BIOETHANOL PRODUCTION FROM BANANA (*MUSA SAPEINTUM)* PEELS WASTE USING CO-CULTURES OF *ASPERGILLUS NIGER* AND *SACCHAROMYCES CEREVISIAE*

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BY

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DEPARTMENT OF MICROBIOLOGY,

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ZARIA, NIGERIA

NOVEMBER, 2016

DECLARATION

I declare that the work in this dissertation entitled bioethanol production from banana waste peels using co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* has been carried out by me, in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Jovita Nevan CHECHET ---------- ----------

Signature Date

CERTIFICATION

This dissertation entitled BIOETHANOL PRODUCTION FROM BANANA PEELS USING CO-CULTURE OF *ASPERGILLUS NIGER* AND *SACCHAROMYCES CEREVISIAE* by Jovita Nevan CHECHET meets the regulations governing the award of degree of Masters in Science (Microbiology) of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to my Late Parents Mr. & Mrs. Alfred .T.S Chechet, for their love and encouragement they gave me which inspired me to enroll on this academic programme.

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ABSTRACT

Bioethanol in this study was produced from banana peels using the process of submerged fermentation, *Aspergillus niger* and *Saccharomyces cerevisiae* were the microorganisms used which were previously isolated from soil and *burukutu* respectively. The fermentation medium was composed of 5% inoculum of *A. niger* and *S. cerevisiae* respectively. The ethanol yield after fermentation was determined by checking its specific gravity after distillation and the value obtained was compared with that of a standard ethanol. The effects of substrate concentration, pH, temperature and time on ethanol yield were determined. After fermentation, ethanol yield was found to be maximum at 8% substrate concentration (9.66%), pH 5.5 (10.52%), 35^{o}C (12.63%) for (3) days. However ethanol yield was influenced by many factors especially temperature, pH, substrate concentration and time.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Bio-ethanol is an alternative source of energy and has received special attention worldwide due to the depletion of fossil fuels, under such circumstances a novel approach is essential to use renewable substrates such as fruit wastes to produce ethanol (Brooks, 2008).

Banana is one of the major foods that constitute the principal food resources in the world and occupied the fourth world rank of the most significant foodstuffs after rice, corn and milk (INIBAP, 2002). Banana peels are not considered very useful and are therefore dried, ground, pelletized, and sold to the feed manufacturers at a low price (Mamma *et al.,* 2008). Though banana peel is a fruit residue, it accounts for about 30-40% of the total fruit weight and contains carbohydrates, proteins, and fibre in significant amounts (Emaga *et al.,* 2008). Banana peels are readily available agricultural wastes that are under utilized as potential growth medium for yeasts, despite their rich carbohydrate content and other basic nutrients that can support yeast growth (Essien *et al*., 2005). Since banana peels contain lignin in low quantities, it could serve as a good substrate for production of value-added products like ethanol (Hammond *et al.,* 1996).

Fermentation is a metabolic process of converting carbohydrates to alcohol or organic acids using microorganisms usually yeasts or bacteria under anaerobic conditions. However similar process takes place in the leavening of bread and in the preservation of sour foods such as sauerkraut and yoghourt with the production of lactic acid (McGovern *et al.,* 2004). In order to make the fermentation method cost effective and to meet the great

demand for ethanol, research studies are now being directed in two areas namely; the production of ethanol from cheaper raw materials and the study of new microorganisms or yeast strains efficient in ethanol production (Akin-Osanaiye *et al*., 2008). In this respect, inexpensive raw materials such as agricultural wastes, cellulosic wastes, fruit wastes, vegetable wastes, municipal and industrial wastes can be used to produce ethanol cheaply. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Joshi *et al*., 2001).

Microorganisms use for bioethanol production must have the ability to withstand high level of acidity, temperature, high concentration of ethanol in fermentation and it must be able to utilize high concentration of sugar and also ferment varieties of sugars (Sean and Johann, 2015).

Commercial amylases are enzymes produced by microorganisms; such enzymes hydrolyze starch molecules to glucose units. Amylases have potential application in a wide number of industrial processes such as fermentation and in some pharmaceuticals industries (Paula and Perola, 2010).

Fruits are edible products of plants usually soft and fleshy, their high moisture content made them perishable in the freshly harvested state due to spillage, physiological decay, water loss, mechanical damage during harvesting and packaging (Shubhra *et al*., 2014).

Agricultural waste that are generated during processing of agricultural products are usually utilize as low cost raw materials for the production other value products such as

citric acid, bioethanol and gluconic acid with the expectancy of reducing the cost of production (Latika *et al.,* 2012).

1.2 Statement of Research Problem

Waste management is a global issue which has become increasingly serious with population and economic growth, particularly in developing countries. It is a major challenge faced by government at all levels in Nigeria. Efforts by the various management authorities towards an effective and efficient waste management have yielded little results. The quantity and rate of solid waste generation in various states of Nigeria depend on the population, level of industrialization, socio-economic status of the citizens and the kinds of commercial activities that is predominant (Abiodun, 2007).

The use of other wastes for Bioethanol production other than banana peels; such as corn cobs, cassava peels, waste paper, sugar cane baggase and sawdust, this waste ends up in open dumps or drainage systems, threatening both surface water and ground water quality. It is therefore necessary to convert these wastes to useful end products rather than allowing them to become a nuisance to the environment.

The choice of microorganisms that are efficient and effective for bioethanol production has imposed another problem, as it is not all microorganisms that have good fermentative and hydrolytic ability suitable for breaking down complex sugars and fermenting the simple sugar for ethanol production (Sambo, 2000).

1.3 Justification of the Research

Since the production of commercial ethanol is expensive, there is the need to utilize cheap agricultural wastes to produce ethanol in order to maximize profit by the industrialist. The Nigerian economy is based mainly on income from fossil fuel which is

non renewable and hence gets depleted on daily basis. Officially, about 2.3 - 2.5 million barrels of crude oil is produced daily and geologists have raised alarm that the Nigerian oil reserves may be totally depleted in 37 years unless alternative sources of fuel are introduced. Meanwhile the developed nations, which are the major consumers of Nigerian crude oil, are making serious efforts to find alternative sources of fuel. Lots of attention is focused on the use of the abundant and renewable waste resources to generate energy in form of bio-fuel (Sambo, 2000).

Therefore, with the trend in technological advancement, leading to the depletion of fossil fuels, the need for alternative sources of energy is inevitable. As an energy resource, plant biomass may be used as solid fuel, or converted via a variety of technologies to liquid or gaseous forms for the generation of electric power, heat or fuel for locomotive power. Biomass resources are considered renewable as they are naturally occurring and when properly managed, may be harvested without significant depletion. The use of renewable biomass wastes to generate energy in form of biofuel will not only provide efficient and inexpensive sources of energy, but also help to solve environmental problems (Sambo, 2000).

1.4 Aim of the Research

The aim of this research was to produce bio-ethanol from banana peels by a fermentation process using co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae.*

1.5 Specific Objectives

The specific objectives of this research were to;

1. isolate and characterize *Aspergillus niger* and *Saccharomyces cerevisiae* used for the fermentation of banana peels
2. prepare the banana peel flour and determine its proximate composition.
3. carryout submerged fermentation of banana peel flour and determine its ethanol content.
4. determine the effects of substrate concentration, pH, temperature and time on the ethanol yield

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background of Bioethanol

Bioethanol is a readily available clean fuel for combustion engines made from plant-based feedstocks. It produces considerably lower emissions on combustion and it only releases the same amount of carbon dioxide as plants bound while growing.

Ethanol or ethyl alcohol has existed since the beginning of recorded history; the ancient Egyptians produced alcohol by naturally fermenting vegetative materials. Also in ancient times, the Chinese discovered the art of distillation, which increases the concentration of alcohol in fermented solutions.

Ethanol was first prepared synthetically in 1826, through the independent effort of Henry Hennel Ford in Britain. Michael Faraday prepared ethanol by the acid-catalyzed hydration of ethylene in 1828, in a process similar to that used for industrial synthesis of ethanol today. In 1893, Henry Ford built a small one cylinder gasoline combustion engine after which he invented a quadricycle; the first horseless carriage that is propelled by bioethanol powered engine. Ford later turn his interest to automobiles where he design a delivery wagon and finally he invented the first Ford Motor Company Automobile which was designed to use corn alcohol called ethanol. After the incoperation of this company, the association of license automobile manufacturers threatened to put Ford out since he was not a licenced manufacturer; however Ford fought the claim and won which open doors for the rapid growth of automobile industries. Ethanol was used as fuel for lamp in

the United States in 1840, but a tax levied on industrial alcohol during the civil war made this use uneconomical, but this tax was repelled in 1906. In 1907, Henry Ford re-introduced ethanol to the Americans motoring public by producing his first vehicle to run on ethanol (John, 2006).

The most common substrate used for nearly 99% of ethanol production in the United States today is starch from agricultural crops, primarily corn (Raneses *et al.*, 1998). In 1940s the first fuel ethanol plant was built in the U.S. army built and operated an ethanol plant in Omaha, Nebraska, to produce fuel for the army and for regional fuel blending. Major quantities were not manufactured until the 1970s due to low cost of gasoline between 1940s and 1970s; however the ethanol industry began to re-emerge when ethanol was used as a fuel extender during gasoline shortages (Raneses *et al.*, 1998). Brazil kept bioethanol production programme alive by investing heavily on renewable fuels leading to the development of extensive bioethanol industry and also the number of ethanol running cars increased to about 90% of all the new cars sold in Brazil thereby making the country the biggest market for an alternative fuel in the world, in 1988 ethanol began to be added to gasoline inorder to reduce CO_2 emission. However on market conditions, all fuels are required to be blended with 20-25% of ethanol. As the production has increased the effect of biofuel on agricultural markets and the environment has become increasingly important topics, yet much uncertainty still remains. Biofuel has the potential to displace the use of petroleum as a transportation fuel and lower toxic emissions, the evolution of new biofuel production technologies could help alleviate some of the concerns regarding the use of food for fuel by facilitating the use of non-food feedstock's, and could alleviate some of the environmental concerns associated with grain

ethanol production. In particular, cellulosic ethanol is believed to hold great promise in this regard, even though there are currently no commercial scale plants in the United State (John, 2006).

2.1.1 Global Bioethanol Production

World production of ethanol was 14.1 billion gallons in 2007 and 65.7 billion litres in 2008 and it is believed that it will soon exceed 100 billion litres. With the largest increase in production in United States of America and Brazil. One of the reasons is that attention has been globally concentrated to ethanol production in the quest for biofuel. United States is producing 46% of the world ethanol and Brazil is producing 37% making them the highest producer of ethanol in the world. There is a continuous increase in global demand for bio-ethanol in the market for this essential commodity is expected to reach 100 $x10^9$ litres. This is necessitated by the use of ethanol for various industrial purposes such as fuel, industrial solvents, cleansing agents and preservatives (Brooks, 2008). Recently, China became one of the largest ethanol producers as a result of enormous investment in the production of ethanol (Ivanova *et al.*, 2011).

Recent analysis of sugarcane and sweet sorghum as bio-ethanol feedstock in Nigeria revealed that sweet sorghum is better suited in terms of its adaptability to harsh climatic conditions and the position Nigeria occupies in the world in terms of cassava production and waste generated annually in cassava processing placed the country in a better position to join the world of bio-ethanol production (Nasidi *et al*., 2010).

2.1.2 Bio-Ethanol Production in Nigeria

Nigeria being eager to meet up with the demand of bio-fuels has a policy to meet ten percent bio-ethanol content in fuel by 2020 (Halilu, 2008). In pursuance of this policy,

the Chief Olusegun Obasanjo's regime marked thousands of hectares of virgin land for cultivation of cassava to supply feedstock for production of bioethanol. The cassava, as it was called, did not however, succeed. In a new development, Global Bio-fuels Limited has embarked on a project which use about 10,000 hectares of virgin land (forest and grasslands). Covering seven states (Osun, Oyo, Kwara, Ondo, Ekiti, Niger and Kogi) in Nigeria, to cultivate sweet sorghum for bioethanol fuel production (Azih, 2007). Global Bio-fuels Limited plans set up seven plants each valued at over 3 billion U.S dollars (345 billion naira) in the seven states, to produce about 1 million litres of ethanol per plant on an ethanol fuel production project in Nigeria.

Nigerian National Petroleum Cooperation (NNPC) as at 2006 has so far worked out plans to acquire farmlands in Anambra, Benue, and Cross River for large scale cultivation of cassava. It has acquired a large plantation site of over 20,000 hectares at Agufa village for the large scale production of cassava and sugarcane in Jigawa State. In addition, the venture will enable the Jigawa Government to acquire world-class technology and farm management best practices in the large scale production of sugarcane (Azih, 2007).

2.2 Uses of Bioethanol

2.2.1 Ethanol as disinfectants

Alcohols are important disinfectants; they evaporate quickly without leaving any residue. They are capable of dissolving lipids which makes them effective against lipid-wrapped viral and bacterial cells but are ineffective against spores. They are inexpensive and relatively easy to handle, although their vapours are flammable, it is usually used in

concentration of 70% because higher concentration evaporates too quickly and lower concentration are ineffective (Pandey *et al.,* 2013).

2.2.2 Ethanol as domestic lighting agent

Alcohol burning stoves based on ethanol can be used for cooking or water boiler for households, institutions and industries. Ethanol that are produced from plant biomass has the advantage of not producing air pollution problems of simple biomass burning for cooking purpose as ethanol produces a higher heat flux with no soot or smoke, cooking and hot water production can take pace faster and pollution free.

The equipment required for ethanol burning stoves is similar to existing kerosene stoves. Some ethanol stoves are made from stainless steel in order to minimize corrosion (Johnson *et al.,* 2012).

