Fermentation forms the basis of production of beer and other alcoholic drinks and now the subject of interest in producing industrial alcohol. Microorganisms which have received attention in industrial alcohol production include a wide range of yeasts, some molds and a number of specialized bacteria. According to Stewart *et al.*(1983), the microorganism being employed in the production of fermentation ethanol should possess the following important characteristics; 1) rapid and relevant carbohydrate fermentation ability, 2) appropriate flocculation and sedimentation characteristics, 3) genetic stability, 4) osmotolerance (i.e., the ability to ferment carbohydrate solutions), 5) ethanol tolerance and the ability to produce elevated concentration of ethanol, 6) high cell viability for repeated recycling and 7) temperature tolerance. In addition to these characteristics a good candidate should be able to use a wide range of sugar sources. Based on these criteria a number of yeasts and bacteria that produce significant quantities of ethanol has been studied.

2.4.3.2 Yeast

The conversion of simple sugars to ethanol can be carried out by many types of yeasts. Different species of yeast require different simple sugar, for example genus the *Saccharomyces; S. cerevisiae* converts glucose, fructose, galactose, maltose, maltotriose and xylulose to ethanol while *rouxii* utilizes glucose, fructose, maltose and sucrose as carbon sources. For the genus *Kluyveromyces*, glucose, galactose and lactose are the carbon source for *K. fragilis* and *K. lactis*. Some species of yeast have also been found to utilize aldopentose and ketopentose. For example *Candida utilis* is able to utilize xylose aerobically for growth but not for anaerobic fermentation (Enari and Suihko, 1983). Gong et al., (1981), reported conversion of xylose by glucose isomerase to xylulose which is utilized by *S. cerevisiae* strain to form ethanol. The problem with this process however is the isomerization introduces an additional cost.

At present over 95% of the ethanol produced globally involves the use of the yeast species *Saccharomyces cerevisiae* and its related species. *Saccharomyces* and a number of other yeast produce ethanol from carbohydrate via the Embden-Meyerhof-Parnas pathway where theroretical yield of 0.51g of ethanol and 0.49g of CO_2 are produced from 1g of glucose. Due to the production of biomass, it is more realistic to consider an ethanol yield of 0.46g `and 0.44g of CO_2 from 1 g of hexose. A number of yeasts, not of the genus *Saccharomyces*, can metabolize xylose through the Heterolactic fermentation pathway. Catabolism of xylose proceeds by its intracellular conversion to xylitol; then to D-xylulose through the mediation of D-xylose kinase and xylitol dehydrogenase (Gong *et al*., 1981). Subsequently D- xylulose is phosphorylated by D-xylulose kinase to an intermediate of the pentose phosphate pathway. One of the major constraints in employing yeast in ethanol production is its limited range of substrate they are able to use. For instance, most oligosaccharides formed during the hydrolysis of starch are not fermented by yeasts; examples of these compounds are

maltodextrins and isomaltose (a α-1, 6-linked dimmer of glucose). Yeasts will require addition of glucoamylases to utilize starch completely. Yeasts cannot also utilize cellulose, hemicelluloses and cellobiose. This inability of yeast to ferment a diversity of cheaper and readily available substrates is a major obstacle to lowering the cost of alcohol.

The utilization of sugar to produce ethanol by yeast starts with either its passage into the cell across the cell membrane, or its hydrolysis outside the cell membrane followed by entry into the cell by some or all of the hydrolysis products (Stewart *et al*., 1983). For example maltose and maltotriose are absorbed directly across the cell membrane while melibioses and sucrose are hydrolysed by extracellular enzymes and the products taken into the cell. The uptake and metabolism of sugars in a mixture occurs in an order determined by regulatory mechanism at the level of gene expression. For instance, glucose is the preferred substrate; therefore the presence of glucose suppresses the induction of other sugar permeases. These substrate are therefore fermented sequentially rather simultaneously. The conversion of glucose to ethanol by yeast is summarized by the Gay-Lussac's equation as

$$C_6H_{12}O_6 \rightarrow \quad 2C_2H_5OH + 3CO_2$$

As indicated by the above equation an equimolalar amount of alcohol and carbon dioxide are produced from each mole of glucose. In a typical fermentation process additional reaction takes place leading to the production of minor products such as glycerol, fusel oils, acetic acid, lactic acid, succinic acid, acetaldehyde, furfral and butanediol. Glycerol is formed as a result of reduction of dihydroxyacetone phosphate to glycerol-3-phosphate. This is then dephosphorylated to glycerol. Ethanol is the major product resulting from *Saccharomyces* sugar fermentation. Yet, at certain concentration, ethanol is very toxic to the yeast cells. Ethanol has been shown to have different and separable effects on the specific growth rate of the microorganism, its viability and rate of fermentation (Casey and Ingledew 1986).

2.4.3.3 Bacteria

Several microorganisms, including *Clostridium* sp, *Zymomonas mobilis*, *Enterobacter arerogenes*, *Escherichia coli*, *Klebsiella oxytoca* have been considered as ethanologenic microbes. However, *Zymomonas mobilis*, a Gram-negative bacterium, is considered as alternative organism to yeast in large-scale fuel ethanol production. Comparative laboratory- and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast by have indicated the suitability of *Z. mobilis* over yeasts due to the following: its higher sugar uptake and ethanol yield, its lower biomass production, its higher ethanol tolerance, it does not require controlled addition of oxygen during the fermentation, and its amenability to genetic manipulations (Gunasekaran and Raj, 1999). However compared to the yeast, its utilizable substrate range is restricted to glucose, fructose, and sucrose. Also under anaerobic conditions, *Z. mobilis* produces by-products such as acetoin, glycerol, acetate, and lactate, which result in reduced production of ethanol from glucose (Gunasekaran and Raj, 1999). It is one of the few facultative anaerobic bacteria which metabolizes glucose and fructose via the Entner–Deudoroff (E–D) pathway, which is usually present in aerobic microorganisms. Glucose, fructose and sucrose are the only three carbon sources that *Z. mobilis* is able to utilize. D-glucose and D-fructose are transported into *Z. mobilis* by facilitated diffusion (Parker *et al.*, 1995). Sucrose however does not need any uptake system due to the fact that it is cleaved extracellularly and its moieties (glucose and fructose) which are subsequently taken up into the cell by facilitated diffusion (Dimarco and Romano, 1985). The uptake of glucose and fructose by facilitated diffusion is through common transport protein and *Z. mobilis* appears to be the only known bacteria that relies solely on such an uniport type for sugar upake; i.e for equilibration of external and internal sugar concentration no phosphoenolpyruvate (PEP)-dependent sugar uptake system has been detected yet in *Z. mobilis*. The effective sugar uptake and metabolism are as a result of the uniporter which

does not require metabolism energy but cannot accumulate substance and subsequent phosphorylation steps distract free hexoses from the equilibrium (Dimarco and Romano, 1985). Concentrated glucose solutions are not inhibitory to the E–D pathway enzymes, since conversion of glucose to ethanol by this organism proceeds rapidly (Scope and Griffiths 1980). Thus, the extracellular osmotic pressure of the glucose solution may rapidly be balanced by corresponding intracellular sugar concentrations. High sugar concentrations decrease the total water potential, and exert osmotic pressures which are comparable to those of relatively strong salt solutions. The low-salt tolerance of *Z. mobilis* poses problems for the fermentation of molasses which usually contains high-salt content (Montenecourt, 1985). In addition to the advantages of *Z. mobilis*, this orgamism can be used along with other hydrolytic organism in simultaneous saccharification and fermentation process. In this process, a hydrolytic organism capable of producing carbohydrate hydrolase is used to saccharify the polymeric substrate. The saccharified products are simultaneously utilized by *Z. mobilis* for ethanol production. Simultaneous saccharification and fermentation (SSF) of cassava starch using *Z. mobilis* or *S. uvarum* ATCC 26602 was investigated by Poosaran *et al.,(*1985). They reported that *Z. mobilis* fermented considerably faster than *S. uvarum*, completing the fermentation in 20 hours yielding 95% of the theoretical yield, while *S. uvarum* required a period of 33 hours to complete fermentation with a yield of 90% of the theoretical value. Ethanol production of 29.7 g ethanol/100 g dry sorghum stalks was achieved by a mixed culture of *Fusarium oxysporum* and *Z. mobilis* (Lezinou *et al.*, 1995). Production of ethanol from malt mash using a mixed culture of *Z. mobilis* and *S. cerevisiae* resulted in 10.1% v/v ethanol which was more than that produced by using boiled and fermented mash (9.3%) (Agrawal and Basappa 1996).

Other microorganisms that have generated a lot of interest are bacteria of the genera clostridia.

Members of these genera include *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. These are thermophilic fermentative anaerobes with an optimal growth between 60 and 65 °C (Lamed and Zeikus, 1980) and are able to degrade lignin-containing materials such as lignocellulosic waste, because of the presence of multiple cellulases and hemicellulases often contained within the cellulosome (Demain *et al.,* 2005). The cellulosome is a multi-enzyme complex located on the outside of the cell membrane and is involved in the enzymatic degradation of cellulosic substances, including crystalline cellulose (Demain *et al.,* 2005). The enzymes found in this complex include endo-β-glucanases, exoglucanases, β-glucosidases, cellodextrin phosphorylases, cellobiose phosphorylases, xylanases, lichenases, laminarinases, pectin lyases, polygalacturonate hydrolases, pectin methylesterase, β-xylosidases, β−galacosidases and β−mannosidases (Demain *et al*,. 2005).

Clostridium thermocellum, a thermophilic, strictly anaerobic Gram-positive bacterium is known to have the highest rate of cellulose utilization of any bacterium, and for this reason it is considered of great significance to the pursuit of biofuel production from the cellulosic materials in plant biomass (Bayer *et al.,* 2004, Lynd *et al.,* 2002). The cellulosome of *Clostridium thermocellum* allows for the degradation of cellulose to cellobiose and cellodextrins, and hemicellulose to xylose, xylobiose and other pentose sugars (Demain *et al*, 2005). Cellobiose and cellodextrins are taken into the cell, where *C. thermocellum* is able to ferment them to ethanol, acetate, lactate, H_2 and CO_2 (Lamed and Zeikus, 1980).

There are, however, a number of disadvantages associated with using *C. thermocellum* in bioethanol production. One of these is that most strains of *C. thermocellum* are sensitive to high ethanol concentrations (Antoni *et al.,* 2007, Demain, 2009). This can be overcome by continuous removal of ethanol as it is being produced (Demain, 2009). Another negative

aspect is the low ethanol yields produced, due to the formation of by-products such as lactate and acetate (Demain, 2009). Another drawback with the use of *C. themocellum* for ethanol production is the fact that despite its ability to degrade lignocellulosic waste to both hexose and pentose sugars, it is only able to utilize hexose sugars from cellulose and not the pentose sugars derived from hemicellulose (Lynd *et al.*, 2002, Taylor *et al.*, 2009). This drawback could be solved by the use of mixed culture of *C. thermocellum* and other thermophilic microorganisms capable of fermenting pentose to ethanol (Lynd *et al.*, 2002).