2.2.3 Ethanol as transportation fuel

Bioethanol is a product of microbial fermentation as opposed to synthetic ethanol produced from petrochemical sources. It is obtained from fermentation of sugars and used as biofuel for internal combustion engines or blend with petroleum. Ethanol has been used as a motor fuel since cars were first manufactured. It is a fuel well-suited for petrol engines; it has a clean combustion process resulting in lower emissions of particulate matter, hydrocarbons and carbon monoxide. The greatest benefit of using bioethanol is the reduction in CO_2 emissions based on life-cycle processes. Bioethanol is a perfect transportation fuel that has high compression ratios to internal combustion engines because of it high octane ratio compare to petroleum. This low volatility enhances engine output per cycle and prevents automobile engine from getting knock. Although, vehicles running on pure ethanol have high fuel consumption rate of one liter per kilometer that is

10-20% less than petrol, the fact that the by-product of its combustion could be used by plants for growth makes it a neutral carbon fuel (Johnson *et al*., 2012).

Bioethanol has been found to have a potential to decrease greenhouse gases, depending on the production method. Bioethanol represents the best alternative transportation fuel because it use is projected to increase significantly and remain high. Plant biomass when used as transportation fuel, it helps to keep engines running smoothly without the need for lead or other chemical additives (Graeme *et al.*, 2010)

2.3 Bio-Ethanol Production Process

Currently, the first generation processes for the production of ethanol from corn use only a small part of ethanol from the plant, the corn kernels are taken from the corn plant and only starch, which represents about 50% of the dry kernel mass, is transformed into ethanol. (Halilu, 2008), stresses that two types of second processes are under development. The first type uses enzymes and yeast to convert the plant cellulose or starch into ethanol while the second type uses pyrolysis to convert the whole plant to either a liquid bio-oil. Second generation processes can be used with plants such as grasses, wood or agricultural waste material such as straw. The basic steps for large scale production of ethanol are:

- Milling and hydrolysis
- Microbial (yeast) fermentation.
- Distillation.
- Dehydration and
- Denaturing (optional).

2.3.1 Milling and Hydrolysis

Milling and hydrolysis which involves the breakdown of starch molecules into glucose by enzymatic process. Prior to fermentation, some crops require saccharification or hydrolysis of carbohydrates such as starch or cellulose into sugars (Leen *et al*., 2007).

2.3.2 Fermentation

Fermentation is the process that converts glucose to ethanol. Ethanol is produced by microbial fermentation of the sugar; microorganism will only work directly with sugars. Two major components of plants are; starch and cellulose, both are made up of sugars and can be converted to sugars for fermentation. Currently, only the sugar (for example, sugarcane) and starch (for instance, corn) portions can be economically converted. However, there is much activity in the area of cellulosic ethanol, where the cellulosic part is broken down to sugars and subsequently converted to ethanol (McGovern *et al*., 2004).

2.3.3 Distillation

Further treatment in order to burn in combination with gasoline in gasoline engines, dehydration of the ethanol to be useable as a fuel must be done. Most of the water is removed by distillation, but the purity is limited to 95-96% due to the formation of a low-boiling water ethanol zoetrope. 95.5% v/v ethanol, 3.3% v/v water mixture may be used as a fuel alone, but unlike anhydrous ethanol, is immiscible in gasoline, so the water fraction is typically removed (Kravchenko *et al*., 2014).

2.3.4 Dehydration

There are various dehydration processes to remove the water from an azeotropic ethanol/water mixture. The first process used in many early fuel ethanol plants is called

azeotroppic distillation and consist of adding benzene or cyclohexane to the mixture. When these components are added to the mixture, it forms a heterogeneous azeotropic mixture in vapor-liquid-liquid equilibrium, which when distilled produces anhydrous ethanol in the column bottom, and a vapor mixture of water and cyclohexane/benzene. When condensed, this becomes a two-phase liquid mixture. Another early method, called extractive distillation consists of adding tertiary components which will increase ethanol relative volatility. When the mixture is distilled, it will produce anhydrous ethanol on the top of the column (Ravagni *et al.*, 2010)

With increasing attention being paid to saving energy, many methods have been proposed that avoid distillation all together for dehydration of these methods. A third method has emerged and has been adopted by the majority of modern ethanol plants. This new process uses molecular sieves to removes water from fuel ethanol. In this process, ethanol vapor under pressure passes through a bed of molecular sieve beads. The bead's pores are sized to allow absorption of water while excluding ethanol. After a period of time, the bed is regenerated under vacuum or in the flow of inert atmosphere (N) to remove the absorbed water. Two beds are used so that one is available to absorb water while the other is being regenerated. This dehydration technology can account for energy saving of 3,000 gallon (840 kj/l) compared to earlier azeotropic distillation (Kravchenko *et al*., 2014).

2.4 Chemistry of Bioethanol

Glucose is synthesized in plant by photosynthesis (Shubhra *et al.,* 2014).

$6CO_2 + 6H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2$

During ethanol fermentation, glucose is decomposed into ethanol and carbon dioxide.

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

During combustion, ethanol reacts with oxygen to produce carbon dioxide, water and heat.

$2C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O + heat$

Plant also contains other sugars apart from glucose such as fructose, sucrose, starch and cellulose that can be fermented. Ethanol may also be produced industrially from ethene (ethylene) by the addition of water to the bond in the presence of acid which can catalyse the reaction but it is not consumed (Shubhra *et al.*, 2014).

$CH_2 = CH_2 + H_2O \rightarrow CH_3CH_2OH$

2.4.1 Properties of Ethanol

Ethanol is a volatile, flammable, colourless liquid with a slight chemical odour. The molecule has an ethyl group linked to a hydroxyl group. The physiochemical properties of ethanols are associated with their chemical structures (Lide, 2012). The summary of the properties of ethanol include the following;

Molecular formula	C_2H_5OH
Molar mass	46.07gmol/mol
Appearance	Colourless liquid
Density	$0.789/cm^3$
Melting point	-114.30C
Boiling point	78.40C
Solubility in water	Miscible
Acidity (Pk_a)	15.9
Basicity (pK_b)	-1.9
Viscosity	1.2 mPa.s (at 20^oC)

Dipode moment 1.69D (Lide, 2012)

2.5. Process Description of Simultaneous Saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) technique is one of the new bioprocess technologies that have been developed that have the potential to lower the cost of ethanol production and improve the competitiveness of ethanol as a fuel additive (Bothas and Saba, 1997). This process has been found to be superior to saccharification and subsequent fermentation due to the rapid assimilation of sugar by the yeast (Krishna *et al*., 2001). SSF involves co-culturing of two organisms with synergistic relationship leading to a rapid fermentation of sugar to ethanol by using *S. cerevisiae* which had been found to keep the sugar concentration low enough to prevent feedback inhibition of amylolytic activity observed in monoculture of *Aspergillus niger*.

However, in some studies, it was observed that monoculture of *A. ni*ger tends to favour higher cell mass production, whereas in the co-culture, most of the substrate carbon is utilized for ethanol production (Cao *et al*., 1996) The process involved the enzymatic hydrolysis of starch to fermentable sugar and subsequent conversion to ethanol in the same fermenter, thereby preventing the inhibiting effects of sugar on amylolytic activity of *A. niger* (Ohgren *et al.,* 2006).

The conversion of biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugars, still suffers severe problems that are economical and technical in nature. This process of SSF was found to increase yield of ethanol not only by eliminating inhibition effects, but also by eliminating the need of separate reactors for saccharification and fermentation and also reduced

economic problems consisting of cost of production and purification of hydrolytic enzymes (Deshpande *et al*., 1983).

2.6 Advantages and Disadvantages of Bio-Ethanol

2.6.1 Advantages of Bioethanol include;

- Exhaust gases from bioethanol are much cleaner, it burns more cleanly (complete combustion)
- So many varieties of plants and plant wastes can be used for the production of bioethanol, it only has to contain sugar and starch.
- It is carbon neutral that is; the carbondioxide released in the bioethanol production process is the same amount plants absorbed during photosynthesis
- The net effect of bioethanol used results in an overall decrease in ozone formation; the emissions produced by burning bioethanol is less reactive with sunlight than those produce by burning gasoline
- It benefits energy security as it shifts the need for some foreign-produced oil to domestically produced energy source especially in countries that don't have access to crude oil resources can grow crops for energy use and gain some economic freedom
- The fuel spills are more easily degraded to non toxic concentrations (Johnson *et al.*, 2012).

2.6.2 Disadvantages of Bioethanol include;

- A large area of land is required to grow crops; this could lead to the destruction of some natural habitat including rainforests.

- The energy content of the petrol is much higher than that of bioethanol; burning 1 liter of ethanol gives 34% less energy than burning petrol of the same amount, hence bioethanol is less efficient as compared to petroleum
- It can negatively affect electric fuel pumps by increasing internal wear and undesireable spark generation
- It is not compatible with capacitance fuel level gauging indicators and may result in erroneous fuel quantity indications in vehicles that employ that system
- Bioethanol is also difficult to vaporize which can make starting a car in cold weather difficult (Johnson *et al.,* 2012).

2.7 Banana Production

Bananas are perennial crops that take the appearance of trees as they mature. Diverse cultivars are grown, *Musa* are believed to have originated in Southeast Asia but their introduction into Africa is unclear. Throughout history *Musa* has provided humans with food, medicine, clothing, tools, shelter, furniture, paper, and handicrafts. It could be termed as the "first fruit crop" as its cultivation originated during a time when hunting and gatherings were still the principal means of acquiring food (Happi *et al.,* 2011).

Bananas are important staple foods in many developing countries, especially in Africa of the numerous edible varieties. They provide food security and income for small-scale farmers who represent the majority of producers. Banana starch, flour, and chips are processed into banana products whose markets are yet to be fully developed (Happi *et al.,* 2011).

Black Sigatoka disease is considered the most economically important disease of banana worldwide, causing typical yield losses up to 50%. The fungus grows on the leaves producing dark spots and causes the fruits to ripen prematurely. Banana *Xanthomonas Wilt* (BXW) attacks almost all varieties of Musa, destroying the fruits and devastating the crop. *Fusarium wilt* has had a huge impact on the world banana trade and is found in every banana producing area, it is spread through the corms used for planting. The major banana pests are the burrowing nematode and the banana weevil. Nematode species attack the plant's roots, resulting in reduced yield. The banana weevil, *Cosmopolites sordidus*, attacks the plant's underground corm, weakening the plant and causing stem breakage.

2.7.1 Banana Peel

Banana peel also known as a banana skin, is the outer covering of the banana fruit. Bananas are popular fruit consumed worldwide with a yearly production of over 145 million tones in 2011. Once the peel is removed, the fruit can be eaten raw or cooked and the peel is generally discarded. Because of the removal of the banana peel, there is a significant amount of organic waste being generated (Babatunde, 1992).

Banana peels are used as feedstock as they have some nutritional value; the peels are widely used for that purpose on small farms in regions where bananas are grown. Banana peels are used as feedstock for cattle, goats, pigs, poultry, rabbits, fish and several other species (Onwuka *et al*., 1997).

The specific nutrient contained in peels depends on the stage of maturity and the cultivar; for example plantain peels contain less fibre than dessert banana peels, and lignin content increases with ripening (from 7 to 15% dry matter). On average, banana peels contain 6-9% dry matter of protein and 20-30% fibre (measured as NDF). Green

plantain peels contain 40% starch that is transformed into sugars after ripening. Green banana peels contain much less starch (about 15%) and ripe banana peels contain up to 30% free sugars. Banana peels are also used for ethanol production, cellulase, and as fertilizer and in composting (Kalemelawa *et al.,* 2012).

2.8 Microorganisms Explored for Ethanol Production

Microorganisms that can ferment varieties of sugars and possess the ability to withstand alcohol and sugar stress conditions during fermentations are expected to be used for ethanol production. Such organisms include *Zymomonas mobilis, Saccharomyces cerevisiae, Saccharomyces uvarum, Candida tropicalis, Candida shehatae, Aspergillus niger,* and *Clostridium* species.

Saccharomyces cerevisiae and *Zymomonas mobilis* are two microorganisms that are efficiently used in industrial production of alcohol. Low ethanol yield was recorded with molasses and the possible reason could be impurities in raw molasses that inhibit yeast growth and fermentation (Bajaj *et al*., 2001). *Zymomonas mobilis* is much more effective than *Saccharomyces cerevisiae* in terms of high ethanol productivity due to its ability to ferment hexoses at very high rate under anaerobic conditions (Davis *et al*., 2005), and can utilize sucrose, glucose, and fructose by Entner-Doudoroff pathway. However, these characteristics can be enhanced when the organism is genetically manipulated (Ramasamy and Ramasa, 2001). Several researchers have metabolically engineered the *Zymomonas mobilis* to broaden its range of fermentable substrates to include the pentose, xylose, hexoses, glucose, fructose, sucrose and arabinose, which are found in renewable biomass feed stocks and agricultural residues (Rogers *et al*., 2007). Recently, it was reported the ability of *Candida tropicalis* in ethanol production.

This organism can utilize starch at low rate and to enhance the carbon source uptake and increase ethanol yield, (Jamai *et al*., 2007).

Other organisms explored in the past for ethanol production include *Trichoderma-Saccharomyces, Clostridium thermocellum and Thermoanaerobium brockii* (Davis *et al*., 2005).

2.8.1 Microorganisms of Interest in this Research

2.8.2 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common species of the genus Aspergillus. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of Starchy botrys (species of which have also been called "black mold") (Verweij *et al*., 2007).

World Health Organization supports the view that *Aspergillus niger* can be cultured for the industrial production of citric acid and gluconic acid that are safe for human consumption (Schuster *et al*., 2002). The organism is also explored for production of enzymes like glucosidase, amylase, cellulase, pectinase and protease.

When *Aspergillus niger* is cultured on Sabouraud dextrose agar or potato dextrose agar and incubated at 25^0C, they tend to produce spores within 5-7 days (Verweij *et al*., 2007). Macroscopic and morphological identification is based on colony pigmentation, and the structure of the conidial head. Microscopic mounts can be done using a cellotape flag or slide culture preparation mounted in lactophenol cotton blue. A drop of alcohol is needed to detach the cellotape flag from the stick, and to act as a wetting agent (Verweij *et*

al., 2007). Colonies on potato dextrose agar are wooly initially white, quickly becoming black with conidial production. Hyphae are septate and hyaline, conidial heads are radiate initially, splitting into columns at maturity. Conidiophores are long, smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle. Conidia are brown to black, very rough, globose, and measure up to 6 or 7µm diameter (Larone, 2002).

According to the research work of Highina *et al.,* (2012), the hydrolysis of wheat using *Aspergillus niger*, shows the optimum range of temperature, pH and particle size are 45 - 50^0C, 4.5 - 5.0 and 75µm - 150µm respectively and that the substrate concentration increases from 1.0gL^{-1} to 10gL^{-1}, after a hydrolysis time of 8 hours, and when cell loading increases, glucose concentration also increases.

2.8.2.1 Amylase Enzymes

Amylase enzymes are enzymes that catalyses the hydrolysis of starch into sugars (oligosaccharides) of different lengths by breaking α-D-(1-4) glycosidic bonds, the enzyme can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in the industrial sectors. The production of this enzyme in submerged fermentation depends on a variety of physicochemical factors such as; temperature, pH and level of aeration (Kandra *et al.,* 2003).

Alpha-amylase produced by fungi have been limited to a few species of mesophilic fungi and most of the fungal sources for commercial scale are confined to terrestrial isolates mostly to *Aspergillus* and *Penicillium* (Kathiresan *et al.,* 2006). *Aspergillus* species (*A. niger and oryzae)* produce a large variety of extracellular enzymes that are of the most significant importance in the industry. *Aspergillus oryzae* has received

increased attention as the favourable host for the production of heterogenous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes (amylase) while *Aspergillus niger* has important hydrolytic capacities in the α-amylase production due to its tolerance of acidity (pH□ 3) which allows it to prevent bacterial contamination (Djekrif *et al.,* 2006).

Generally, the saccharification of starch in most of the bio-processing industries is mostly carried out by enzymes compared to acid this is because the conversion of raw starch by amylase is better compared to the conventional methods that make use of pre-gelatinized starch as substrate. (Couto *et al.,* 2006).

2.8.2.2 Uses of Amylase

Microbial amylases have been exploited in a variety of purposes which include;

- Addictive to detergents for removing stains
- Saccharification of starch for alcohol production and in brewing processes
- For the production of high fructose corn syrup (Couto *et al.,* 2006).

2.8.3 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a eukaryotic microbe that posses a globular-shaped, creamy-white yeast belonging to the fungi kingdom, which includes multicellular organisms such as mushrooms and molds. Natural strains of the yeast have been found on the surfaces of plants, the gastrointestinal tracts and body surfaces of insects and warm-blooded animals, soils from all regions of the world and even in aquatic environments. Most often it is found in areas where fermentation can occur, such as on the surface of

fruit, storage cellars and on the equipment used during the fermentation process (Mortimer, 2000).

Saccharomyces cerevisiae is famously known for its role in food production; it is the critical component in the fermentation process that converts sugar into alcohol an ingredient shared in beer, wine and distilled beverages. It is also used in the baking process as a leavening agent; yeast releasing gas into their environment results in the spongy-like texture of breads and cakes. Because of its role in fermentation, humans have known about and have been using *Saccharomyces cerevisiae* for a long time (Landry *et al*., 2006). Morealso, *S. cerevisiae* is also considered to be a "model organism" by scientists because of its big advantage of being both a unicellular and eukaryotic organism (Legras *et al*., 2007).

2.8.3.1 Macroscopic morphology

Colonies of *Saccharmyces cerevisiae* on solid media are usually white or creamy in colour, smooth or semi matt up to 5mm in diameter while in liquid media; sediment is formed, sometimes with ring growth at the air-liquid-glass junction but pellicle is not formed.

2.8.3.2 Microscopic morphology

Cells of Saccharomyces *cerevisiae* when stained with lactophenol cotton blue are found to be spherical or short ovoid cells in shape under the microscope with a multilateral type of budding.

2.8.4 Metabolic pathway of fermentation by Saccharomyces cerevesiae

Saccharomyces cerevisiae can live in both aerobic as well as anaerobic conditions. In the presence of oxygen, yeast can undergo aerobic respiration, where glucose is broken to CO_2 and ATP is produced by protons falling down their gradient to an ATPase. When oxygen is lacking, yeast only get their energy from glycolysis and the sugar is instead converted into ethanol, a less efficient process than aerobic respiration. The main source of carbon and energy is glucose, and when glucose concentrations are high enough, gene expression of enzymes used in respiration are repressed and fermentation takes over respiration. However, yeast can also use other sugars as a carbon source. Sucrose can be converted into glucose and fructose by using an enzyme called invertase, and maltose can be converted into two molecules of glucose by using the enzyme mannase (Bekatorou *et al*., 2006).

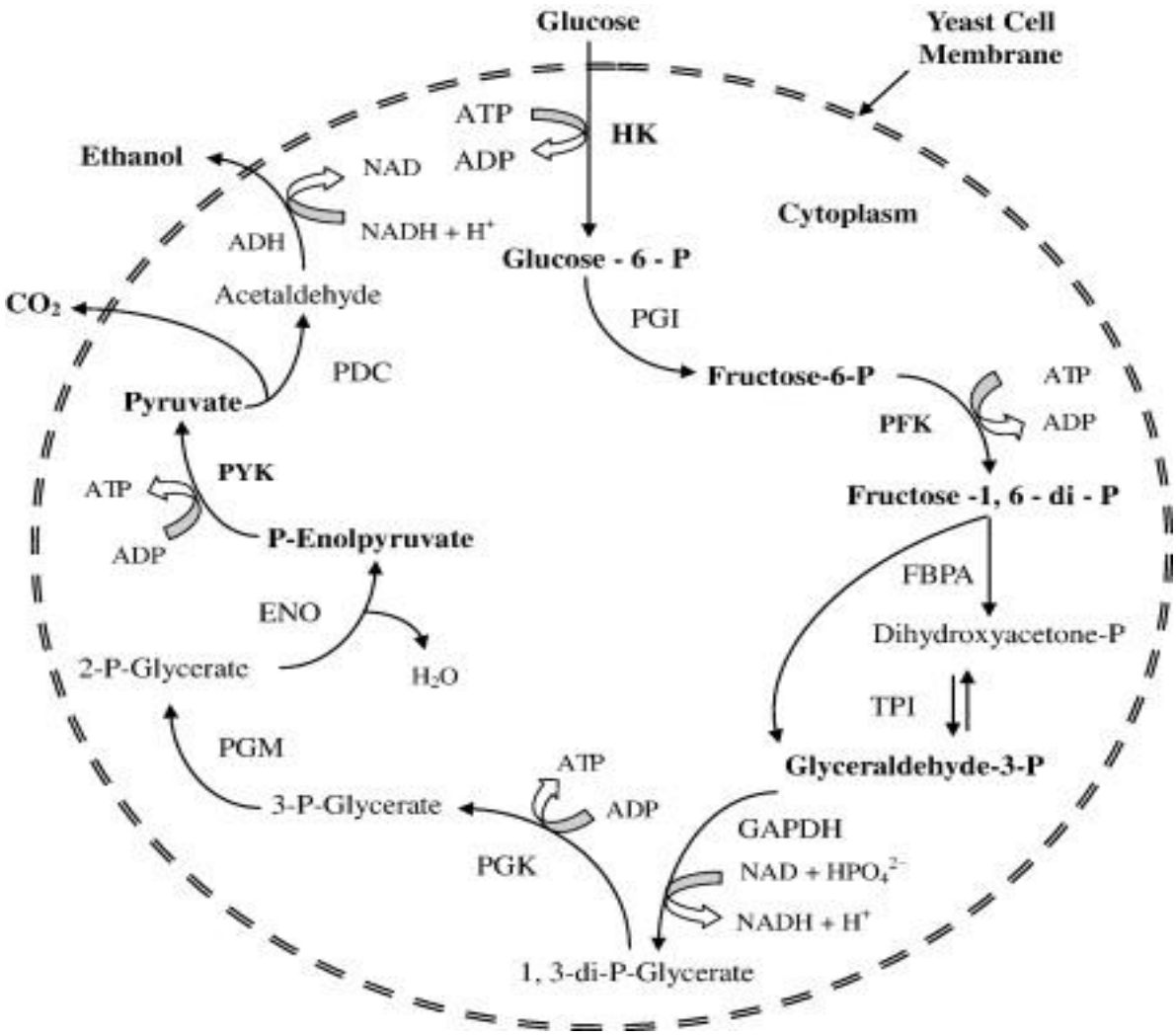


Figure 2.1: Metabolic Pathway of Ethanol Fermentation by *Saccharomyces cerevisiae* (Bai *et al*, 2008).

KEY: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

2.8.5 Mechanism of Yeast Fermentation

Monosaccharides and disaccharides can be metabolized by yeast but usually preferred glucose and fructose as substrates. Yeast is known to employ glycolytic, tricarboxylic acid and pentose pathways in the metabolism of hexoses and disaccharides and differ only in the initial basic steps of metabolism. The sugar breakdown may occur anaerobically or aerobically and the anaerobic step is called fermentation while the aerobic processes occur in the presence of oxygen. Alcoholic fermentation of glucose is the most common of yeast and the process yields ethanol and CO_2. When yeast sense sugar in its environment, it utilizes the sugar by transporting it across the plasma membrane (Kruckeberg *et al*., 2004).

The amount of glucose influence the enzyme levels through several processes like alteration of mRNA translation rates; mRNA stability or protein degradation, and the concentration of intracellular metabolites. This processes lead to the extensive transcriptional regulation of a large number of genes leading to adaptation to fermentative metabolism. These processes is required for the release of genes for the utilization of glucose, such as genes for encoding glycolytic pathway enzymes, whereas genes required for the metabolism of alternative substrates, and those encoding proteins in the gluconeogenic and respiratory pathways are repressed by glucose (Gancedo, 2008). *Saccharomyces cerevisiae* consists of more than 20 members for hexose transporters which includes; 18 genes encoding transporters, being the most relevant, with a low affinity for glucose and high transport capacity genes with a high affinity and two genes encoding sensors (SNF3, RGT 2), all these sensors and transporters are therefore the primary interveners on sugar metabolism.

After glucose uptake, it enters into glycolytic pathway in order to be metabolized to pyruvate, whereby production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for biosynthetic pathways (Rodrigues *et al*., 2006). The first step of the glycolytic pathway consists on the phosphorylation of glucose to glucose 6-phosphate by the action of the enzyme hexokinases and the glucokinase; which are linked to high-affinity glucose uptake. Then glucose-6-phosphate is isomerized by the phosphoglucose isomerase, to fructose-6-phosphate. The next step, done by the phosphofructokinase requires energy, in the form of ATP, to convert fructose-6-phosphate into fructose 1,6-biphosphate (Fábio *et al*., 2013).

2.9 Factors Affecting Fermentation of Ethanol

2.9.1 Effect of temperature on ethanol fermentation

The temperature of the fermentation medium is one of the major factors that have direct effect on many industrial processes. Ethanol production by thermotolerant yeasts has been extensively studied, because thermotolerant yeasts are capable of growth and increase rate of fermentation (Ueno *et al*., 2002).

Considerable difficulties are associated with lack of heat tolerance yeast strains that are been used in fermentation. These difficulties include high ambient temperature, coupled with an exothermic fermentation reaction, the compound effect of which leads to inhibition of yeast fermentation ability (Anderson *et al*., 1986). As the temperature increases, productivity decreases sharply because of greater ethanol inhibition (Sree *et al*., 2000). This was attributed to the fact that high temperature can cause denaturation of enzymes such as amylase thereby decreasing ethanol yield. Most of the time, the yeast performances are researched to stabilized biomass in this way it is necessary to find

thermo tolerant yeast.

Low temperature was also observed to reduce the activity of enzymes, giving no significant impact on ethanol yield. However in some study, low temperature was found to favour survival of yeast cells in rapid fermentation process (Verma *et al*., 2000).

2.9.2 Effect of ethanol concentration on fermentation

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Birch *et al.,* 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu *et al*., 2007).

The main sites for ethanol effects in yeast are cellular membranes, hydrophobic proteins, hydrophilic proteins and endoplasmic reticulum (Amutha and Paramasamy, 2001). For both ethanol stress and heat shock, vacuole morphology is altered from segregated structures to a single large organelle (Meaden *et al*., 1999). Membrane structure and function appear to be a predominant target of ethanol, exposure of yeast cell to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity. A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these proteins may be denatured (Hallsworth *et al*., 1998).

2.9.3 Effect of pH on ethanol fermentation

Control of pH during ethanol fermentation is important for two reasons:

- The growth of harmful bacteria is retarded by acidic solution.
- Yeast grows well in acidic conditions. With increase in pH, yeast produces acid rather than alcohol. Substrates that have naturally alkaline pH must be acidified prior to fermentation (Kourkoutas *et al.*, 2004).