2.5 Fermentation Parameters

2.5.1 Fermentation time

An experiment was conducted for conversion of raw starch to fuel ethanol which was 72.2 g l^{-1} ethanol produced in 120 minutes (Krishnan *et al.*, 1999). Damaged sorghum and rice grains were utilized by Suresh *et al.*,(1999) for ethanol production and obtained ethanol yield of 2.90 percent (V/V) at 30º C after 5 days of fermentation. Higher ethanol yield was achieved in 3 days during fermentation of yam to ethanol by *S. cerevisiae* as observed by Ramanathan (2000). The effect of four different fermentation periods *viz*., 24, 48, 72 and 96 hours on ethanol production from starch medium was studied by Verma *et al.*, (2000). A maximum ethanol concentration of 24.8 g l^{-1} at 48 hours was achieved as compared to 13.7 and 21.6 g l^{-1} at 24 and 96 hours respectively.

2.5.2 Effect of pH

Most bacteria grow in the range of pH 6.5 to 7.5. Yeast and fungi tolerate a range of 3.5 to 5.0 pH. The ability to lower pH below 4.0 offers a way for operators using yeast in aseptic equipment to minimize loss due to bacterial contaminants. Srivastava *et al.*,(1997) showed

that the optimum initial pH of guava pulp medium for the production of ethanol was 5.0 for all three strains of *S. cerevisiae* employed and obtained maximum yield of 5.8 percent during 36 hour fermentation. Simultaneous saccharification and fermentation with *Aspergillus niger* and *Zymomonas mobilis* was carried out at pH of 4.5 using guinea corn husk and millet husk. Ethanol yield from guinea corn husk (26.83 g/l) and millet husk (18.31 g/l) was maximum at 120^{th} hour and with ethanol concentrations of 67.7 and 63.8%, respectively.

2.5.3 Effect of temperature

All organisms function best at a particular optimum temperature. The optimum temperature depend on whether the organism is mesophilic or thermophilic. Mesophilic organisms function between 30° to 38°C while thermophilic function between 60-65^{o}C (Lamed and Zeikus 1980).

Operating at greater temperatures is desirable for a number of reasons: high fermentation temperature increases growth rate and productivity exponentially when the ethanol producing organism can thrive at the high temperature; 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 is able to operate up to 55°C and may be combined with fermentation, further reducing capital and glucose inhibition (Hettenhaus, 1998). Krishnan *et al.*,(1999) studied a simultaneous saccharification and fermentation and separation hydrolysis followed by fermentation in the fluidized bed reactor system. The hydrolysis and fermentation steps were performed at the

optimum temperature of 55 and 30º C respectively and were able to obtain ethanol productivity of 19 to 25 g l^{-1}respectively.

2.5.4 Osmotic Tolerance

The semi-permeable membrane surrounding the microbial 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.

2.5.5 Alcohol Tolerance

The majority of organisms cannot tolerate ethanol concentrations above 10 to15% (w/v). Enzymatic proteins become denatured. Higher temperature lowers the tolerance of the organism. High alcohol concentration disrupts the structure of the lipid bilayer membrane and makes it less stable. Although *S. cerevisiae* and related species produce ethanol with apparent ease, it is toxic to the cell at levels ranging between 8 and 18% (w/v) ethanol, depending on the strain of yeast and the metabolic state of the culture. One of the groups of chemicals that have been known to affect the tolerance of yeast to ethanol has been unsaturated fatty acids. Susan *et al.*, (1978) have shown that cells grown in the presence of linoleic acid are more tolerant to added ethanol than those grown in the presence of oleic acid. Moreover, the cell viability was higher when the cells were cultured in a medium supplemented with ergosterol which has an unsaturation in the side chain at C_{28}, than with campesterol which has a saturated side chain at C_{17}. *Z. mobilis is* more tolerant to ethanol, as the cell-free system of *Z. mobilis* can rapidly consume glucose and produce ethanol more than 15% (w/v) (Algar and

Scoppes 1985). The cell membrane of *Z. mobilis* has fatty acid content to counteract the adverse effects of ethanol. The major fatty acids occurring in *Z. mobilis* are myristic acid, palmitic acid, and *cis*-vaccenic acid. Among the phospholipids, phosphotidyl ethanolamine is the most abundantly present. The high concentrations of cis-vaccenic acid and unusual hopanoids in the membrane are responsible for the high ethanol tolerance (Buchholz *et al*., 1987).

2.6 ECONOMIC IMPACTS OF ETHANOL PRODUCTION

Ethanol production from renewable sources will bring significant benefit to the entire Ghana economy. More specifically, production of ethanol will prove to be extremely beneficial to the economies of the local communities where the production facilities will be located and farmers in general. For the local communities investment associated with constructions production facilities will create new high-paying jobs for the people and generate additional income for the households and also stimulate the general economic activities of the area. One way of improving rural economies is adding value to farm product locally rather than selling those products raw. The rationale according to Swenson, (2008) is very straightforward: processing of the agricultural products in the area helps to substantiate and stabilize local production and, through the processing, adds value to the commodity as additional income to farmers and workers in the area. The use of agricultural waste for the production of ethanol will increase market opportunities for the farmers in those areas as in addition to selling their produce, the wastes from their crops can also be sold to the ethanol producing companies. Farmers can also become stakeholders in the ethanol producing companies, thus creating more income for them (Swenson 2008).

For the whole country, ethanol producing industry will stimulate capital investment. In 2007, the ethanol industry is said to have provided employment for 238,000 workers in all sectors of the U.S economy, added $47.6billion to the nation's GDP, and added additional $12.3 billion into the pockets of American consumers (Mrbanchuk, 2008). According to Mrbanchuk, (2008) the increase in good paying jobs as a result of the facility boosted local household incomes by more than $100 million.

2.7.0 Ethanol as Fuel

Ethanol can be used as fuel in many forms: ethanol is blended with gasoline to produce an oxygenated motor fuel, it is used to improve octane in conventional fuel. Ethanol can be added to gasoline to add oxygen to meet clean air act requirement and can also be used as an extender for gasoline. As a motor fuel, ethanol has lower energy content than gasoline. According to a report by Oak Ridge National Laboratory, ethanol provides on the average 79863.5 KJ per 3.79 litres compare to 121,325KJ for gasoline (Al-Hasan, 2003). This implies 5.76 litres of ethanol provides the same energy as a 3.70 litres of gasoline. The ethanol consumption in an engine is therefore approximately 51% higher than for gasoline since the energy per unit volume of ethanol is 34% lower than for gasoline. Ethanol has a higher compression ratio, therefore in an only ethanol engine, this will allow for increase in power output and better fuel economy than gasoline (Bailey, 1996). Ethanol has high octane rate compared to gasoline (Bailey, 1996).

2.8.0 Environmental Impact of Bioethannol Production

When ethanol is burnt, the released carbon dioxide is recycled into plant material because plants use CO_2 to synthesize cellulose during photosynthesis cycle; ethanol production is therefore a closed carbon dioxide cycle (Wyman, 1999). The ethanol production process uses only 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 Hinman, 1990). Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the greenhouse gas effect (Foody, 1988). The threat of global warming and its consequence which include transformation of forest into desert is a major concern. The possibility of global warming is attributed to a variety of current practices that include burning of fossil fuel, use of CFC, and destruction of vegetation. The release of carbon dioxide from combustion of fossil fuel in automobiles is the biggest single concern. Therefore as energy demand increases, the global supply of fossil fuels will cause more harm to human health by contributing to the greenhouse gas (GHG) emission.

The main advantage of utilizing biomass conversion into ethanol is the reduction of GHG pollution of the atmosphere (Demirbas, 2007). Ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that pose health hazard to living things. According to Wyman and Hinman, (1990), the amount of carbon dioxide released when fuel is produced and then burned is equal to the amount of ethanol needed to replenish the plant needed to produce the ethanol. The ethanol blended diesel (E10 and E30) combustion at different loads found that addition of ethanol to diesel fuel simultaneously decreases octane number, high heating value, aromatics fractions and kinematic viscosity of ethanol blended diesel fuels and changes distillation temperatures (Bang-Quan *et al.*,2003). These factors, according to Chandel *et al.*, (2007), lead to the complete burning of ethanol with less emission. Bioethanol can also play significant role in reducing the harmful gasses in the cities. Bioethanol has the ability to reduce ozone precursors by 20-30%. Ethanol blended diesel (E-15) causes 41% reduction in particulate matter and 5% NOx emission (Subramanian *et al.*, 2005). However, one limitation with the use of ethanol as fuel is the emissions of

aldehyde, predominantly acetaldehydes, which are higher in ethanol than those of gasoline. However, acetaldehydes emissions generate less adverse health effects in comparison to formaldehydes emitted from gasoline engines (Gonsalves, 2006).

Another issue of environmental benefit of the production of ethanol is the energy balance of ethanol. An amount of energy is required to produce ethanol; i.e. energy is required to grow, harvest, transport and prepare the feedstock for ethanol production. Energy is also required for fermenting the feedstock to ethanol. The total amount of energy input into the ethanol production process compared to the energy released by burning the resulting ethanol fuel is known as energy balance. According to critics, the non-renewable energy required to grow and convert feedstock into ethanol is greater than the energy value present in the ethanol fuel (Pimentel, 1991; Pimentel and Pimentel, 1996; Pimentel, 2001). However work by Shapouri*et al.*, (2002) revealed that production of corn-ethanol is energy efficient, in that it yields 34 percent more energy than it takes to produce it, including growing the corn, harvesting, transporting, and distilling it into ethanol.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Sample collection

Fresh palm wine samples were obtained from 20 different palm wine tappers in four different towns [Effiduas, Ntonso, Appiadu and Pakyi] in the Ashanti region. The freshly tapped palm wine samples were collected using pre-sterilized 500ml capacity bottles. The sample were transported to the laboratory in a cooler equipped with packs of ice-blocks to slow down fermentation. The samples were taken immediately to the laboratory for analysis, which was carried out in three replicates. *Pito* marsh was collected from local *pito* brewers in the Ashanti region. The samples were dried and ground to a powdered form using attrition milling machine.

3.1 Media preparation

(a) Standard solid media

Five hundred milliliter (500 ml) of standard media was prepared by dissolving 2.5 g of yeast extract and 10 g of glucose in 500 ml conical flask containing 150 ml distilled water. Exactly 10 g of agar was added and topped up to 500 ml mark and autoclaved at 121°C for 15 minutes.

(b) Preparation of synthetic media

Synthetic media consisted of these chemicals per litre: 1 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$ and 20 g glucose. Synthetic media was prepared by dissolving the above chemicals except glucose in a litre volumetric flask containing about 750 ml of tap water. Glucose was added and topped up to 1000 ml mark with water and autoclaved at 121°C for 15 minutes

(C) Malt yeast peptone glucose media

Five hundred milliliter (500 ml) of MYPG was prepared by dissolving 1.5 g of yeast extract, 2.5 g peptone, 1.5 g malt extract, and 10 g of glucose in 500 ml conical flask containing about 150 ml distilled. Exactly 10 g of agar (melted) was added and topped up to 500 ml mark and autoclaved at 121°C for 15 minutes

The pH of each medium was adjusted to the appropriate pH values using 1N NaOH and 1N HCl.

(D) Sorghum base medium

This medium was prepared similar to MYPG medium except glucose and malt extract were substituted with *pito* mash.

3.2 Isolation of Microorganisms

3.2.1 Isolation of bacteria

A 1ml aliquot of each palm wine was taken aseptically into test tubes. From 10-fold serial dilution in peptone, 0.1 ml portion was surface-spread onto MYPG agar (Cheesbrough, 2003). The inoculated plates were incubated aseptically at 30^{o}C for 3 days. Fourteen colonies were randomly selected from plates with distinct colonies, recultivated in MYPG agar at 30^{o}C for 1 day and further purified on MYPG agar.

3.2.2 Isolation of yeasts

A 1ml aliquot of each palm wine was taken aseptically into test tubes. These samples were serially diluted 10-fold in sterilized distilled water. One ml of the serially diluted sediment was inoculated by streaking on plates of standard media (media were supplemented with chloramphenicol (0.05 mg/l) (Nwachukwu, 2001) and incubated at 28°C for 24 hours (Okafor, 1975).

3.3 Identification of Isolates:

3.3.1 Identification of bacteria isolate

Purified isolates from fresh plates of MYPG medium were subjected to the following tests for the characterization of the isolates: Gram stain, catalase, motility, oxidase, urease, indole, and carbohydrate fermentation using API^{TM}test kit.

3.3.2 The principles of API 20 test for bacterial identification

The API 20 strip consists of 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces colour changes that are spontaneous and revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to reading table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

3.3.3 Carbohydrate (sugar) fermentation

The ability of the bacterium to ferment various carbohydrates using glucose, fructose, sucrose, maltose, lactose and arabinose were determined by growing the isolate in liquid standard medium containing 1% (w/v) of the particular carbohydrate as described (Obire, 2005). Durham-tubes were inverted into the culture tubes for gas collection. Incubation was at 30°C for 24 hours and uninoculated broths were used as control.

3.3.4 Identification of yeast isolates

Colonies suspected to be yeast were isolated from the spread plates and purified by streaking on freshly prepared media and incubated for 1 day at 30°C. Isolates from such fresh plates were subjected to the following tests for the characterization; morphology, surface

characteristics, presence of pseudohyphae, ascospore formation and vegetative reproduction. Fermentative tests included sugars such as glucose, lactose, sucrose and maltose.

3.4 Evaluation of yeast and bacterial isolate for ethanol fermentation

To evaluate ethanol fermentation by different strains of *Klebsiella pneumoniae, Enterobacter aerogenes*, *Saccharomyces cerevisiae* and *Z. mobilis*, 100 ml of rich medium (RM) containing 5 g/L of glucose was places into 100 ml of Erlenmeyer flasks. After inoculation with 5% (v/v) seed culture, the cultures were incubated at 30°C without agitation for 24 hours. The yield of ethanol was used to assess the fermentation performance. The ethanol yield was determined by gas chromatography.

3.5 Preparation of substrate

3.5.1 Malting of sorghum

One hundred and fifty grams (150 g) of sorghum cultivar grains were washed well with tap water to remove dirt and foreign bodies. The grains were steeped in 0.2% sodium hydroxide for 8 hours at room temperature. The sodium hydroxide was drained off and the grains were air dried for an hour. The grains were then steeped in tap water for 16 hours with 2 changes of water at 8 hours interval before steep out. Germination was carried out at room temperature (approximately 27-30^{o}C) on a jute sac. The grains were spread out on pre-wetted jute sac and another pre-wetted jute sac was used to cover the grains. Germination was carried out for 4 days with water sprinkled on the grains daily. At the end of the four days period, the germinated grains were hand-rubbed to break the rootlets and kept in oven for 24 hours at 50^{o}C.

3.5.2 Mashing

For the mashing process, 10g of the ground sorghum malt and 40g of the powdered *pito* mash (i.e. 20% malt and 80% mash) and placed into a beaker and mixed well with the spatula. Two hundred milliliter (200 ml) of distilled water kept at the temperature of 45^{o}C was added to the sample in the beaker and stirred with glass rod to avoid the formation of lumps. The slurry was then placed in a water bath pre-heated to 45^{o}C and maintained at this temperature for 30 minutes. The temperature of the mash was raised to 100^{o}C and kept at this temperature for one hour. Complete saccharification was determined every ten minutes from the time the temperature reached 85^{o}C by the iodine test.

3.6 Optimization

3.6.1 Optimization of pH

Ten ml (10 ml) of the mashed sorghum mash was placed in different test tubes and optimization was carried out by adjusting the pH ranges from 4.0, 4.5, 5.0, 5.5 and 6.0 of fermentation media. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH.

3.6.2 Optimization of temperature

Ten ml of the mashed sorghum mash was placed in different test tubes and optimization of temperature was carried out by incubating the fermentation media at 30, 35, 40 and 45°C.

3.6.3 Optimization of fermentation period

Fermentation time was optimized by putting various tests, which contain the fermentation medium, at 30^{o}C from 1 day to 5 days.

3.7 Fermentation process

The procedures were adopted from Dowe and McMillan (2008). Fermentation was carried out in 500 ml Erlenmeyer flasks. The fermentation lock or bubble trap consisted of rubber stopper (with hole) through which a tube was inserted. A cotton plug was inserted in the tube and the tube was connected to silicone tubing. The other end was submerged in a test tube containing water. All mashes were cooled to a temperature between 27-30^{o}C after liquefaction and saccharification and the pH adjusted with HCl. Saccharified mashes were then inoculated with 10 ml pre-culture *Saccharomyces cerevisiae* and *Zymomonas mobilis* in separate set ups. Fermentation was performed in an incubator with intermittent shaking at optimized conditions. The fermentation process was monitored by measuring the sugar content and ethanol content.

3.8 Chemical Analysis

3.8.1 Estimation of reducing sugars

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959). DNSA and Rochelle salt were prepared as described in appendix B. About 0.5 ml of powered *pito* mash was drawn from every treatment into test tubes. The volume was made up to 3 ml using distilled water. Three milliliters (3 ml) of DNSA reagent of was added to each sample, and mixed well. The reagent blank containing 3 ml of distilled water and 3 ml of DNS reagent was also prepared. Similarly, standards were also included whose glucose concentration ranged from 10 µg to 100 µg. All tubes *viz.*, samples, standards and blank were kept on boiling water bath for 5 minutes. After this one (1) ml of 40% Rochelle salt solution was added when the reaction mixture was still warm and then cooled. The absorbance in terms of optical density of the standards and samples were read at 510 nm using Systronics UV Spectrophotometer-117. The standard glucose was also prepared similarly with concentration ranging from mg to mg ml-

3.8.2 Estimation of starch

Procedure: one (1) gram of the powered *pito* mash was homogenized in hot 80% ethanol. The sample was centrifuged and the supernatant was discarded while the residue was retained. The residue was thoroughly washed with hot 80% ethanol and dried over water bath. Five (5) milliliters of water and 6ml of 52% perchloric acid were added to the residue and kept at 0^{o}C for 20 mins. The sample was centrifuged and the supernatant kept. The extraction was repeated with fresh perchloric acid. The supernatant was pooled together and 0.2 ml was pipetted into test tube and the volume was made up to 1 ml. Standard solutions were prepared by taking 0.2, 0.4, 0.6, 0.8 and 1 ml and the volume made up to 1 ml in each test tube with water. Five ml of anthrone reagent (Appendix B) was added to each test tube and heated in boiling water bath for 8 mins. The samples were cooled to room temperature and the absorbance taken at 630 nm. The starch was calculated using the formula

Starch (%) = % glucose x 0.9.

3.8.3 Estimation of cellulose

Procedure: Three (3) milliliters of acetic/nitric reagent (Appendix B) was added to 1 g of the powered *pito* mash and mixed in vortex mixer. The sample was placed in water bath and heated at 100^{o}C, cooled after 30 mins and centrifuged for 20 mins. The supernatant was discarded and the residue washed with distilled water. Ten (10) milliters of 67% sulphuric acid was added and allowed to stand for an hour. One (1) milliliter of the solution was diluted with water to 100 ml. To 1 ml of the diluted sample, 10 ml of anthrone reagent was added and mixed well. The sample was then heated in boiling water bath for 10 mins and cooled to room temperature. Absorbance was measured at 630 nm. One hundred (100) milligram of cellulose solution was prepared in a test tube as standard and the procedure above was repeated for the standards.

3.8.4 Estimation of Ethanol Concentration

Ethanol concentration was determined using a Perkin Elmer, Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 Pen recorder. A chromopak K 80/100 CRS column was used. The flow rate of the carrier gas, N_2, was 42 ml/min. H_2 and air were the fuel used. The oven temperature, injector temperature and detector temperature were 130˚C, 200˚C and 200˚C respectively. The injected volume was 1 µl and the retention time was 8.5 minutes. Identification and quantification were based on direct comparison of the gas chromatogram response to ethanol standards.

The expected ethanol amount was calculated after fermentation stoichiometry, assuming that 1.0 g of total sugars produced 0.511 g of ethanol. The ethanol yield was calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (g g^{-1}). The volumetric ethanol productivity as actual ethanol yield g l^{-1} /time (h) (Onsoy *et al* 2007)

The efficiency of reducing sugar conversion into ethanol by both microorganisms (%) expresses the amount of produced ethanol relative to the theoretical quantity expected based on the sugar content of the malted sorghum, and it was calculated accordingly with the following equation;

$$\textbf{Efficiency (\%)} = \frac{\textbf{Ethanol produced (g/l)}}{\textbf{TRS}_i - \textbf{TRS}_f} \times 100$$

Where, TRS_i is the initial sugar content (before fermentation) and TRS_f is the final sugar content (after fermentation) Alvarenga *et a*l., (2011).

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and identification of bacteria

Microscopic examination of palm wine samples showed that palm wine serves as a good medium for the growth of numerous microorganisms which included Gram-positive and Gram-negative bacteria mostly in chains and in clusters (rods and cocci). According to the different morphology of the colony and fermentation of different carbohydrates, seven strains of bacteria were isolated and three were identified from four localities in Ashanti region; Effiduase). The codes represent the locality from which the isolate was obtained. From the Gram staining examination, three isolates (WE1, S4, and D3) were Gram-negative and bacillus. Four isolates (S2, SP1, SP3 and SP4) were Gram-positive. Isolates SP1, SP3 and S2 were bacilli, SP4 was cocci. The ability of the bacterial isolates to ferment various carbohydrates aerobically and anaerobically is presented in table 1. All the bacterial isolates were able to ferment glucose and fructose with only three isolates (WE1, D3 and S4) which produced gas from the fermentation both aerobically and anaerobically. Isolates D3 and S4 were able to ferment all the four carbohydrate both aerobically and anaerobically with gas production. Isolate WE1 was able to ferment glucose, fructose and sucrose but not lactose both aerobically and anaerobically with gas production. The results therefore indicated that all the three isolates (WE1, D3 and S4) were facultative anaerobe. API^{TM} analysis revealed that the isolate WE1 was urease, oxidase and indole negative, catalase positive. D3 was urease and catalase positive, oxidase and indole negative while S4 was urease, oxidase and indole negative and catalase positive. Confirmatory test with API^{M}20 revealed isolate WE1 to be *Zymomonas mobilis*, S4 to be *Klebsiella pneumoniae*, D3 to be *Enterobacter cloacae.*

Table 1: Characteristics of isolates

Isolate	Colour of colony	shape	Gram-stain
WE1	Cream/white	Rod	-
S4	Cream/white	Rod	-
S2	White/cream	Rod	+
D3	Cream/white	Rod	-
SP1	Cream/white	Rod	+
SP3	Cream/white	Rod	+
SP4	Cream /white	circular	+

Table 2: Fermentation of different sugars by bacteria isolates under aerobic and anaerobic conditions.