The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. More acidic and basic conditions, both retard the yeast metabolic pathways and hence the growth of cells (Willaert and Viktor, 2006). Productivity may decrease by increase and decrease in pH due to the lower metabolic rate of the yeast cells. It may also be due to the growth of other microbes with the increase in pH, as the fermentation carried out without sterilization (Kourkoutas *et al*., 2004). In addition, pH of the surrounding medium change the configuration and permeability of the cell membrane thus reduced the rate of sugar fermented enzymes. There are some evidences that the rates of yeast growth and fermentation are decreased as the initial pH is decreased towards 3.0 however the yeast strains examined in another study, it exhibited similar growth behaviour at either pH 3.0, 3.5 or 4.0, and there was no firm evidence that initial pH over the range 3.0 to 4.0, would provide a selective growth advantage to any one particular species. (Heard and Fleet, 1988).

However, enzymes are very sensitive to pH, therefore the selection of optimum pH has been recommended for the production of alpha amylase and glucoamylases (Alva et al., 2007).

2.9.4 Effect of Substrate Concentration on Ethanol Fermentation

High substrate concentrations are inhibitory to fermentation (Almeida, 2007) due to osmotic stress. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. Under fermentative condition, the rate of ethanol production is related to the available sugar concentration. At very low substrate concentration, the yeast starved and productivity decreases. An important secondary effect of higher sugar content is catabolite repression of the oxidative pathways. Higher concentration of sugars in case of sugarcane juice media causes metabolic repression of yeast which results reduced the yield of ethanol. Amount of sugar utilization and ethanol production varied in the presence of different sugars like mono & disaccharides. This is because monosaccharides such as glucose is transported across cell membrane by a simple facilitated transport mechanism whereas disaccharides like sucrose is converted to glucose & fructose at the cell surface using invertase which is present in yeast cell wall. In this way variation in ethanol content shown by yeast cells as a result of variable sugars used as a carbon source is due to their different pathways of metabolism. It is observed that higher utilization of sugars corresponds to higher protein utilization by yeast cells, and ultimately higher ethanol yield, (Johannes, 1994).

2.9.5 Effect of Time on Fermentation

The optimum time of incubation of fermentation varies both with the organism and fermentation condition. The production usually starts after the lag phase of about 18 hours ad reached maximum at the onset of stationary phase. Further increase in incubation period, do not enhance production of ethanol (Arzumanov *et al*., 2000).

2.9.6 Inhibition of Ethanol Productivity in Yeast Cells

During ethanol fermentations, yeast cells are negatively affected by various environmental factors which includes; nutrient deficiency, high temperature and contamination, others are the yeast cell metabolism such as high sugar content tolerance, ethanol tolerance and production (Elena *et al*., 2009).These factors causes structural and metabolic changes in an organism acting as expression activator for genes involved in the synthesis of specific compounds that protect cells. This living and non-living factors can trigger the synthesis of compounds that changes the gene expression of the organism thereby inhibiting ethanol productivity. The inhibition is favoured by the presence of other fermentation by-products such as acetaldehyde and acetate, and other stresses such as high temperature (Bai *et al*., 2008).

The research work of Bai *et al.*, (2009) critically looked at some key aspects of ethanol fermentation technologies that have been neglected or misunderstood. In comparing the ethanol yield and productivity of Zymomonas *mobilis* with *Saccharomyces cerevisiae*, they found that *Zymomonas mobilis* produces higher ethanol with less biomass when higher metabolic rate of glucose is maintained through its special Entner–Doudoroff pathway. They however, recommend that due to its specific substrate spectrum as well as the undesirability of its biomass to be used as animal feed, this species cannot readily replace *Saccharomyces cerevisiae* in ethanol production. The dynamic behavior of the continuous ethanol fermentation under high gravity or very high gravity conditions has been neglected, which needs to be addressed in order to further increase the final ethanol concentration and save the energy consumption.

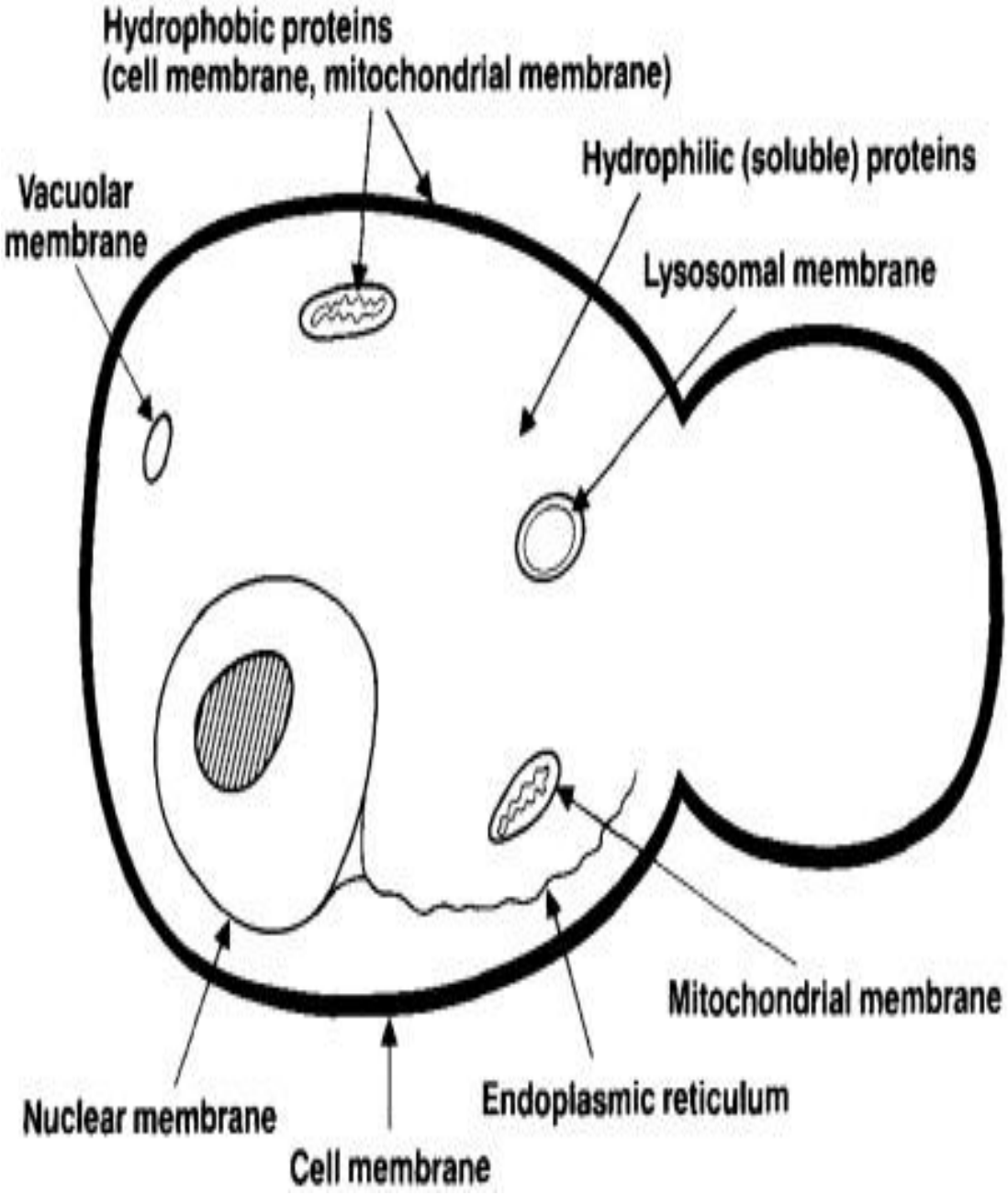


Figure 2.2:Target Sites for Ethanol Inhibition in Yeast Cells (Zhao *et al.*, 2007)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Collection of Samples

3.1.1 Isolation of *Aspergillus niger* and *Saccharomyces cerevisiae*

The local beverage (burukutu) used in the research for the isolation of *Saccharomyces cerevisiae* was obtained from *mami* market in Basawa barracks, Zaria, Kaduna state. While the soil sample used for the isolation of *Aspergillus niger* was obtained from Ahmadu Bello University botanical garden and also from beneath flower beds within the Department of Microbiology.

3.1.2 Isolation and Characterization of *Aspergillus niger*

Aspergillus niger was isolated from humus garden soil. The soil samples were collected randomly from the top 2cm of the soil profile at four different points (beneath the flower beds from Department of Microbiology A.B.U Zaria and from A.B.U Botanical garden). Approximately 30g of the soil was collected from each point and was put into clean polythene bags and brought to the laboratory. The soil samples were air-dried at room temperature for 48h. The dried soil samples were then processed to remove stones and plant residues using a sized sieve. One gram of each soil sample was transferred into labeled test tubes containing 9mls of distilled water. Streptomycin (0.005g) was added in order to suppress bacterial growth; the test tubes containing the soil sample solution were shaken in order to obtained homogenous mixture. Aliquot of 0.1ml of 10^{-5} serial dilution of the soil sample solution was spread on the surface of sterile solidified Sabouraud Dextrose Agar (SDA) plate and incubated at 28^{0}C for 5 days. *Aspergillus niger* isolates were differentiated on the basis of their cultural and morphological characteristics

obtained after incubation. The isolates were further subcultured on sterile SDA plates and incubated at 28°C for 5 days in order to obtain pure isolates (Knudsen *et al*., 1995).

The isolates were characterized based on their cultural, microscopic examination and amylase producing ability as follows:

3.1.2.1 Cultural Examination of Fungal Isolates

Morphological features were observed include; colour, growth pattern and physical appearance on SDA (Verweji *et al*., 2007).

3.1.2.3 Microscopic Examination of Fungal Isolates

A loopful of the fungal colony was taken and teased on a grease free slide containing a few drops of Lactophenol cotton blue stain, and was viewed under the microscope using 10x and 40x objective lens of the microscope. The microscopic view obtained was compared with that found in the "Atlas of Mycology" by Barnett and Hunter (1972).

3.1.2.4 Screening for alpha –amylase production

The fungal isolates were tested for amylase production by starch hydrolysis test in order to check if the fungal isolate have amylase production potential which is essential for the breakdown of complex sugar contained in the substrate. Modified starch agar medium consisting of soluble starch (2g), peptone (2g), yeast extract (1g) and agar (2g) was used and the isolates were inoculated and incubated for 72h at 28^0C. After incubation, the plates were flooded with iodine solution and observed for blue-black colour around colonies to change to brown or milky colour which accounts for their ability to digest the

starch and thus indicates the presence of alpha-amylase. The isolate with the largest zone of clearance was sub-cultured and maintained on fresh sterile Sabouraud dextrose agar (SDA) slant at 4^0C for further studies (Ado *et al.,* 2009).

3.1.3 Isolation and Characterization of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae that was used in the research was isolated from a local fermented beverage (*burukutu*). Aliquot of 0.1ml of 10^{-5} serial dilution of the beverage was spread on the surface of a solidified Malt Yeast Peptone Glucose (MYPG) agar plate and was incubated for 48h at 30^{o}C. Colonies suspected to be *S. cerevisiae* based on their colonial characteristics were sub-cultured on sterile MYPG slants. A smear of the isolate was examined microscopically after staining with lactophenol cotton blue. Stress tolerance test which include; thermotolerance, ethanol tolerance, sugar utilization test as well as fermentation of sugar test by the yeast isolates were carried out (Ameh *et al*., 1989).

3.1.3.1 Microscopic observation of *Saccharomyces cerevisiae*

A drop of lactophenol cotton blue was placed on a clean slide, using a sterile wire loop a portion of the colony from the yeast culture was placed in the drop of the lactophenol cotton blue and teased. Then a clean cover slip was gently placed and observed for microscopic morphology under the microscope using oil immersion (100×) objective lens (Guimaraes *et al*, 2006)).

3.1.3.2 Thermotolerance Test

The yeast isolates were inoculated on SDA plates and incubated at 25, 30, 35, 40 and 45^{o}C and the inoculated plates were observed for growth after 48h (Babiker *et al.,* 2010).

3.1.3.3 Ethanol Tolerance Test

A 24h washed yeast cells was used, aliquot of 0.1ml of the washed cells was inoculated into yeast extract broth containing 3, 6, 9, 12, 15 and 18% ethanol. The tubes were incubated at 30^{o}C for 48h, the optical density of the cells were taken using a spectrophotometer to determine the level of growth (Breisha, 2010).

3.1.3.4 Growth in 10-50% Glucose

A basal medium containing 10, 20, 30, 40 and 50% (w/v) glucose, 1.0% (w/v) yeast extract broth, 0.75% (w/v) peptone and 2% (w/v) agar respectively were used to grow the yeast isolates. The testubes were incubated at 30^{o}C for 48h and the optical density of the cells was determined using a spectrophotometer (Breisha, 2010)

3.1.3.5 Fermentation of sugars

A basal medium containing 0.5% (w/v) yeast extract and 0.75% (w/v) peptone was used with bromothymol blue as indicator. Nine millilitres of the medium was dispensed into test tubes containing Durham tubes. The tubes were plugged in with cotton wool and sterilized at 121^{o}C for 15 minutes, after cooling; 2ml of filter sterilized solutions of sugars of glucose, maltose, sucrose, lactose, fructose and galactose were added into the different test tubes after which 1ml of the washed yeast cells suspension was added. The tubes were incubated at 30^{o}C and observed for colour change and gas production in the Durham tubes. The isolates were selected and maintained on fresh SDA slants at 4^{o}C for further studies (Ameh *et al*., 1989).

3.2 Preparation of Banana Peel flour Substrate

Banana peels were obtained from three (3) local markets in Zaria, Kaduna, Nigeria. The ripe waste banana peels, were washed with distilled water to remove sand

and then chopped into 3cm. It was then sun dried for 14 days before which it was ground to fine powder (Ajay *et al*., 2014).

3.2.1 Proximate Composition of Banana Peels

Proximate analysis of food is the determination of the major components of food, which include: moisture, ash, lipid (fats), protein, fibre and carbohydrates content.

The proximate analysis was carried out at Institute of Agricultural Research (IAR) Samaru, Zaria. In carrying out the analysis practically; methods used vary according to the food material being studied.

3.2.1.1 Determination of Moisture Content

The moisture content was determined according to the standard of AOAC (2010). The crucibles were washed and dried in an oven at 100^{o}C for one hour and the weight was noted as W_1. Two grams of each of the sample was separately weighed into crucibles and their weights were taken and noted down as (W_2) before and after drying at 100^{o}C to a constant weight ($W_{3)}$

Moisture content= $W_2 - W_3 \times 100$ = weight of moisture × 100 (AOAC, 2010).

Where;

W_1 = weight of empty crucible

W_2 =weight of crucible and sample before drying

W_3= weight of sample after drying to a constant weight

3.2.1.2 Determination of Ash Content

The ash content of the samples was determined according to the standards of AOAC (2010). A pre-heated and cooled crucible was weighed. The sample was charred on a Bunsen flame inside a fume cupboard. The charred sample was placed in a muffle

furnace set at 550°C for two hours until a white or light grey ash was obtained. The sample was removed and cooled in desiccators before it was weighed.

Ash content = $\frac{W_3 - W_1}{W_2 - W_1} \times 100$ (AOAC, 2010).

Where w_1= weight of empty crucible

W_2= weight of crucible + weight of sample

W_3= weight of crucible + weight of sample after ashing

3.2.1.3 Determination of Protein Content

The protein content of the sample was determined according to the standards method of AOAC (2010) using Kjeldahl's method.

Two grams of the sample was weighed into kjeldahl's flask and 5g of anhydrous sodium sulphate was added. Twenty five millilitre H_2SO_4 was added with few boiling chips, the content of the flask was heated in a fume chamber until a clear solution was obtained. The solution was cooled and transferred into 250ml volumetric flask and was made up to the level with distilled water. The digest was washed down with distilled water followed by addition of 5ml of 60% sodium hydroxide solution; it was distilled until the volume of the distillate (ammonium sulphate) reaches 100ml. The solution in the flask was then titrated with 0.04 HCl until the first permanent pink colour appeared. The blank was also titrated in the same way.

The distillation unit was carried out using a well cleaned kjedahl apparatus. One hundred millilitres conical flask containing 5ml of 2% boric acid and two drops of methyl red indicator was placed under the condenser. Five (5ml) of the digest was pipetted into the apparatus through the small funnel on the distillation unit

% nitrogen= vs-vb × N acid × 100 (AOAC, 2010).

Where;

vs = volume (ml) of acid required to titrate sample

Vb = volume (ml) of acid required to titrate the blank

N acid = normality of acid (0.04N)

Note: most proteins contain about 16% nitrogen, so that 16mg nitrogen =100mg protein.

1mg Nitrogen = 6.25

Therefore protein (%) = N × 6.25 (conversion factor for protein)

3.2.1.4 Determination of Fat Content

The fat content was determined by using the standard AOAC (2010) method.

Five hundred millilitres round bottom flask together with a soxhlet extractor and a reflux condenser was set up and 300ml of petroleum ether was poured into the round bottom flask. Two (2g) of the banana peel flour was weighed into a labeled thimble and sealed with cotton wool, before fitting it into the extraction tube of the soxhlet extractor. The soxhlet extractor after assembly was allowed to reflux for about 6 hours, after which the thimble was removed with care and the petroleum ether collected on top was drained into a container for re-use. After the flask was free of ether, it was then removed and dried in desiccator before it was weighed.

$$\text{Fat (\%) content} = \frac{W_2 - W_1}{W} \quad \text{(AOAC, 2010)}$$

Where;

W= weight of sample used

W_1 = weight of empty extracting flask

W_2= weight of flask and extracted oil

3.2.1.5 Determination of Crude Fibre

This was done according to AOAC (2010) method:

The sample was dried in an oven at 105°C, 2g of the dried sample was placed in a 500ml beaker and 200ml of boiling 1.25% H_2SO_4 was added. The beaker was then placed on a hot plate and boiled for 3 minutes with occasional rotation of the beaker. After boiling, the beaker was allowed to cool before filtering it by suction through a Buchner funnel. The beaker was rinsed twice with 50ml of boiling water. The residue was carefully transferred into a beaker and 200ml of 1.25% NaOH was added and allowed to boil for 30 minutes, cooled and filtered before it was washed twice with 50ml of boiling water.

Finally, the residue was washed with 25ml of 95% alcohol. The residue was dried in an oven for 2 hrs at 130°C and cooled in a desiccator and weighed.

$$\text{Crude fibre} = \frac{\text{weight of residue} \times 100}{\text{Weight of sample}} \quad \text{(AOAC, 2010)}$$

3.2.1.6 Determination of Carbohydrate Content

The carbohydrate content was determined as illustrated by AOAC (2010) as follows: it was the remainder after accounting for all; moisture, ash, crude fibre, protein, and fats.

Total carbohydrates contents =100-% moisture +%ash +%fat content +%protein

Cp = crude protein

Cf = crude fibre

Ash = ash content

Fat = fat content

Moisture = moisture content

3.3 Fermentation of Banana Peel flour

3.3.1 Fermentation Medium

The fermentation medium that was used for ethanol production in the research contained (per 100ml); Soluble Starch1g, Glucose 1g, Peptone 0.1g, Malt Extract 0.1g, Yeast Extract 0.2g, Magnesium Chloride 0.1g, Calcium Carbonate 0.2g, Ammonium Phosphate 0.2g and Ferrous Sulphate 0.001g. One normal HCl and one normal NaOH was used to adjust the pH of the medium to 6.0 (Ado *et al*., 2009).

3.3.2 Inoculum Preparation:

A. niger and *S. cerevisiae* inocula were prepared by using slant cultures to inoculate 50ml of sterile growth medium contained in 200ml Erlenmeyer flasks. The flasks were incubated with shaking (200 rpm) at 30^{o}C for 2 days (*S. cerevisiae*) and 3 days for *A. niger* (Byaruagaba-Bazirake *et al* 2013)

3.3.2.1 Preparation of spore inoculum of *Aspergillus niger*

Inoculum of *A. niger* was prepared from fully sporulated (5 day old) culture grown on Sabaoraud Dextrose Agar slant. The colonies were flooded with 10ml of sterile distilled water containing 2.5% Tween 80 (polyoxyethlene sorbitan monooleate), added to facilitate the uniform distribution of *Aspergillus niger* inoculum. The spores were dislodged by vigorous shaking for 15 minutes. One millilitre of the stock suspension was diluted into 99ml sterile distilled water, 0.5ml of the diluted culture suspension was dropped on a haemocytometre and counted under the microscope using 10× and 40× objectives inorder to know the number of spores/ml introduced into the fermentation medium. Ten milliliter of the inoculum was transferred into the fermentation medium (Byaruagaba-Bazirake *et al.,* 2013).

3.3.2.2 Preparation of Yeast cells of *Saccharomyces cerevisiae*

The yeast isolate was grown in yeast peptone dextrose (YPD) broth (1% Yeast extract, 2% Peptone and 2% dextrose) containing 0.05mg/ml of chloramphenicol. Ten millilitres of the YPD medium was inoculated with a single selected colony of the yeast isolate using a wire loop and then incubated overnight at 30^{o}C with an agitation rate of 200 rpm. After incubation, 1ml of the yeast cell culture was transferred into 200mls YPD medium. The yeast cells were allowed to grow at 30^{o}C for 24h on a shaker-incubator. The cells were then harvested by centrifugation at 3000 rpm for 5 minutes. The pellets formed in the centrifuge was re-suspended in 30mls sterile distilled water and washed three times, after which it was prepared according to Macfaland standard one. One millilitre of the yeast suspension was introduced into the growth medium; ten milliliter of the inoculum was transferred into the fermentation medium (Byaruagaba-Bazirake *et al.*, 2013).

3.3.3 Experimental Set-Up and Fermentation

A batch of 200ml medium in 500ml Erlenmeyer flask with 4% of banana peel flour was used during the experiment. The flasks were sterilized by autoclaving at 121^{o}C for 15 minutes, then 2.5 x 10^{6} spores/ml of *A. niger* and 3 x 10^{8} cells/ml of *S. cerevisiae* were used as the inocula and incubated at 30^{o}C, 200 rpm using a rotary shaker incubator for 5 days (Ado *et al*., 2009).

3.3.4 Determination of Ethanol Content

After 24h interval during fermentation, 30mls aliquot from the flask was taken after thorough shaking of the flask in order to homogenize the contents. This was used for the analysis of ethanol concentration (Ado *et al*., 2009)

Ethanol concentration was determined by measuring its specific gravity after distillation; the specific gravity values obtained were used to determine ethanol concentration from a standard curve prepared using known concentration of ethanol (Oyeleke *et al*., 2012).

3.3.4.1 Distillation

The fermented liquid was transferred into a round bottom flask and placed on a heating mantle fixed to a distillation column enclosed in a running tap water. Another flask was fixed to the other end of the distillation column to collect the distillate at 78^0C (Oyeleke *et al*., 2012).

3.3.4.2 Specific gravity

A clean and dry 50ml specific gravity bottle was weighed with its stopper. It was filled with distilled water at 20^oC equal to the volume of the distillate to be measured. The excess water was emptied and dried before reweighing it. It was then filled with the distillate at 20^oC and reweighed and the specific gravity was calculated as:

$$\text{Specific gravity} = \frac{\text{Density of distillate}}{\text{Density of equal volume of water}}$$

The specific gravity values obtained were used to determine ethanol concentration from a standard curve prepared using known concentrations of ethanol (100%) as reported by Emeka, (2001) – as follows:

3.3.5 Preparation of ethanol standard curve

Six stocks of varying concentrations of ethanol, 5, 10, 15, 20, 25 and 30% were used. Aliquots of 5 ml of each of these concentrations was taken into the specific gravity bottle and weighed against equal weight of de-ionized distilled water. The values obtained

were used to plot a standard ethanol curve against the six known concentrations previously determined (Emeka, 2001).

3.3.6 Confirmatory Test for Ethanol Produced by the Co-culture Using Banana Peels

One millilitre of ethanoic acid was added to the distillate and then heated; production of sweet ester smell indicated the presence of ethanol (Jimoh *et al.*, 2009).

3.4 Optimization of Culture Conditions

The effect of substrate concentration was determined by carrying out fermentation at varying substrate concentration of 2, 4, 6, 8 and 10% for 5 days each, with the initial pH adjusted to 6.0.

In order to determined the effect of pH on ethanol production, the substrate with the maximum ethanol yield was taken and used for fermentation with varying pH values with the initial pH 6.0 adjusted to 5.0, 5.5, 6.0 and 7.0 by using one normal HCl and one normal NaOH to adjust the pH.

The pH with the maximum ethanol yield was taken and used for fermentation at varying temperature range of 25, 30, 35 and 40^{o}C for 5days each.

The effect of time on fermentation was determined by extending the length of fermentation to 10 days under optimum culture parameters of substrate concentration, pH and temperature (Ado *et al*., 2009).

3.5 Statistical Analysis of Data

Results obtained were presented in form of tables and graphs and were further subjected to statistical analysis using standard deviation at P□ 0.05 significance level

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and Characterization of *Aspergillus niger* and *Saccharomyces cerevisiae*

4.1.1 Macroscopic and Microscopic Observation of *Aspergillus niger*

The growth of *A. niger* on SDA plate produced black colonies consisting of a compact dark mass colony as shown in Plate I. The microscopic observations of the isolates revealed the presence of a transparent tabular conidiophore with a dark globose conidial head as shown in Plate II.

4.1.2 Amylase production test

The result obtained after flooding *Aspergillus niger* colonies with iodine on starch agar plates, showed zone of clearance on the plate indicating starch hydrolysis potential of the isolate. However from the experiment, out of the four (4) isolates only isolate BS_1 showed the highest zone of clearance (6.2mm) as shown in Table 4.2 and Plate III.

4.2 physico-chemical/biochemical characterization of the yeast isolate

4.2.1 Macroscopic and microscopic observation of the yeast isolate

The macroscopic observation of the yeast isolate showed raised colonies that were milky in colour, spherical in shape, moist and also have alcoholic odour typical of *Saccharomyces cerevisiae* (Plate IV). The yeast isolate when stained lactophenol cotton blue was found to be transparent and spherical in shape with a multilateral type of budding under the microscope using 100× objectives as presented in Table 4.3.