	Isolates													
	WE1		S4		S2		D3		SP1		SP3		SP4	
Sugar	aerobic	anaerobic	aerobic	anaerobic	Aerobic	Anaerobic	aerobic	anaerobic	Aerobic	Anaerobic	Aerobic	anaerobic	aerobic	anaerobic
Glucose	+ Gas	+ Gas	+ Gas	+ Gas	+ No Gas	–	+ Gas	+ Gas	+ No Gas	–	+ No Gas	–	+ No Gas	–
Fructose	+ Gas	+ Gas	+ Gas	+ Gas	–	–	+ Gas	+ Gas	+ No Gas	–	+ No Gas	–	+ No Gas	–
Lactose	–	–	+ Gas	+ Gas	–	–	+ Gas	+ Gas	+ No Gas	–	+ No Gas	–	+ No Gas	–
Sucrose	+ Gas	+ Gas	+ Gas	+ Gas	–	–	+ Gas	+ Gas	+ No Gas	–	+ No Gas	–	+ No Gas	–

No gas – no gas was produced during fermentation

Gas - gas was produced during fermentation

+ - substrate was fermented indicated by colour change

- substrate was not fermented
- WE – Effiduase
- SP – Pakyi
- D - Appiadu
- S - Ntonso

4.2 Isolation and identification of yeast

A total of seven yeast isolates were identified. Based on the colony and cell morphology including the growth of isolates in liquid medium as well as the assimilation and fermentation of carbohydrates, four different types of yeasts were recognized in the palm wine samples. Nearly, all the yeast isolates fermented glucose, fructose and sucrose, but not lactose. Results presented in Table 2 indicated that all the isolates were aerobes. They were tentatively identified as *Saccharomyces cerevisiae* based on the API database. Five (D1, D2, WE4, SP2 and S3) out of the 7 isolates identified were *S. cerevisiae*, one (WE2) was identified as *Kloechera apiculata*, and one (WE3) could not be identified. The results indicated that *S. cerevisiae* is the dominant microorganism at the four locations sampling was done.

Table 3: Fermentation of different sugar by yeast isolates under aerobic condition.

Isolate	Glucose	Fructose	lactose	Sucrose
D 1	+ Gas	+ Gas	_	+ Gas
D 2	+ Gas	+ Gas	_	+ Gas
S 3	+ Gas	+ Gas	_	+ Gas
WE 2	+ Gas	+ Gas	_	+ Gas
WE 3	+ Gas	+ Gas	_	+ Gas
WE 4	+ Gas	+ Gas	_	+ Gas
SP 2	+ Gas	+ Gas	_	+ Gas

No gas – No gas was produced during fermentation
Gas - Gas was produced during fermentation
+ - substrate was fermented indicated by colour change

- substrate was not fermented
- WE – Effiduase
- SP – Pakyi
- D - Appiadu
- S - Ntonso

4.3 Evaluation of selected yeast and bacteria strains for ethanol production

The two yeast strains and three bacterial strains were selected to evaluate the ethanol fermentation capacity in RM medium containing 5 % of glucose. The results are presented in Table 3. There was significant difference in ethanol produced by the different microorganisms. *Z. mobilis* produced the highest concentration 1.4 v/v (± 0.03), followed by *S cerevisiae* which produced ethanol concentration of 0.834 v/v (± 0.02). In brief these results indicated that, among the isolated bacterial strains, *Z. mobilis* had an advantage in glucose utilization over *Klebsiella pneumonia* and *Enterobacter cloacae*. *Saccharomyces cerevisiae* was also showed to have an advantage in glucose utilization over *Kloechera apiculata*.

Table 4: Evaluation of yeast and bacterial strains for ethanol fermentation

microorganism	Actual ethanol produced g/l	Ethanol yield $g\ g^{-1}$	Volumetric ethanol productivity $g\ l^{-1}\ h^{-1}$	Conversion efficiency %
Zymomonas mobilis	0.140	0.28	0.0058	54.90
Klebsiella pneumonia	0.029	0.057	0.0012	11.17
Enterobacter cloacae	0.032	0.064	0.0013	12.54
Saccharomyces cerevisiae	0.083	0.166	0.0035	32.62
Kloechera apiculata,	0.028	0.056	0.0012	10.98

RM medium containing 5% of glucose

4.4 Initial starch content and reducing sugars of *pito* mash (spent sorghum)

Initial starch and reducing sugars contents of *pito* mash were estimated and results are presented in Table 4. The starch content of *pito* mash was (15.96%), the initial reducing sugars content estimated before saccharification was 11.1mg g^{-1}. Total reducing sugar recorded after saccharifiaction was 19.75mg ml^{-1}.

Table 5: Initial cellulose, starch and reducing sugars content of spent sorghum mash

Component	Composition
Cellulose	6.41mg g^{-1}
Starch	15.96%
Initial Reducing sugar	11.1mg g^{-1}
Final reducing sugar	19.75 mg ml^{-1}

4.5 Optimization results

Plate 1: optimization of pH of fermentation for *Z. mobilis* and *S. cerevisiae.*

4.5.1 Optimization of pH

As shown in Figure 1, effect of pH on ethanol production was determined at pH values of 4.0, 4.5, 5.0, 5.5 and 6.0, 6.5 with both *S. cerevisiae* and *Z. mobilis*. Generally, ethanol concentration increased with increased pH in both *S. cerevisiae* and *Z. mobilis*. However the increase was more pronounced in *S. cerevisiae* than *Z. mobilis*. For *S. cerevisiae*, fermentation took place at pH of 4 but gave low ethanol concentration (Table 5). Ethanol concentration began to increase with increased pH till it reached maximum at pH of 6, and then decreased at pH 6.5 (Fig 1). In the case of *Z. mobilis*, fermentation took place at pH of 4 and gave higher ethanol concentration compared to ethanol concentration produced by *S. cerevisiae* at the same temperature. Ethanol concentration reached maximum at pH of 5.5, beyond which it began to decrease (Fig 1). In the case of *S. cerevisiae* there was significant ($p < 0.05$) difference between the ethanol produced at all pH vaues. Optimum pH for ethanol was between 6.0 and 6.5 with pH of 6.0 producing the maximum ethanol volume of 0.948 mg ml^{-1} for *S. cerevisiae*. For *Z. mobilis*, there was significant difference in ethanol produced all pH values. The optimum pH was between 5.0 and 5.5 with 5.5 producing the highest ethanol of 1.85 mg ml^{-1}. In all cases the ethanol produced by *Z. mobilis* was higher compared to *S. cerevisiae.*

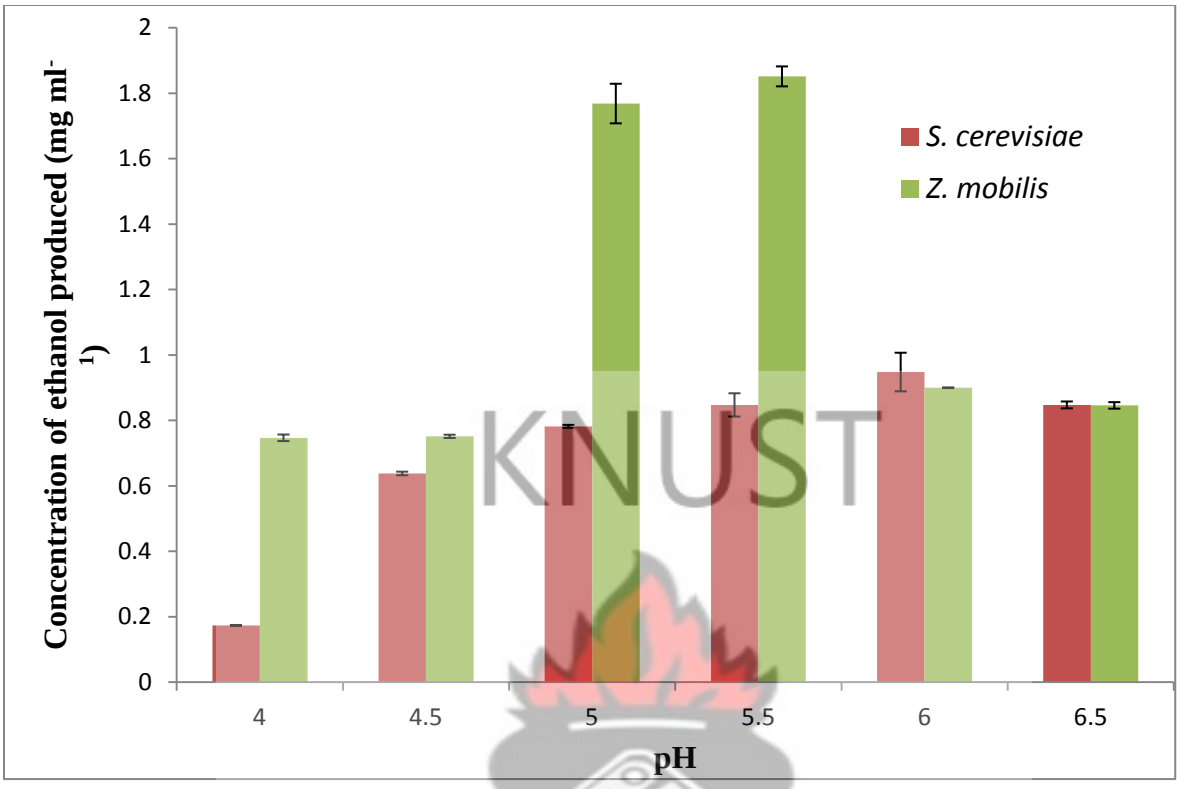

Fig 1. Effect of pH on ethanol production by *S. cerevisiae* and *Z. mobilis* using *pito* mash as substrate

4.5.2 Optimization of temperature

Optimization of temperature was carried out by incubating the fermentation flask at 30, 35, 40 and 45°C. The results (Table 6 and Fig 2) indicated that temperature affected ethanol production with increasing temperatures generally decreasing ethanol concentration in both organisms. In *Z. mobilis* there was initial increase in ethanol concentration with temperature increase from 30^{o}C to 35^{o}C; however beyond 35^{o}C increasing temperature became inhibitory to ethanol production (Fig 2). The decrease was more pronounced at 45^{o}C. *S. cerevisiae* produced maximum amount of ethanol at 30^{o}C and further increase in temperature (35^{o} -45^{o}C) was inhibitory to its ethanol production ability (Fig 2). Analysis of variance indicated that for *S. cerevisiae* there was significant ($p < 0.05$) difference in the ethanol produced at each temperature. However there was no significant ($p < 0.05$) difference in ethanol produced at the temperature of 35-45^{o}C. The

highest concentration (0.951 mg l^{-1}) was produced at temperature of 30^{o}C for *S. cerevisiae*, followed by 0.849 mg l^{-1} at 35^{o}C. The lowest volume (0.323 mg l^{-1}) was produced at 45^{0}C (Table 6). In the case of *Z. mobilis*, there was significant ($p < 0.05$) difference between ethanol produced at all temperatures. However there was no significant ($p < 0.05$) difference between ethanol produced at 30- 35 and 40-45^{o}C. The highest concentration of 1.951 mg l^{-1} was produced at temperature of 35^{o}C followed by 1.889 mg l^{-1} at the temperature of 30^{o}C (Table 6). At all temperature, the concentration of ethanol produced at each fermentation examined using *Z. mobilis* was significantly different from that of to that using *S. cerevisiae.*