Plate I: Five day-old pure culture of *Aspergillus niger* on Sabouraud Dextrose (SDA) agar plate

Table 4.1: Microscopic Characteristics of *Aspergillus niger*

Feature	Description
Hyphae	septate
Conidiophore	transparent tubular appearance
Head	black, globose head

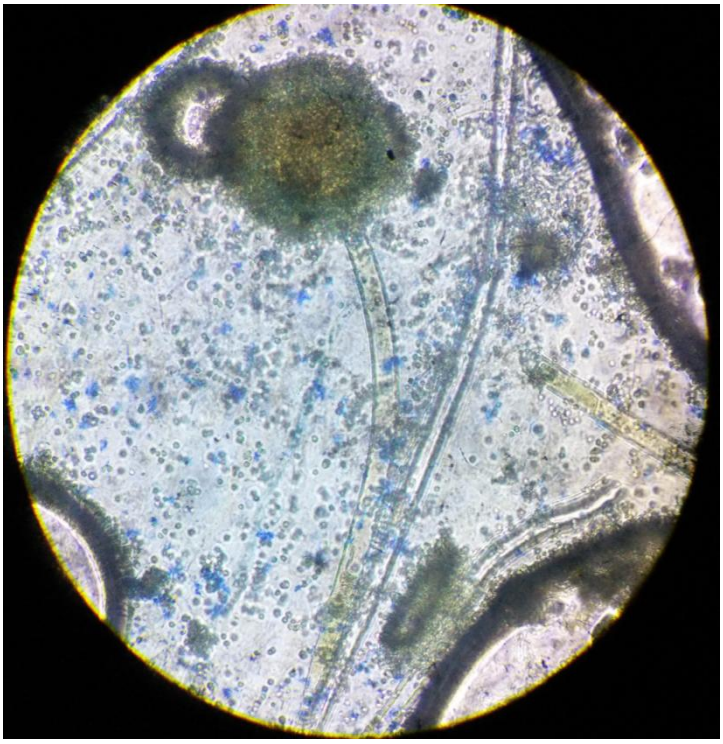


Plate II: A portion of a young culture of *Aspergillus niger* viewed under the microscope using 40× objectives

Table 4.2: Amylase producing potential of *Aspergillus niger* on starch agar plates

Isolate	Zone of clearance (mm)
FS_1	5.2
FS_2	4.5
BS_1	6.2
BS_2	6.0

Key:

FS: soil from beneath flower beds of the Department of Microbiology, A.B.U Zaria

BS: Soil from botanical garden of A.B.U Zaria



Plate III: Zone of clearance due to hydrolysis of starch by the screened *Aspergillus niger*

Table 4.3: Macroscopic and Microscopic Characteristics of Yeast Isolates Observed After 48 hr of Incubation at 30^{o}C on Malt Yeast Peptone Glucose agar Plates

Macroscopic	**Description**
Colony	creamy white
Edges	entire
Consistency	moist
Colony shape	spherical
Colony surface	smooth surface
Microscopic	**Description**
Cell shape	ovoid and spherical cells
Arrangement of cells	multilateral budding
Colour	transparent

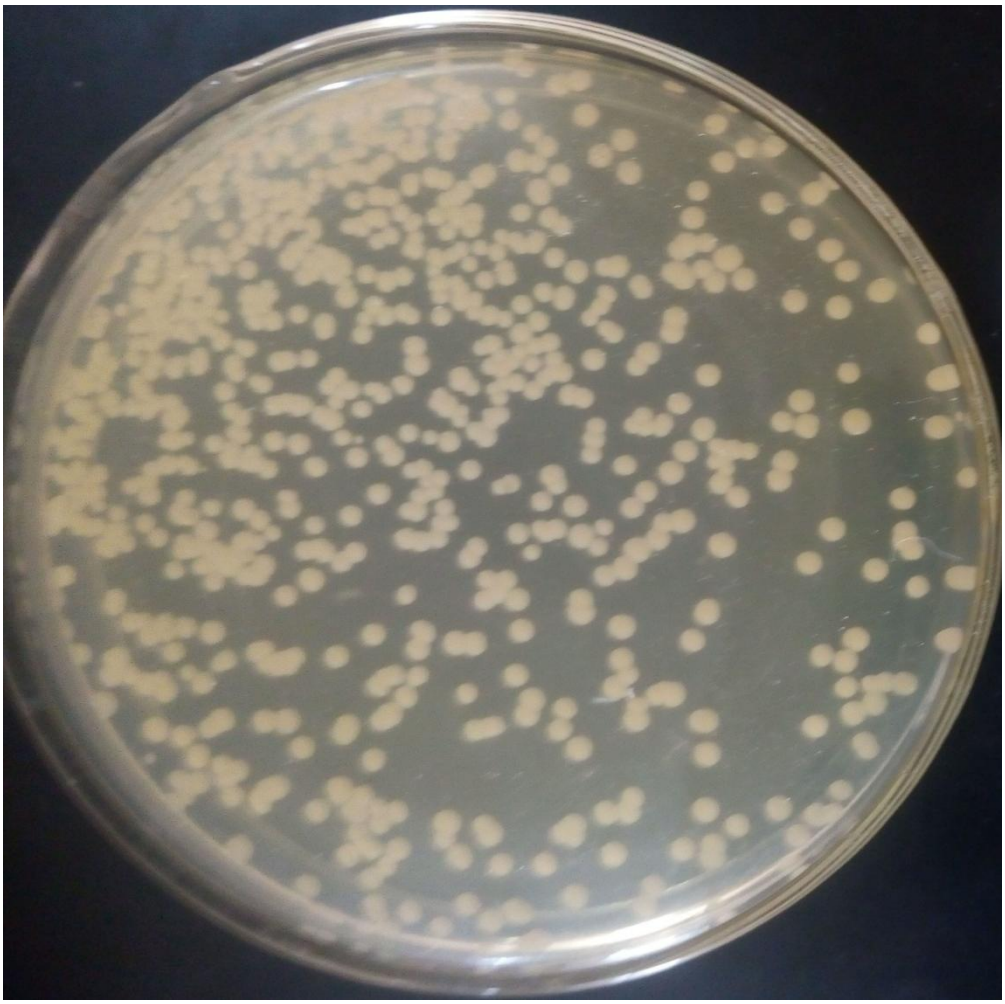


Plate IV: Colonies of *Saccharomyces cerevisiae* on Malt Yeast Peptone Glucose (MYPG) agar Plate after 48h

4.1.3.2 Thermotolerance test

The yeast isolate was incubated at varying temperatures of 25, 30, 35, 40 and 45^{o}C. Evidence of growth was observed on plates incubated at 25-40^{o}C while plates incubated at 45^{o}C showed little or no growth. The relative thermotolerance of the yeast isolates is presented in Table 4.4.

4.1.3.3 Ethanol tolerance test

The yeast isolate was found to grow on media containing ethanol concentration of 3, 6, 9, 12, 15 and 18% at 30^{o}C for 48 hours with the optical density of the yeast cells decreasing with increasing ethanol concentration as shown in Table 4.5**.**

4.1.3.4 Growth in 10-50% glucose test

The yeast isolate was found to grow in 10-50% glucose with the optical density of the yeast cells increasing with a decrease in glucose concentration as presented in Table 4.6

4.1.3.5 Sugar fermentation test

The yeast isolate was found to ferment different sugars of glucose, maltose, sucrose, galactose, fructose with the evolution of gas in the durham tubes except lactose, as presented in Table 4.7

4.3 Proximate Composition of the Banana Peels

The proximate composition of the banana peels used in this research was found to have high carbohydrate content of 70.09% with significant amount of moisture, ash, protein, fat and fibre content. The results of the proximate composition is shown in the Table 4.8

Table4.4: Relative Thermotolerance of the Yeast Isolate observed after 48h

Temperature (^{o}C)	Level of growth
25	++
30	+++
35	+++
40	++
45	+

Key: + = evidence of growth

++ = good growth

+++ = excellent growth

Table 4.5: Ethanol Tolerance Test of the Yeast Isolate observed after 48h

Ethanol concentration (%)	optical density of cells (600nm)
3	2.465
6	2.263
9	2.118
12	1.986
15	1.752
18	1.564

Table 4.6: Growth in 10-50% Glucose After 48 Hours of Incubation

Glucose concentration (%)	optical density of cells (600nm)
10	2.511
20	2.368
30	2.172
40	1.786
50	0.988

Table 4.7: Sugar Fermentation by Yeast Isolate at Three Different Time Intervals in duplicates

	SUGARS					
	Glucose	**Maltose**	**Lactose**	**Sucrose**	**galactose**	**fructose**
Time(hrs)						
Before 48	-	-	-	-	-	-
	+	-	-	-	+	+
At 48	+	+	-	+	+	+
	+	+	-	+	+	+
After 48	+	+	-	+	+	+
	+	+	-	+	+	+

Key: + = presence of acid and gas

\- = absence of acid and gas

Table 4.8: Proximate Composition of Banana Peels

Parameters	Composition (%)
Moisture	12.58
Ash	7.70
Fat	4.65
Protein	5.22
Fibre	9.10
Carbohydrate	70.09

4.4 Substrate Fermentation for Ethanol Production

Aspergillus niger and *Saccharomyces cerevisiae* isolated were used to carry out submerged fermentation of banana peel flour at 30^{o}C, pH 6.0 and 4% substrate concentration for five days. From the result, there was a gradual increase in ethanol yield from the first day (1.48%) to the fourth day (6.22%), but a decrease in yield was observed on the fifth day (5.78%) indicating that under this condition of temperature (30^{o}C), pH (6.0) and substrate concentration (4g); the maximum ethanol yield was obtained on the fourth day to be 6.22% as shown in Figure 4.1.

4.4.1 Confirmatory test for ethanol produced by the co-culture using banana peels

The ethanol produced when confirmed with ethanoic acid produced a sweet ester smell that is typical of ethanol as presented in Table 4.9.

4.5 Optimization of Culture Conditions for Ethanol Production

4.5.1 Optimization of substrate concentration

Figure 4.2 shows the result of ethanol yield at varying substrate concentration from 2 to 10%, at 30^{o}C, pH 6.0 and at 200rpm for five days. There was a gradual increase in ethanol yield as the substrate concentration increases, but at 10% substrate concentration; the ethanol yield decreased. The maximum ethanol yield was obtained at 8% substrate concentration on the fourth day of fermentation (9.66%v/v) while 2% substrate concentration gave the least ethanol yield with it maximum yield obtained on the fourth day (4.93%v/v).

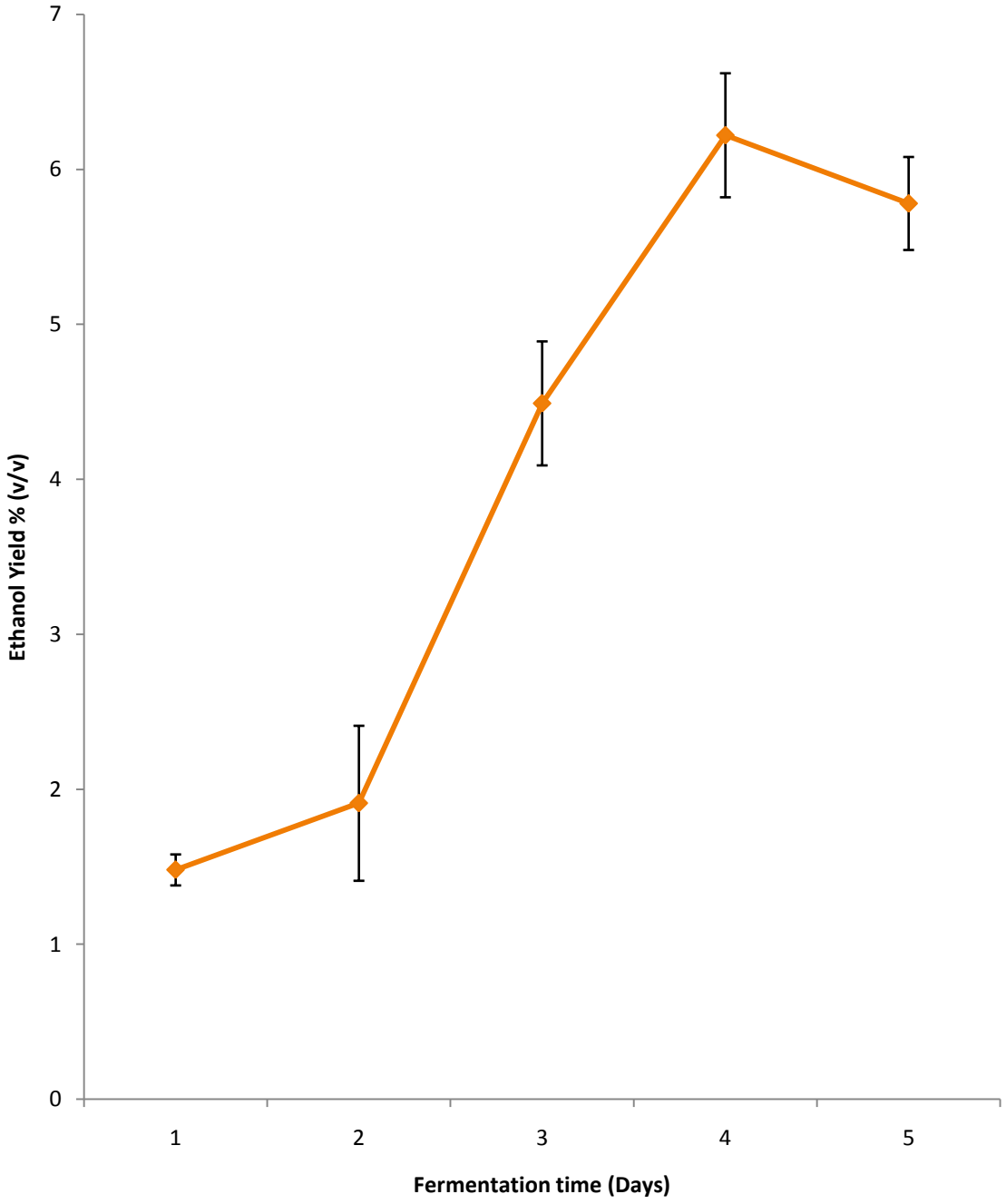


Figure 4.1: Ethanol Production by Co-Cultures of *Aspergillus niger* and *Saccharomyces cerevisiae* for Five Days at pH 6.0, 30^{o}C and 4g of the Substrate.