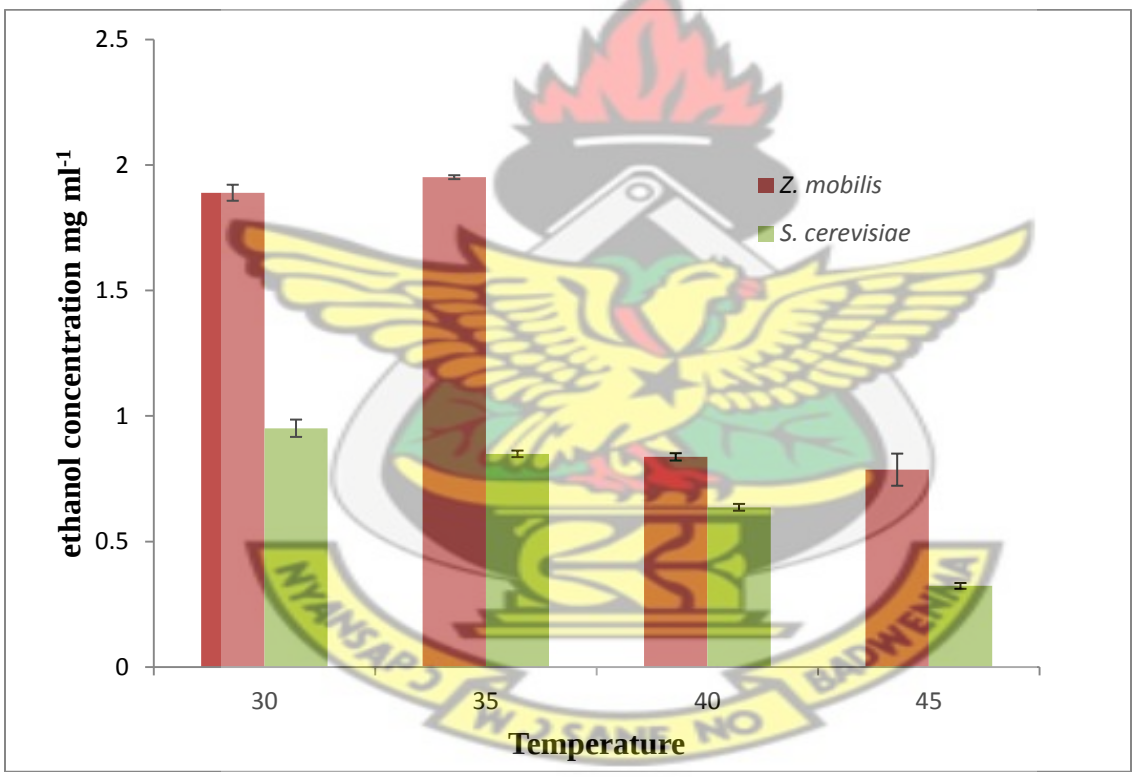

Fig 2: Effect of temperature on ethanol production by *S. cerevisiae* and *Z. mobilis* using *pito* mash as substrate

4.5.3 Optimization of fermentation period

As seen in Fig 3, the flasks were incubated for different time duration; 1, 2, 3, 4, 5 days. On the fourth day maximum ethanol production of 0.847mg l^{-1} was observed for *S. cerevisiae*. Maximum ethanol production of 1.223 mg l^{-1} was produced on the third day for *Z. mobilis* (Table 7*)*. The concentration of ethanol produced at each fermentation time examined using *Z. mobilis* was higher compared to that using *S. cerevisiae* (Table 7).In both organisms, there was a sharp increase in ethanol concentration within the first two days. Slight increase occurred between day 2 and day 4 for *S. cerevisiae* and day 3 for Z. mobilis. It production began to decrease after the maximum concentration was reached (Fig 3).

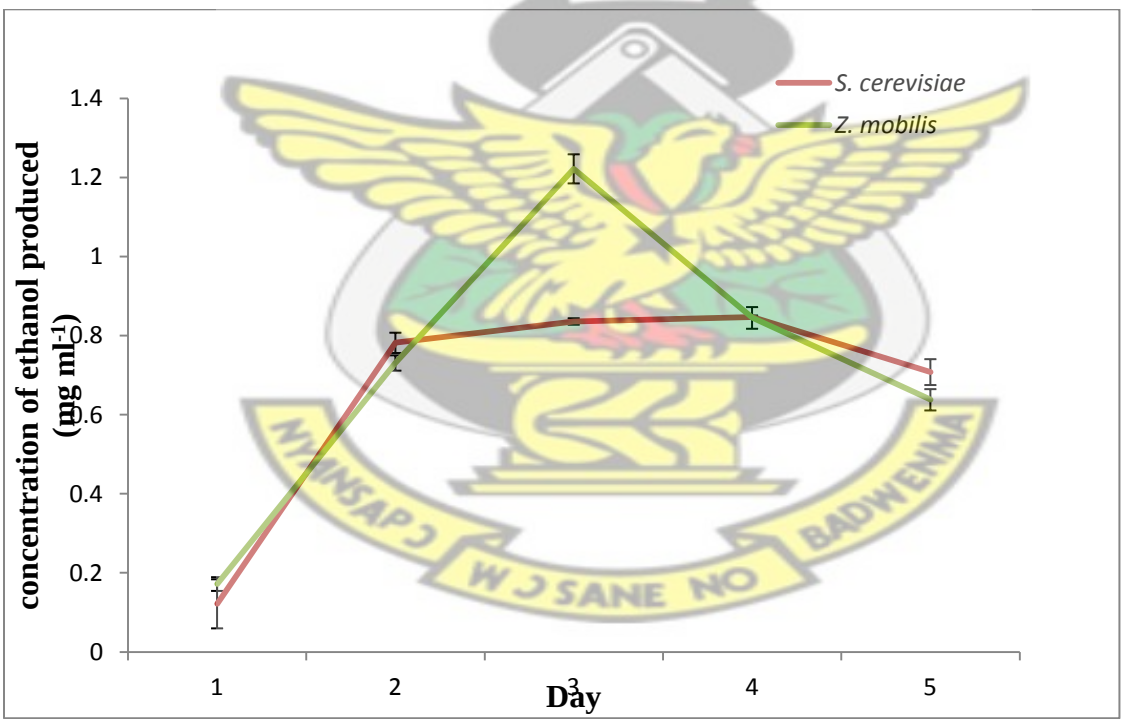

Fig 3: Effect of time duration on ethanol production by *S. cerevisiae* and *Z. mobilis* using *pito* mash as substrate.

4.6 Ethanol produced from spent sorghum using *S. cerevisiae* and *Z. mobilis* separately

Plate 2: Experimental setup for batch fermentation from spent *pito* mash using *S. cerevisiae* and *Z. Mobilis* separately.

Based on the above optimization experimental result, *S. cerevisiae* and *Z. mobilis* were employed in fermenting *pito* mash hydrolysate containing 19.75 g ml^{-1} reducing sugar. In both organisms, a continuous increase in ethanol yield was accompanied with decreased in reducing sugar concentration during the whole period of fermentation (Fig 4 and 5). The fermentation with *Z. mobilis* proceeded very rapidly and was essentially completed in three days with maximum yield of 3.63 g l^{-1}. Fermentation with *S. cerevisiae* required three days to complete with a yield of 3.03g l^{-1}. However in contrast with early optimization results (Fig 3) for *S. cerevisiae* which required four days to reached maximum ethanol concentration. In all cases the sugar utilization was faster in *Z. mobilis* than in *S. cerevisiae.* T-test analysis showed significant difference between the amounts of sugar utilized by Z. *mobilis* and *S. cerevisiae* on each day at 95% confidence interval. This indicates that the utilization of reducing sugar on each day is dependent on the microorganism used. Fig 5 shows the concentration of ethanol produced from *pito* mash

after separate fermentation with *S. cerevisiae* and *Z. mobilis*. The maximum yield of 3.63 g l^{-1} was observed on third day with *Z. mobilis*. In the case of *S. cerevisiae*, the maximum yield of 3.03 g l^{-1} was also observed on the third day. In both cases, the percentage yield of ethanol produced at each fermentation time examined using *Z. mobilis* was higher compared to that using *S. cerevisiae.*