Table 4.9: Confirmatory Test for Ethanol Produced by the Co-culture Using Banana Peels

Test	Observation	Inference
1ml of distillate + 1ml of ethanoic Acid + heat	sweet ester smell perceived	ethanol present

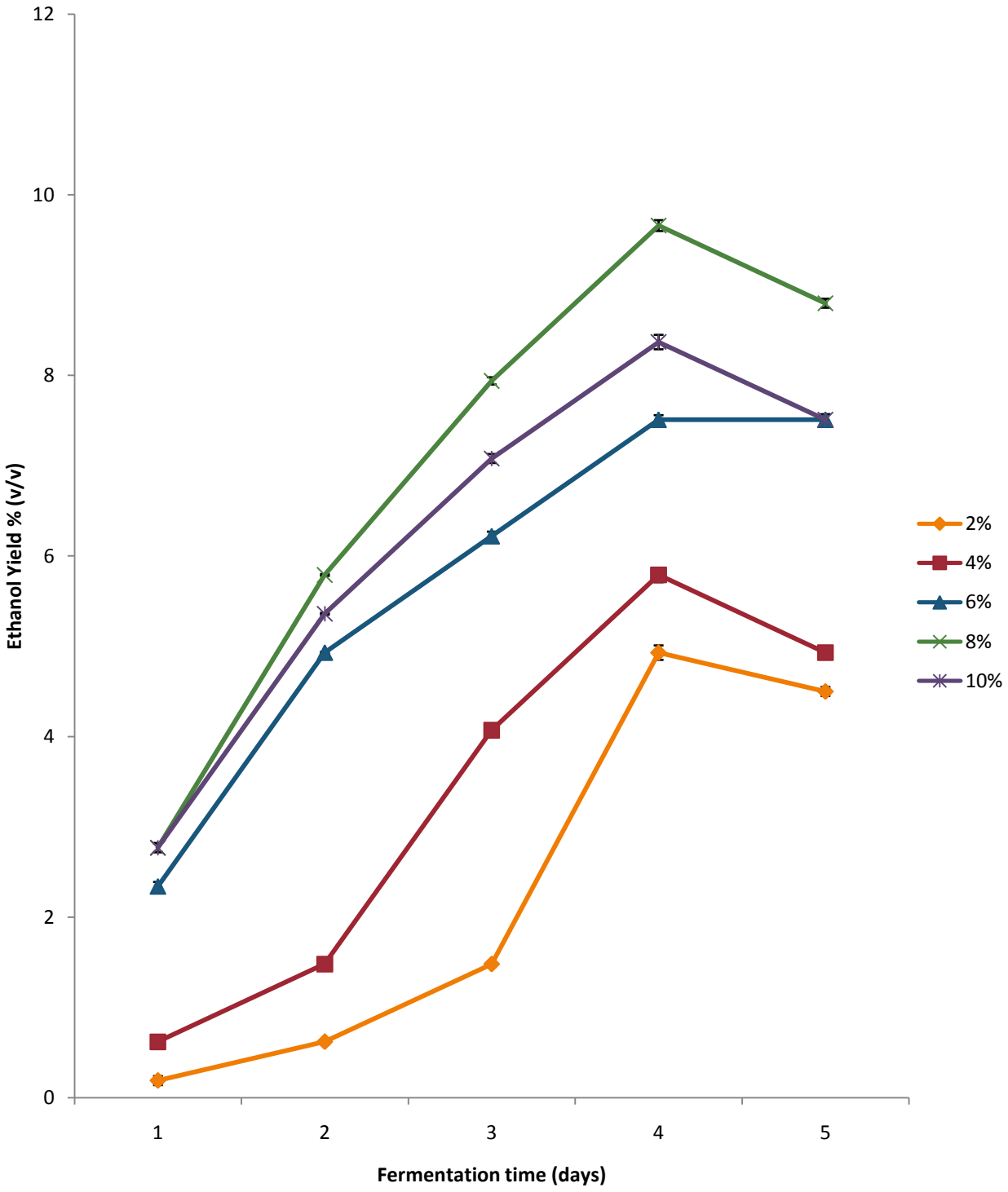


Figure 4.2: Effect of Substrate Concentration on Ethanol Yield for Five Days at 30^{o}C and pH 6.0 for five days

4.5.2 Optimization of pH

Figure 4.3 shows the effect of pH on ethanol yield at 30^{o}C, 8% substrate concentration which was the maximum ethanol yielding concentration and at 200rpm after five days of fermentation. Maximum ethanol yield was obtained at pH 5.5 which was found to be 10.52%(v/v) on the fourth and fifth day, above pH 5.5, there was a continuous decrease in ethanol yield to 7.51%v/v, 6.65%v/v and 5.48%v/v for pH 6.0, 6.5 and 7.0 respectively.

4.5.3 Optimization of Temperature

Figure 4.4 shows the effect of temperature on ethanol yield using 8% substrate concentration and pH 5.5 at varying temperature range of 25, 30, 35 and at 40^{o}C.The ethanol yield increased with increased in temperature but the yield maximized at 35^{o}C with a decline at 40^{o}C. The maximum ethanol yield obtained at 35^{o}C was 12.63%(v/v) on the third day of fermentation while the least ethanol yield obtained was 10.52%(v/v) at 40^{o}C on the fourth day of fermentation, maximum ethanol yield obtained at 25^{o}C and 30^{o}C were found to be 10.95%(v/v) and 11.88%(v/v) respectively on the fourth day of fermentation as shown in Figure 4.4.

4.5.4 Optimization of Length of Fermentation

Fermentation was carried out for 10 days inorder to determine the effect of time on ethanol yield at 35^{o}C, pH 5.5 and at 8% substrate concentration. Maximum ethanol yield of 12.67% (v/v) was obtained on third day of fermentation; there was no observable increase in ethanol yield beyond the third day of fermentation as shown in Figure 4.5.

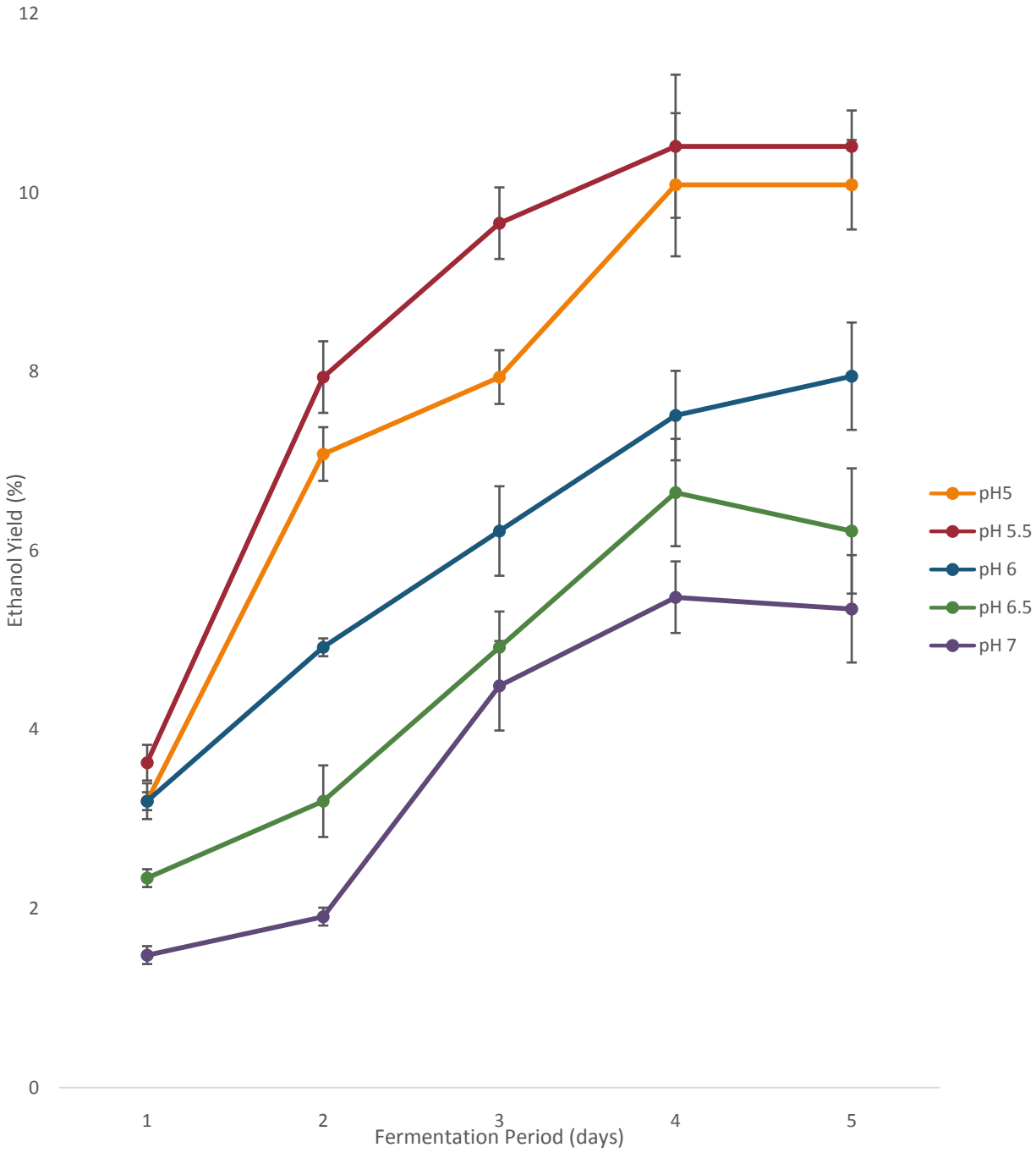


Figure 4.3: Effect of pH on Ethanol Yield at 30^{o}C and 8% Substrate Concentration for Five Days

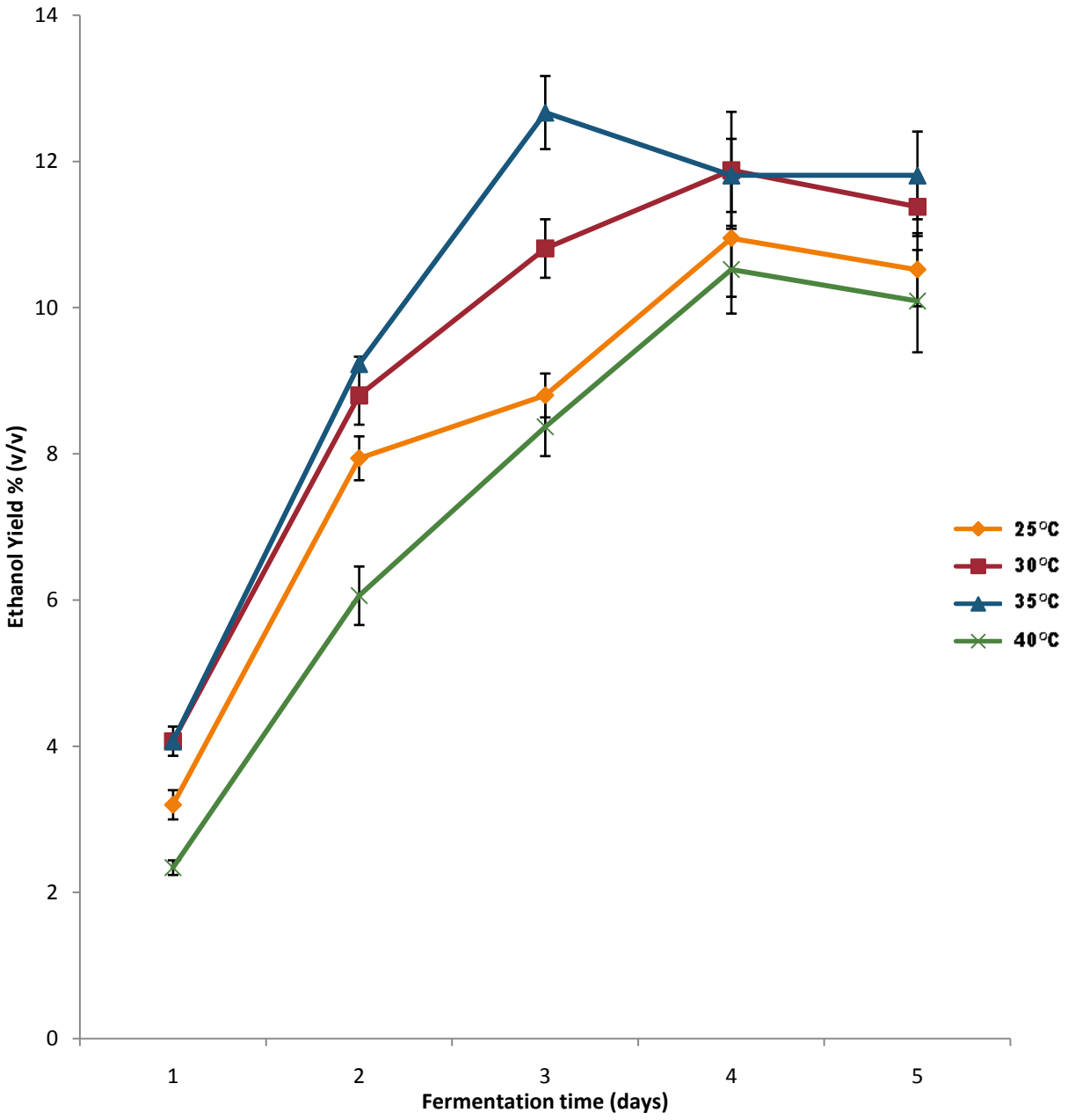


Figure 4.4: Effect of Temperature on Ethanol Yield at 8% Substrate Concentration, pH 5.5 and at 200rpm for Five days Using Co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae*