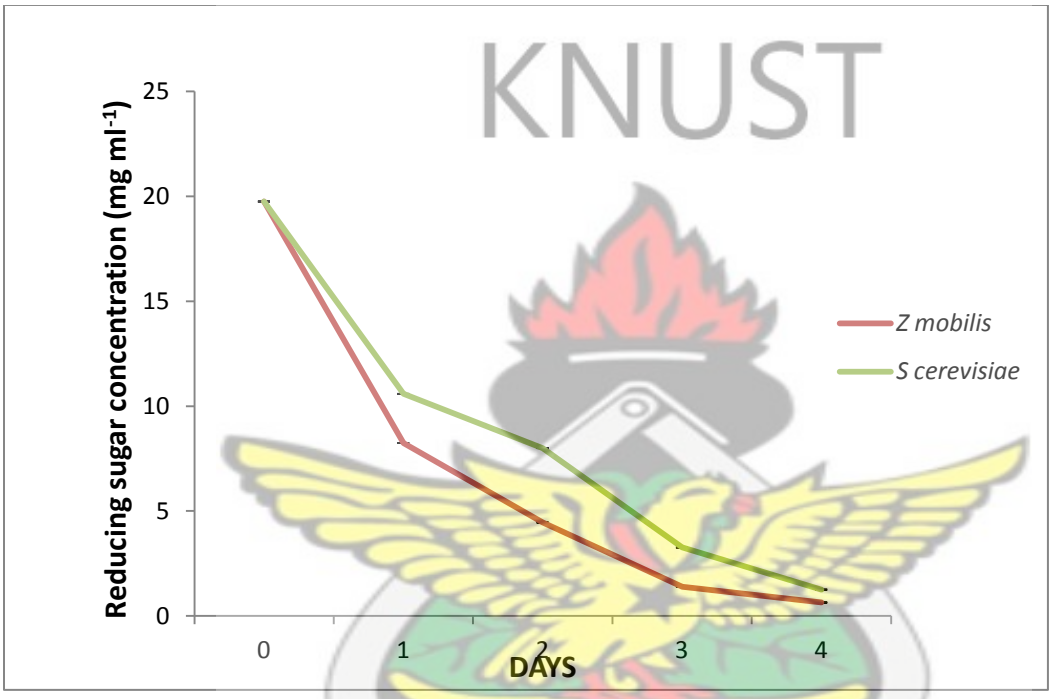

Fig 4: Sugar utilization by *Z. mobilis* and *S. cerevisiae*

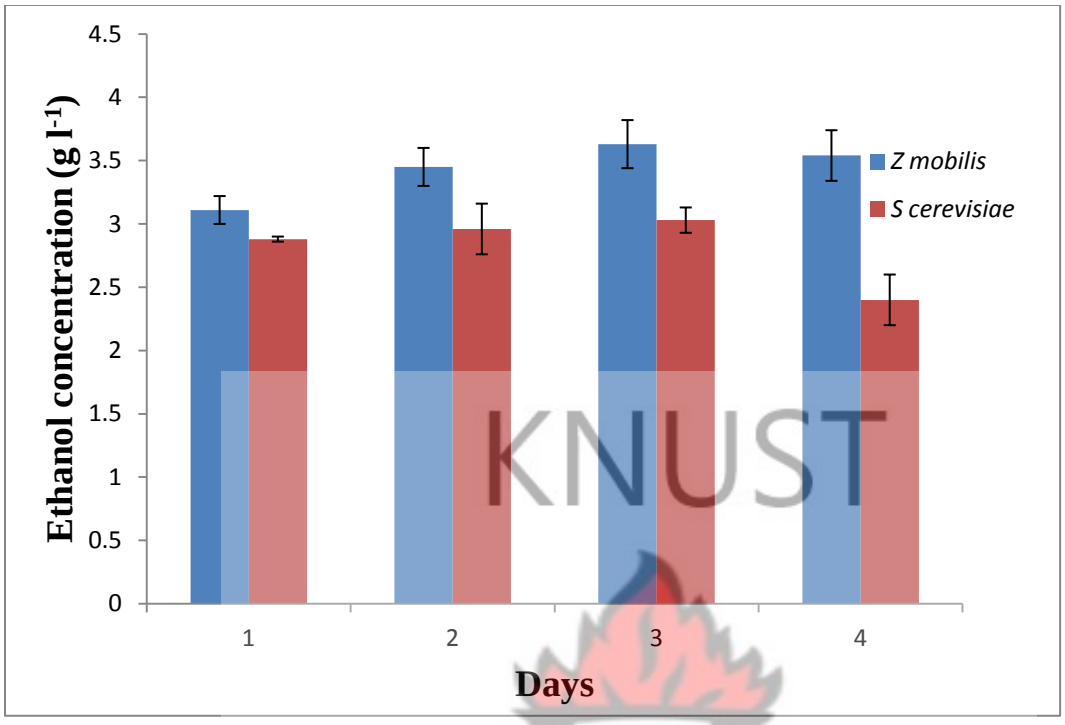

Fig 5: Ethanol produced from *pito* mash using *S. cerevisiae* and *Z. mobilis* separately

Under the operative conditions, the efficiency of reducing sugar conversion to ethanol was found to be 62% in *S. cerevisiae* and 74.2% in *Z. mobilis*. The efficiency of conversion of sugar to ethanol by *Z. mobilis* was higher compare to *S. cerevisiae* (Table 8). T-test analysis showed that at 95% confidence interval, there was significant difference between the amount of ethanol produced by *Z. mobilis* and *S. cerevisiae* on each day. From the data below (Table 8), it is clear that the overall performance of *Z. mobilis* was superior to *S. cerevisiae* making it attractive for large scale ethanol production.

Table 6: Comparison of ethanol production and efficiency between *S. cerevisiae* and *Z. mobilis.*

Day	Ethanol produced (g l^{-1})		Microorganism conversion efficiency %	
	Z. mobilis	*S. cerevisiae*	*Z. mobilis*	*S. cerevisiae*
1	3.11 ± 0.15[a]	2.88 ± 0.02[b]	62.13	57.69
2	3.45 ± 0.21[a]	2.96 ± 0.28[b]	68.68	59.13
3	3.63 ± 0.26[a]	3.03 ± 0.14[a]	72.0	60.24
4	3.54 ± 0.28[a]	2.40 ± 0.28[b]	70.23	47.65

Numbers in column followed by different superscript are significantly different at P<0.05

CHAPTER FIVE

5.0 DISCUSSION

5.1 Isolation and identification of microorganism

This study investigated bioethanol production using *Zymomonas mobilis* and *Saccharomyces cerevisiae. Pito* mash (waste from sorghum brewing with no appreciable value to industries or competitive use as food) was examined as alternative and cost-effective feed stock for the production of bioethanol.

Palm wine samples were inhibited by numerous micro-organisms as seven strains of bacteria and seven strains of yeast were isolated from four localities in Ashanti Region; Effiduase, Ntonso, Appiadu, and Pakyi. The bacterial isolates from the palm wine were dominated by Gram-positive with four out of the seven being Gram-positive and three isolate being Gram-positive. Fermentation test showed three isolate to be ethanogenic as they produced gas from fermentation of their substrate. API^{TM} test kit showed the three isolate to be *Enterobacter cloacae, Klebsiella pneumonia* and *Zymomonas mobilis.* The isolation of *Klebsiella and Zymomonas* sp from the fresh palm wine agrees with the work done by Obire, (2005) and Okafor (1975). Obire, (2005) reported isolation of *Zymomonas mobilis* from fresh palm wine while Okafor, (1975) reported the isolation of *Klebsiella* and *Zymomonas* from fresh palm wine. *Enterobacter cloacae* and *Klebsiella pneumonia* were found to be facultative anaerobic producing gas from glucose, fructose, lactose and sucrose. *Z. mobilis* was found to be facultative anaerobic since it fermented glucose, fructose and sucrose both aerobically and anaerobically; heterofermentative, producing gas from glucose, fructose and sucrose. These observations were similar to those reported by Swings and De Ley (1977) and Obire, (2005).

Saccharomyces cerevisiae and Kloechera apiculata were also isolated from the palm wine. *Saccharomyces cerevisiae* had creamish colony with spherical shaped. The organism was glucose, fructose, sucrose and lactose fermentor with gas production. The results agree with Chilaka *et al*., (2010) who reported the isolation of *Saccharomyces cerevisiae* and other yeast from palm wine. Elijah *et al*., (2010) also reported isolation of *Saccharomyces cerevisiae* from palm wine.

5.2 Evaluation of isolates for ethanol production

Enterobacter cloacae, Klebsiella pneumonia, Zymomonas mobilis, Saccharomyces cerevisiae and *Kloechera apiculata* were selected and their performance in ethanol production was assessed. The result indicated that *Z. mobilis* had the highest ethanol concentration of 1.4 v/v, followed by *S. cerevisiae* with ethanol concentration of 0.834 v/v. The rest of the isolates; *Enterobacter cloacae, Klebsiella pneumonia* and *Kloechera apiculata* produced ethanol concentration below 0.5 v/v. The difference in ethanol concentration produced by the different isolates might be due to the different pathways employed by the isolates in conversion of glucose to ethanol and the major product of such pathway. In the case of *Z. mobilis* and *Saccharomyces* the major products from fermentation were ethanol and carbon dioxide. In *Enterobacter cloacae* and *Klebsiella pneumonia* the main fermentation products are acetoin, 2, 3 butanediol, ethanol, lactic acid, formic acid and acetic acid reported by Johansen *et al.*, (1975). The proportion of these compounds varies with oxygen supply and the pH (Harrison and Pirt 1967). In the absence of air, growth was slow in this organism and most of the carbon was converted into ethanol, formate, butanediol acetoin and acetate (Maddox 1988). These observations might account for the low ethanol concentration in these organisms.

5.3 Hydrolysis of substrate (*pito* mash) for ethanol production

If ethanol is to serve as a motor fuel and compete well with petroleum, the requirement of an abundant and inexpensive substrate is particularly an important issue (Wayman *et al.,* 1988). Agriculture biomass containing starch can be used as potential substrate for bioethanol production. These substrates include corn, wheat, oats, rice, cassava, potatoes, and sorghum (Lin and Tanaka, 2006). However, continuous use of food as a source of fuel may have serious implications for the demand and prices of food. Some of the alternative biomass substrate that have been successfully tried for bioethanol production include starch (Verma *et al.,* 2000), canary waste (Nigam, 1999), kitchen garbage (Wang *et al.*, 2008). Waste from the *pito* brewing industry is alternative substrate that can be used in the production of bioethanol. Apart from being in abundance, it only serve as feed for animals such as pigs, rabbits, sheep and goat and can therefore serve as cheap source of fermentable sugars. However this substrate has high content of insoluble fibres and other nonstarch generated as a result of processing the *pito* drink. The aim of this work was to develop the technology of converting this low cost *pito* mash to produce ethanol by fermentation using efficient glucose fermenters *Z. mobilis* and *S. cerevisiae.* However due to inability of *Z. mobilis* and *S. cerevisiae* to convert starch directly to fermentable sugars which can then be converted to ethanol, the substrate had to be saccharified to glucose. The starch is heterogeneous polysaccharide composed of two high molecular weight component; amylose and amylopectin linked by glycosidic linkage. As presented in Table 4, the starch content of the *pito* mash was 15.96, this is relatively low but expected. *Pito* brewing, like any conventional beer production, basically involves malting, mashing and fermentation. Malting essentially consist of steeping, germinating, and limiting sorghum grains growth when enzymes have been produced for degradation of starch and proteins in the grains. Mashing involves

enzymatic hydrolysis of the starch to fermentable sugars which are subsequently fermented by lactic acid bacteria and yeast to produce *pito* drink. The mash which is waste from the *pito* brewing therefore contain starch which was not hydrolysed by the mashing process, fermentable sugar which was not utilized by the microorganism and has some enzyme activities (Table 4). Malted sorghum was therefore added to hydrolysed starch left over starch to sugar which can be utilized by *Z. mobilis* and *S. cerevisiae*. After the mashing the reducing content was increased to19.75 mg ml^{-1}

5.4 Optimization of fermentation parameters

Both *S. cerevisiae* and *Z. mobilis* exhibited different properties in fermentation potentials. pH is regarded as one of the most important fermentation parameters due to its effect on growth of microorganism, fermentation rate and by-product formation (Pramanik, 2003). Maintenance of pH is therefore of great very importance in fermentation. This study evaluated the efficiency of *Saccharomyces cerevisiae* and *Zymomonas mobilis* in the pH range of 4.0 to 6.5. In both organisms ethanol concentration increased with increasing pH till optimum pH was reached (Fig) and then decreased. The decrease could be due to lesser enzyme activity at that pH. The result agrees with observation by Hwang *et al.*, (2004) who reported that the activities of ethanol producers are slightly suppressed at pH below 4.5. Analysis of variance indicated the difference in ethanol concentration at different pH in both *S. cerevisiae* and *Z. mobilis* was significant $P < 0.05$. The low ethanol concentration at low pH can be attributed to the fact that at low pH enzyme activity was not activated as enzymes are pH specific. Maximum ethanol concentration was achieved at pH of 6.0 for *S. cerevisiae* and pH of 5.5 for *Z. mobilis*. It however decrease at pH of 6.5 and this could be possibly due to the formation of undesired products like organic acids, glycerol etc at the expense of ethanol. In all levels of pH *Z. mobilis* produced more

ethanol. Swing and De-Ley (1977) described pH 4-7.5 as optimum pH condition for *Z. mobilis*. This result is consistent with the observation by Falcào De Moraes *et al.,* (1981), that *Z. mobilis* possesses wide tolerance at pH variation from 3.5 to 7.5. This result also agrees with findings by Buzato (1984), that there was no substantial oscillation on the alcohol yield at pH range of 5.0 to 6.0, showing that there is was major influence of this factor when *Z. mobilis* is cultured on molasses.

Effect of temperature on fermentation was accessed in the present study. Fleet and Heard (1993) reported that the endurance of different species of yeast during alcoholic fermentation, is conditioned by temperature. Temperature is also known to affect the metabolism of microorganism and as a result, the formation of secondary metabolites such as glycerol, acetic acid, succinic acid etc (Lacfon- Lafourcade, 1983). In the present work the highest ethanol concentration of 1.95g l^{-1} was achieved at 35^{o}C for *Z. mobilis* (Fig 2). Further increase in temperature had shown an inhibitory effect on the ethanol production. It was also observed that the decrease was very sharp between 35^{o}C-40^{o}C in contrast to less decrease between 40^{o}-45^{o}C. The decrease might be due to thermal sensitivity of the cells. Similar observations were made by Panesar *et al*., (2007). Panesar *et al*., (2007) indicated that, decrease in the membrane phospholipids content may be responsible for the unique thermal sensitivity of *Z. mobilis* cells grown at higher temperature. There is leakage of magnesium nucleotide and proteins from the cells grown at temperatures above 30^{o}C. The protein loss from the cell was interpreted as disruption of membrane integrity (Benschoter and Ingram 1986). For *S. cerevisiae* the highest concentration of 0.951 mg ml^{-1} was achieved at 30^{o}C beyond which increase in temperature decreased ethanol concentration. This could be attributed to low enzyme activity at higher temperature. Many research works have shown that temperature above 37^{o}C is detrimental to

ethanol production (Lee *et al*., 1981; Lyness and Doelle, 1981). Also the higher temperature can lead to formation of other secondary metabolites leading to low ethanol production. Nanba and Najai (1987), observed excessive enzyme degradation and loss of cell viability at temperatures above the optimal in *S. cerevisiae*. Torija *et al*., (2002), observed there is high yeast mortality at 35^{o}C which might have induced slowdown fermentation and decreased ethanol yield. Other reports have also suggested yeast viability decreased as temperature increases (Nagodawithana *et al*., 1974, Casey *et al*., 1984). This decrease was attributed to a greater accumulation of intracellular ethanol at high temperature which would produce cell toxicity (Nagodawithana *et al*., 1974) and would alter the structure of the membrane decreasing its functionality (Lucera *et al*., 2000). The optimum temperature for *S. cerevisiae* and *Z. mobilis* were 30^{o}C and 35^{o}C respectively

The experiment revealed that the time course of ethanol production by both *Z. mobilis* and *S. cerevisiae* followed an initial upward till the third day for *Z. mobilis* and fourth day for *S. cerevisiae* after which there was a decline in ethanol production. The decline in ethanol concentration after the third day might be due to the build-up of toxic by in the fermentation medium as reported previously by Zakpaa *et al.*, (2009). Changes in the pH of the medium could have also affected the fermentation enzymes thereby reducing ethanol yield.

The optimum conditions achieved for ethanol production from *pito* mash was pH of 6.0, temperature of 30^{o}C and fermentation period of four days for *S. cerevisiae* and pH of 5.5, temperature of 35^{o}C and fermentation period of three days for *Z. mobilis*. Zheng and Feng, (2010) reported ethanol yield of 66.4g/l from sweet potatoes at optimal parameters of pH 4, and fermentation period of 24 hours using *Z. mobilis*. Le-Man *et al.*, (2010), obtained maximum ethanol concentration of 24.17g/l at the optimum condition of temperature 38^{o}C, pH 5.45 using

S. cerevisiae as the microorganism and Korean food waste leachate as the substrate. The different optimizations obtained by different researchers using different substrates give an indication that the optimization conditions for optimum ethanol production depend on the substrate used. This was confirmed by Gunasekaran *et al.*, (1986), who reported that fermentation pattern for strains of *Z. mobilis* on different substrates (synthetic medium, cane juice and molasses) were different with different strains producing maximum ethanol concentration on different substrates. Alvarenga *et al.*, (2011), also showed the fermentation parameters for different strains of *S. cerevisiae* were significantly different when grown on banana pulp.

5.5 Productivity of ethanol from *pito* mash

Different microorganisms have been used in bioethanol production and each organism has exhibited different fermentation properties. In this work *Z. mobilis* and *S. cerevisiae* showed different fermentation properties. In both organisms, a continuous increase in ethanol yield was accompanied with decrease in reducing sugar concentration during the whole period of fermentation (Fig 4 and 5). The fermentation with *Z. mobilis* proceeded very rapidly and was essentially completed in three days with maximum yield of 3.63 g l^{-1}. Fermentation with *S. cerevisiae* was also successful and also required three days to complete with a yield of 3.03g l^{-1} in contrast to the fermentation period obtained in the optimization test. *Z. mobilis* therefore showed quick rate of substrate utilization (Fig 4) and could utilize glucose and other hydrolysate from the *pito* mash faster than *S. cerevisiae* and thus achieved higher fermentation efficiency than .*S* cerevisiae (Table 8). Bacteria are known to multiply faster than yeast thus *Z. mobilis* might reached the lag phase faster than *S. cerevisiae* and therefore utilized its substrate faster. The ethanol yield for *Z. mobilis* was higher than that of *S. cerevisiae* at all fermentation periods.

S. cerevisiae is known to employ the EMP pathway to metabolize glucose producing 2 moles of ATP from 1mole of glucose whereas *Z. mobilis* employing the ED pathway produces 1mole of ATP from 1 mole of glucose (Bringer *et al.*, 1984). Rogers et al., (1982) reported that approximately 2% of the carbon source is converted into biomass as a result of the E-D pathway used by this microorganism. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein (Sprenger 1996). *Z. mobilis* maintain a high level of glucose flux through the pathways to compensate for its low yield (Barnell, *et al.*, 1990). Parker *et al.*, (1995) reported that the low biomass production, and cell growth and fermentation are not linked. As a consequence, *Z. mobilis* perform less biomass formation and efficient production of ethanol compares to *S. cerevisiae* (Roger *et al.*, 1980). The low ethanol conversion efficiency by *S. cerevisiae* might therefore be due to the fact that a portion of the substrate was converted to cell mass and other products. Although liquefaction and saccharification might probably kill some microorganism that might cause contamination, both organisms were able to metabolize their substrate faster thus competitively inhibiting the growth of other microorganisms. They can therefore be used to produce ethanol using non-sterile substrate. This could reduce energy cost involved in sterilizing the substrate.

According to Tao *et al.*, (2005), and Aggarwal *et al*, (2001), cheap raw material, low processing cost and high productivity are the main considerations for most ethanol production. This work therefore shows that under appropriate conditions *pito* mash can be used as alternative and cost-effective feed stock for the production of bioethanol without supplementing the fermentation broth with other nutrients. The substrate was able to support the growth of both organisms without the addition of nutrients is indication that *pito* mash has high starch and protein content.

The products of saccharification of the *pito* mash also did not inhibit ethanol production by *Z. mobilis* and *S. cerevisiae* as indicated by the high fermentation efficiencies. This is advantageous compared to lignocelluloses materials which require pretreatment which produce inhibitory compounds that decrease the productivity in ethanol production (Chandel *et al.*, 2007).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

Bioethanol was produced from *pito* mash (waste from sorghum brewing) using *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from palm wine. The study revealed that palm wine is a good medium for growth of ethanogenic microorganisms. *Zymomonas mobilis*, *Klebsiella pneumoniae, Enterobacter aerogenes, Saccharomyces cerevisiae* and *Kloechera apiculata* were isolated from fresh palm wine. Evaluation of four strains for ethanol fermentation showed *Saccharomyces cerevisiae* and *Zymomonas mobilis* as better candidates for ethanol production. The results indicated that ethanol could be produced from industrial waste such as *pito* mash using *Saccharomyces cerevisiae* and *Zymomonas mobilis.* Optimum parameters for ethanol fermentation by *Zymomonas mobilis* are pH 5.5, temperature 35^{o}C, and fermentation period of three days. Optimum parameters for ethanol fermentation by *Saccharomyces cerevisiae* are pH 6.0, temperature 30^{o}C and fermentation period of four days. *Zymomonas mobilis* was found to produce higher concentration of ethanol and efficiency than *Saccharomyces cerevisiae*. Maximum ethanol concentration using *Zymomonas mobilis* was 3.63 g l^{-1} whilst *Saccharomyces cerevisiae* was 3.03 g l^{-1}. In addition to being cost effective way of producing ethanol, ethanol production from *pito* mash is a means of controlling environmental pollution, it can therefore be considered as good venture to undertake.

RECOMMENDATIONS

1. Different pretreatment must be tested on the *pito* mash to increase the yield of reducing sugars with less inhibitor concentration. This is due to the fact that *pito* mash also contains cellulose (Table 4) which was not hydrolysed by the mashing process.
2. It can also be possible to increase yield by using improved strains of the two microorganisms. *Z. mobilis* cannot ferment any sugar apart from glucose, fructose and sucrose; therefore any other sugar in mash will not be ferment.
3. It possible by-product produced by these organism could reduce ethanol yield; it will therefore be interesting to investigate the type and amount of by-product produced.

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APPENDIX

Appendix A: List of Equipments

Balance: AdventurerPro., Ohaus; Gram precision electronic balance.

Centrifuge: Centrikon T-42K, Kontron instruments.

Dry oven: Gallenkamp.

Magnetic stirrer/hot plate: Staurt scientific UK.

Micropipette: Accupette (0650012).

Microscope Reichert Neova (serial No. 372112) and Olympus (401458) Tokyo.

pH meter: Basic pH meter (840087) Spec Scientific Ltd.

Spectrophotometer: Helios UV Visible Spectrometer. Thermospectronic UVG 121108. Thermo Electron Cooperation.

Water bath: Grant Instruments (Cambridge) Ltd.

APPENDIX B: Standard preparation

Preparation of DNSA reagent

DNSA: one gram of 3, 5-dinitrosalycylic acid (DNSA), 200 mg of crystalline phenol and 50 mg of sodium sulphite were dissolved in 100 ml of one percent NaOH and were stored at 40^{o}C. As the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite was added at the time of use.

Rochelle salt solution (40%)

It was prepared by dissolving 40 g of potassium sodium tartrate in 100 ml distilled water.

Preparation Acetic/nitric Reagent

Acetic/nitric reagent was prepared by dissolving 150 ml of 80% acetic acid in 15 ml of concentrated nitric acid.

Preparation of Anthrone reagent

Two hundred milli gram of anthrone was dissolved in ice-cold 95% sulphuric acid. The solution was stored at 4^{o}C.

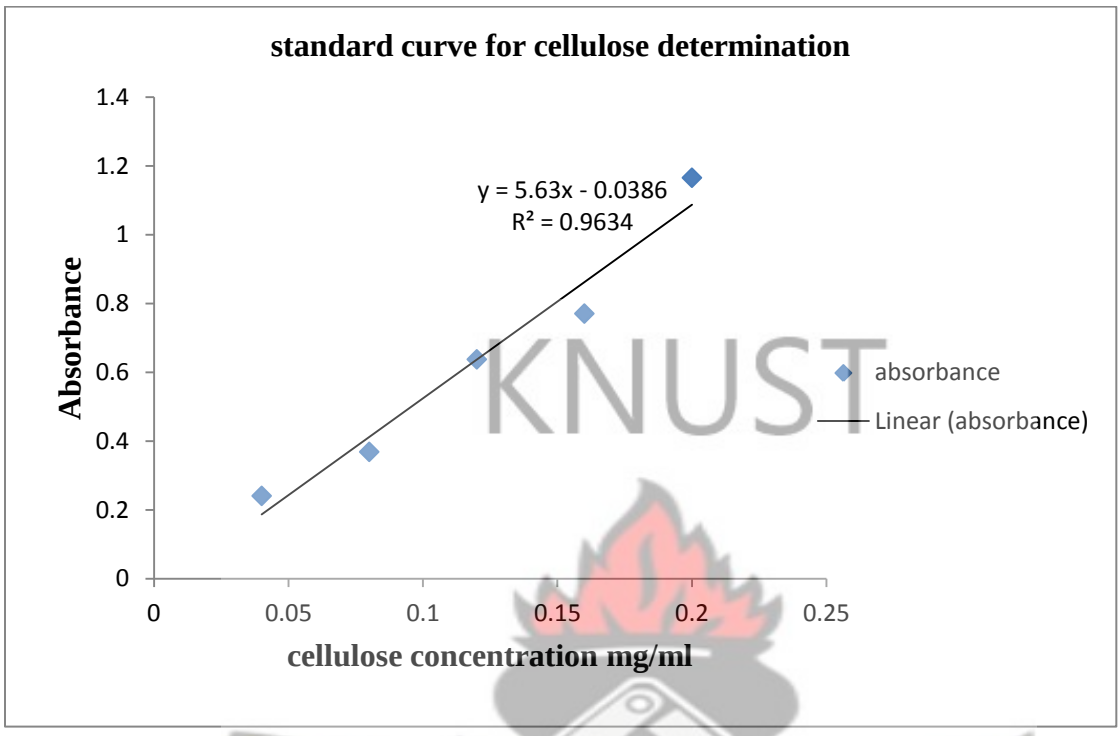
Preparation of stock solution of glucose

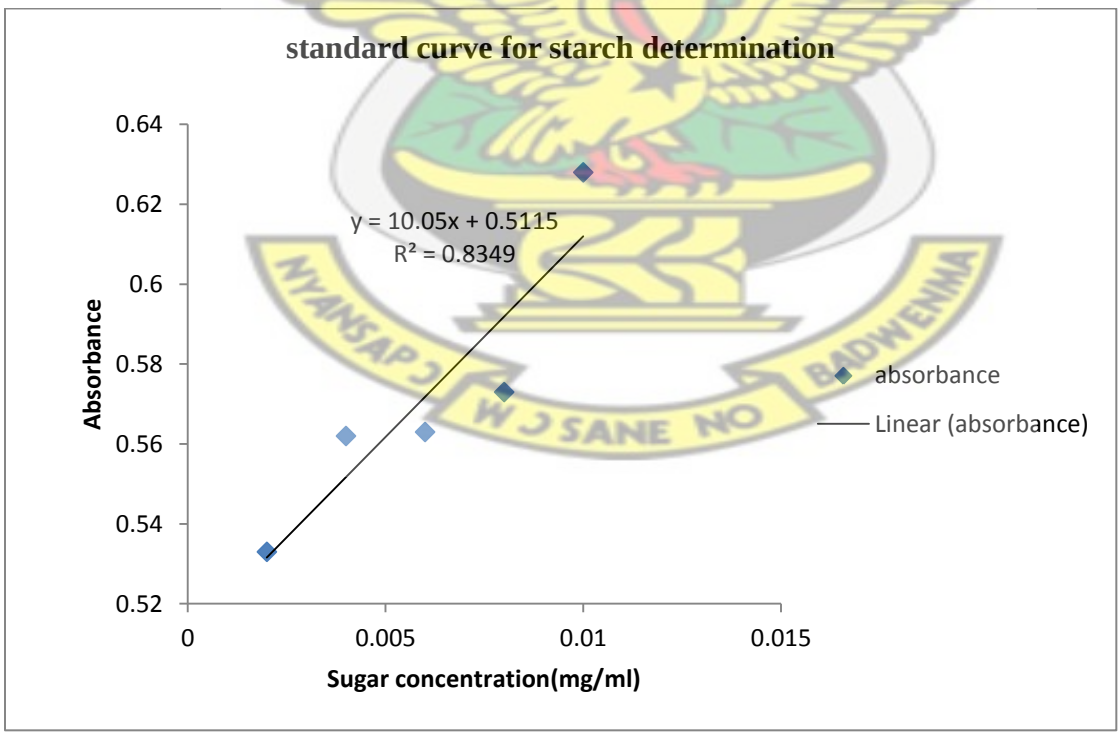
Standard stock solution having the concentration of 1 mg glucose ml^{-1} was prepared by dissolving 100 mg of D-glucose in small amount of distilled water and final volume was made up to 100 ml with distilled water.

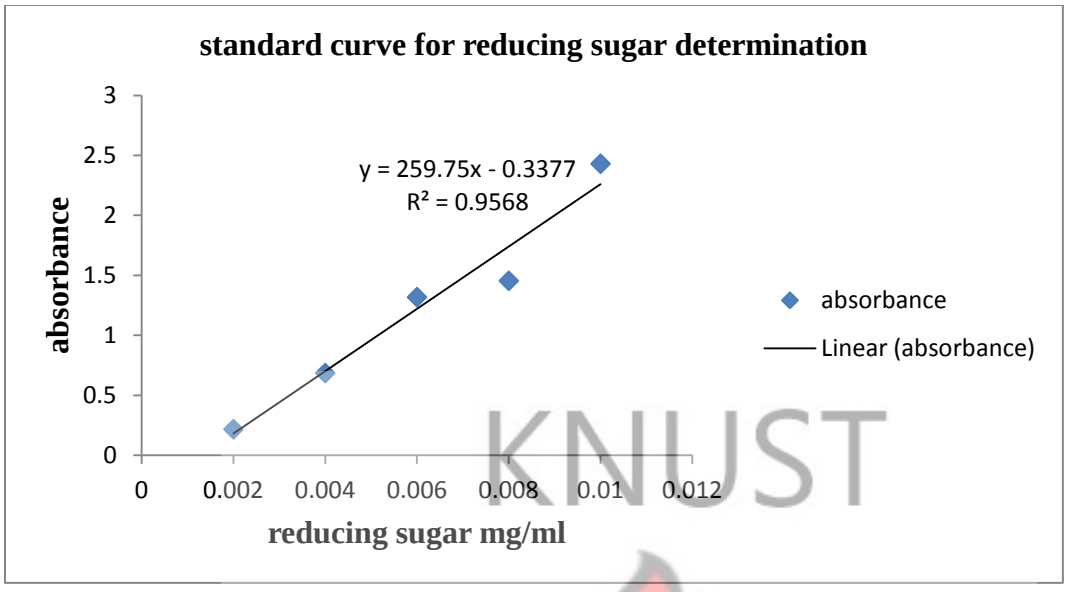
Preparation of working standard of Glucose

About 10 ml of the stock was diluted to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 100 μg glucose ml^{-1}.

APPENDIX C: Standard curves






standard curve for reducing sugar determination
absorbance
reducing sugar mg/ml
y = 259.75x - 0.3377
R² = 0.9568
absorbance
Linear (absorbance)
0
0.5
1
1.5
2
2.5
3
0.002
0.004
0.006
0.008
0.01
0.012

APPENDIX D: ANOVA and T-test Analysis

ANOVA table for effect of pH an ethanol production for *Saccharomyces cerevisiae*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	0.8086	5	0.16172	98.93	0.0000
Within groups	0.0098085	6	0.00163475		
Total (Corr.)	0.818409	11			

ANOVA table for the effect of pH on ethanol production by *Zymomonas mobilis*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	2.70034	5	0.540068	336.39	0.0000
Within groups	0.009633	6	0.0016055		
Total (Corr.)	2.70997	11			

ANOVA table for effect of Temperature an ethanol production by *Saccharomyces cerevisiae*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	0.183676	3	0.0612252	48.57	0.0000
Within groups	0.0100853	8	0.00126067		
Total (Corr.)	0.193761	11			

ANOVA table for the effect of Temperature ethanol production by *Zymomonas*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	3.77846	3	1.25949	311.67	0.0000
Within groups	0.0323287	8	0.00404108		
Total (Corr.)	3.81079	11			

ANOVA table for the effect of fermentation period on ethanol production by *Saccharomyces cerevisiae*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	0.887272	4	0.221818	65.33	0.0000

Within groups	0.0339513	10	0.00339513		
Total (Corr.)	0.921224	14			

ANOVA table for the effect of fermentation period on ethanol production by *Zymomonas mobilis*

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	1.76865	4	0.442163	210.19	0.0000
Within groups	0.0210367	10	0.00210367		
Total (Corr.)	1.78969	14			

T-test Table for sugar utilization between *Saccharomyces cerevisiae* and *Zymomonas Mobilis*

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day 2	Equal variances assumed	.	.	22.000	2	.002	.04469	.00203	.03595	.05343
	Equal variances not assumed			22.000	1.000	.029	.04469	.00203	.01888	.07050
Day 3	Equal variances assumed	7.096E-10	1.000	69.296	2	.000	.09953	.00144	.09335	.10571
	Equal variances not assumed			69.296	2.000	.000	.09953	.00144	.09335	.10571
Day 4	Equal variances assumed	8.550E+15	1.110E-16	5.973	2	.027	.13305	.02227	.03721	.22889
	Equal variances not assumed			5.973	1.389	.059	.13305	.02227	-.01704	.28314
Day 5	Equal variances assumed	2.456E+16	.000	27.412	2	.001	.22446	.00819	.18922	.25969
	Equal variances not assumed			27.412	1.031	.021	.22446	.00819	.12757	.32134

T-test Table for sugar utilization between *Saccharomyces cerevisiae* and *Zymomonas Mobilis*

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Day 2	Equal variances assumed	.	.	22.000	2	.002	.04469	.00203	.03595	.05343
	Equal variances not assumed			22.000	1.000	.029	.04469	.00203	.01888	.07050
Day 3	Equal variances assumed	7.096E-10	1.000	69.296	2	.000	.09953	.00144	.09335	.10571
	Equal variances not assumed			69.296	2.000	.000	.09953	.00144	.09335	.10571
Day 4	Equal variances assumed	8.550E+15	1.110E-16	5.973	2	.027	.13305	.02227	.03721	.22889
	Equal variances not assumed			5.973	1.389	.059	.13305	.02227	-.01704	.28314
Day 5	Equal variances assumed	2.456E+16	.000	27.412	2	.001	.22446	.00819	.18922	.25969
	Equal variances not assumed			27.412	1.031	.021	.22446	.00819	.12757	.32134