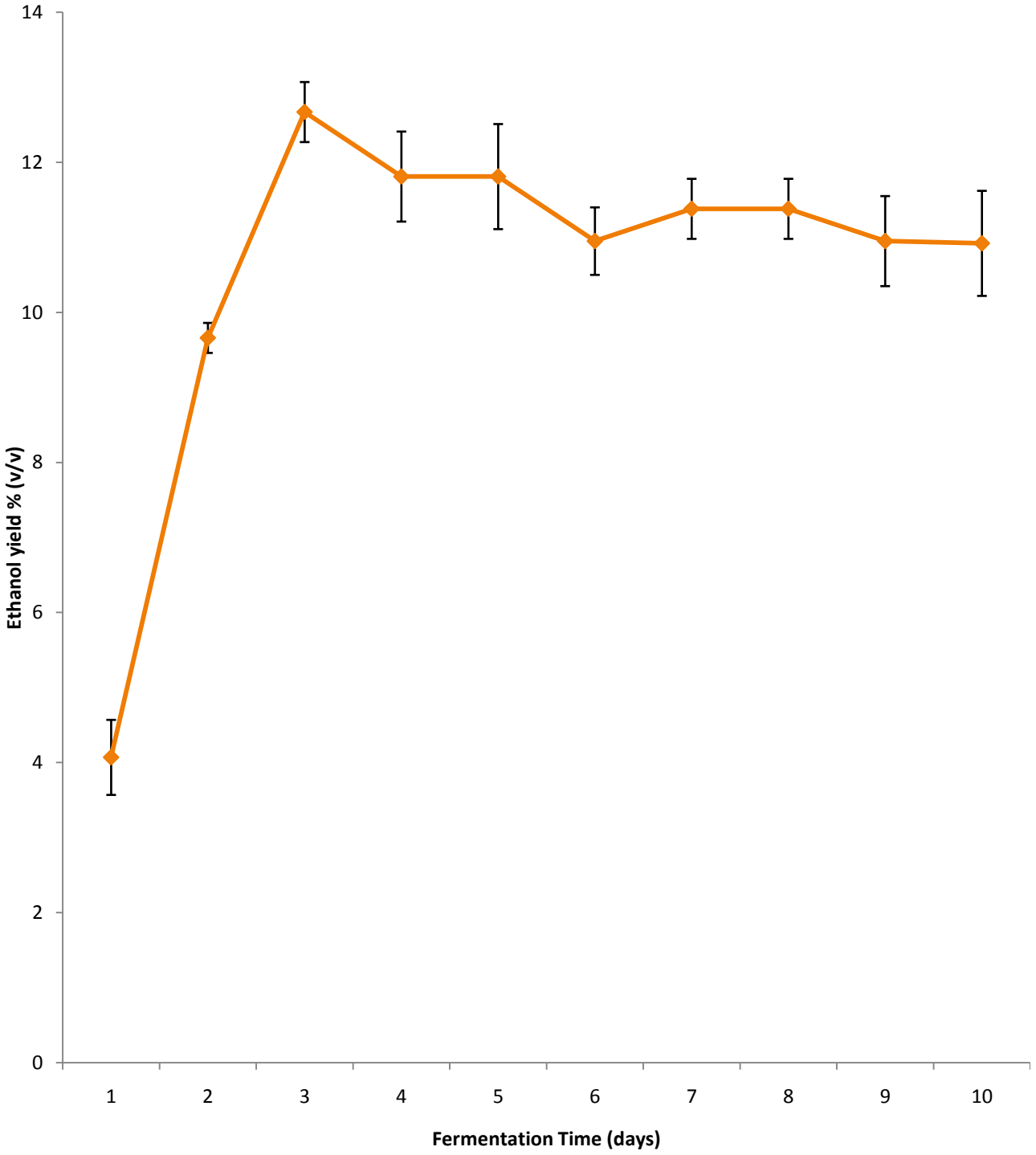


Figure 4.5: Effect of time of Fermentation on Ethanol Yield by Co-Culture of *Aspergillus niger* and *Saccharomyces cerevisiae* Grown on 8% Substrate Concentration at 35^{o}C and pH 5.5 for 10 Days

CHAPTER FIVE

5.0 DISCUSSION

This finding reveals the ubiquitous nature of *Aspergillus niger* as they are universally found where environmental conditions are conducive especially in the soil and plant debris (Habib *et al.,* 2015) which suggest that the soil is a natural habitat for amylase producing organisms especially *Aspergillus niger*. The macroscopic and microscopic identification of the organism was based on colony pigmentation and the structure of the conidial head as classified by Verweji *et al.*, (2007) who reported that colonies of *A. niger* are carbon black with a dark globular conidial head. *Aspergillus niger* strain that was isolated from soil samples was found to have good amylase production potential which is important in the hydrolysis of starch, this is in agreement with the work of Omemu *et al.*, (2005) who reported that *A.niger* can be used for industrial production of ethanol, citric acid and gluconic acid because of it hydrolytic capacities in amlylase production and it ability to have high tolerance to acidity thereby enabling it to prevent bacterial contamination.

The yeast isolate (*Saccharomyces cerevisiae)* based on its microscopic and macroscopic identification was found to belong to the *Saccharomyces* type of unicellular ascomycetes according to (Vanda *et al.,* 2013). The isolate was able to withstand various stress conditions of temperature, ethanol concentration, high glucose concentration; it was also able to utilize various sugars of glucose, maltose, fructose, sucrose and galactose with the exception of lactose, this agrees with the work of Andrietta *et al.*,(2007) who reported that *Saccharomyces cerevisiae* is a yeast that is capable of withstanding stressful conditions and have high fermentation efficiency, effective sugar used, tolerance to high

ethanol concentration, osmotolerance and thermotolerance which are fundamental for industrial used. However from this research, the yeast isolate was able to withstand this various stress conditions as a result of acquisition of stress from the environment (*burukutu)* it have been isolated thereby stimulating an adaptive response resulting in a transient resistance to higher levels of the same stress (Ueno *et al.,* 2002).

Banana peels contain various ingredients with major amount in carbohydrates and significant amount of protein, fats, fibre and ash content; this agrees with the work of Dhanasekaran *et al.*,(2011) and Sanker *et al.,* (2011), who reported that banana peels contained various ingredients and may be used as carbon and energy source for the growth of fungi. The carbohydrate and protein content of the banana peel from this research are indication that the waste peels can be used as an alternative substrate for the cultivation of fungi and hence for the production of bioethanol since the raw materials for bioethanol production are sugary, starch and fibrous materials (Essien *et al*., 2003).

The ethanol yield from Figure 1; there was increase in yield and this may be because ethanol yield increase with increase in time but begins to decrease when the organism becomes starved and die thereby leading to a decrease in the metabolic activity and subsequent decrease in ethanol yield. This research is in agreement with the work of Shilpa *et al.,* (2013) and Zainal *et al*., (2014) who carried out a similar research using banana peels and cassava peels respectively, where the optimum day of ethanol yield was the fourth day.

Optimization of culture conditions for the fermentation of banana peel was carried out in order to obtain better conditions that will enhance ethanol yield. The effect of

substrate concentration on ethanol yield was carried out using varying substrate concentration of 2, 4, 6, 8 and 10%, from this research the ethanol yield increased with increase in substrate concentration which was statistically significant (P□ 0.05) but maximized at 8% (4.93% - 9.66%) in Figure 4.2. The increase in ethanol yield may be because, at low substrate concentrations the yeast tends to starve and productivity decreased. Also, increase in ethanol yield could be due to the presence of substrates that can readily be hydrolysed to sugar by the amylolytic activity of *A. niger* and subsequent sugar conversion to ethanol by the yeast cells in the medium (Stanberg *et al*., 2001). This work is in agreement with the work of Wen *et al.,* (2004) and Ado *et al.,* (2009) who carried a similar work using cassava starch. However, there was a decrease in ethanol yield at 10% substrate concentration (8.37%) which was found to be statistically significant (at P□ 0.05). The decrease in ethanol yield maybe because, high substrate concentration causes catabolite repression of the oxidative pathway which causes metabolite repression of the yeast that leads to decrease in ethanol yield (Verma *et al*., 2000). More also, high substrate concentration can cause end product inhibition and subsequent decrease in rate of hydrolysis (Willaert and Viktor, 2006). The decrease in ethanol yield due to high substrate concentration may also be because of increase in the substrate concentration resulted in more sugar available for hydrolysis and since the amount of cells were not proportionally added, this probably means that the sugar quantities will become excess for the cells digestive capacity which are left inactive thereby resulting to a low ethanol yield, (Verma *et al*., 2000). This work is not in agreement with the work of Jimoh *et al., (*2009) and Ajay *et al.,* (2014) who reported that

ethanol yield increased with increase in substrate concentration where the optimum concentration for ethanol yield was recorded to be 10% and 12% respectively.

From this research maximum ethanol yield of 10.52% v/v was obtained at pH 5.5 on the fourth day with pH 7.0 having the least ethanol concentration (5.65%v/v). These favourable pH may be because the yeast isolate preferred mildly acidic environment, hence with the increase in pH the yeasts tends to produce more acid than alcohol thereby leading to a decrease in ethanol yield (Kourkoutas *et al*., 2004). Also a decrease in the ethanol yield at high pH may be because enzymes (amylase) are active in mildly acidic medium and hence amylase activity of *A. niger* are sensitive to pH, (Alva *et al*., 2007). This is in agreement with the work of Thippareddy *et al.,* (2010), Jannani *et al.,* (2013) and Shilpa *et al.*,(2013) who carried out a similar research using fruit waste, grape fruit and banana peels respectively. However, this work is not in agreement with the work of Neelakanda *et al*., (2009) and Shafaghat *et al*., (2010) and Ajay *et al*.,(2014) who reported optimum yield of ethanol at a higher pH of 6.5 from cellulosic waste and banana peels. This may be because of the nature or specie of the substrate been used (Alva *et al*., 2007).

The effect of temperature variation on ethanol yield was found to increase with increase in temperature which was statistically significant ($P \square 0.05$) and maximized at $35^{o}C$, however the yield decreases at $40^{o}C$. This work is in agreement with the work of Ueno *et al*., (2002) and Ado *et al*., (2009) who carried a similar work using corn cobs and cassava starch respectively. The increase in ethanol yield maybe because enzyme activity and other chemical reactions in the cells which are favoured by temperature increase but to a tolerable level and also enzymes activity tends to be low at lower temperatures, and the decrease in ethanol yield at temperature above $35^{o}C$ may be because increasing

temperatures beyond the optimal level might dispel the available oxygen or increase the toxic effect of ethanol in the medium due to dissociation of the molecules and also decrease enzyme activity due to denaturation of the enzymes giving no significant impact on ethanol yield (Kiran *et al*., 2000). The ethanol yield at different temperatures of 25, 30 and 40^{o}C produced maximum yield of 10.95%, 11.88% and 8.37% respectively on the fourth day, except for temperature 35^{o}C that produced the highest ethanol yield of 12.63% on the third day which may be due to the fact that ethanol production is highly dependent on temperature which is one of the critical factors that have direct effect on many industrial processes and hence optimum ethanol yield tends to maximized within a shorter time period.

The optimization of culture conditions was completed by extending the length of fermentation from 5 to 10 days using optimum culture conditions. Maximum ethanol yield of 12.67% was obtained on the third day. However, there was no any increased in ethanol yield beyond the third day. This work agrees with the work of Ohgren *et al.,* (2007) and Ado *et al*., (2009) who carried a similar work using corn and cassava starch respectively and reported that maximum ethanol yield was obtained within a shorter time period under optimum culture conditions. This maybe because optimum culture conditions tends to give a better ethanol yield which can be achieved within a short time period which is desirable as it decrease time input. From this work maximum ethanol yield of 12.67% was obtained under optimum conditions which was less than the maximum yield obtained by Ohgren *et al.,* (2007) who carried a similar work using corn, the researcher obtained a maximum yield of ethanol under optimum conditions of 26%, this may be as a result of the substrate

been used as corn was found to have high carbohydrate content as compared to banana peels.

Morealso, ethanol yield obtained under optimum conditions of substrate concentration, pH, temperature and time was higher (12.67%) as compared to the yield obtained under un-optimized culture conditions. This may be because different substrate may have different conditions that favour their ethanol production capacity.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The soil is a good habitat for the isolation of amylase producing organisms especially *Aspergillus niger* due to its ability to hydrolyse complex plant materials found in the soil as it derived its growth nutrient from there. Also, the local beverage (burukutu), maybe a good source of high quality yeast in terms of its ability to produce ethanol.

From this study, banana peel wastes was found to be highly rich in carbohydrate content (70.09%) and other basic nutrients that could support microbial growth. therefore, it can be effectively used for the production of value added products like ethanol.

Despite the ability to use banana peels for ethanol production, the yield can be influenced by several factors especially temperature, pH, time and substrate concentration. Therefore, the process with optimized culture conditions as described in the research could be used in scaling up of the process to a pilot scale or commercial fermenter level thereby making the process more cost effective.

The maximum ethanol yield (12.67%) obtained from the fermentation process using co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* was carried out at 35^{o}C, pH 5.5 and 8% substrate concentration for three (3) days.

6.2 RECOMMENDATIONS

- Further studies about other microorganisms that can be efficiently used for ethanol production better than the ones used in this research should be considered.
- Studies on different banana species with higher carbohydrate content should be carryout to ensure better ethanol production.
- Government at all levels should encourage small scale production of ethanol from agricultural wastes for domestic use.
- Efforts should be made towards the provision of up to date equipments like autoclaves, incubators and distillation set-up used for ethanol production and also ensure constant electric power supply through the provision of stand-by generators or inverter by the government.
- Government research institutes should award grants to students so as to encourage more realistic approaches to solving environmental pollution by replacing fossil fuels with biofuels.
- Further research about the economic cost analysis of converting banana peels to bioethanol should be carried out.
- If all these recommendations are fully considered by government, non-governmental organizations and individuals, large scale production of Bioethanol will soon be a thing of reality in Nigeria which is of great future use.

Contribution to Knowledge

- Banana peels waste was found to have high carbohydrate content (70.09%) which is an essential raw material for bioethanol production.
- Production of bioethanol from banana peels wastes has help to solve the problem of wastes management.
- From this research, the maximum ethanol yield was found to be 12.67% which was obtained under optimal culture conditions (8% substrate concentration, pH 5.5 and at 35^{o}C for 3 days).

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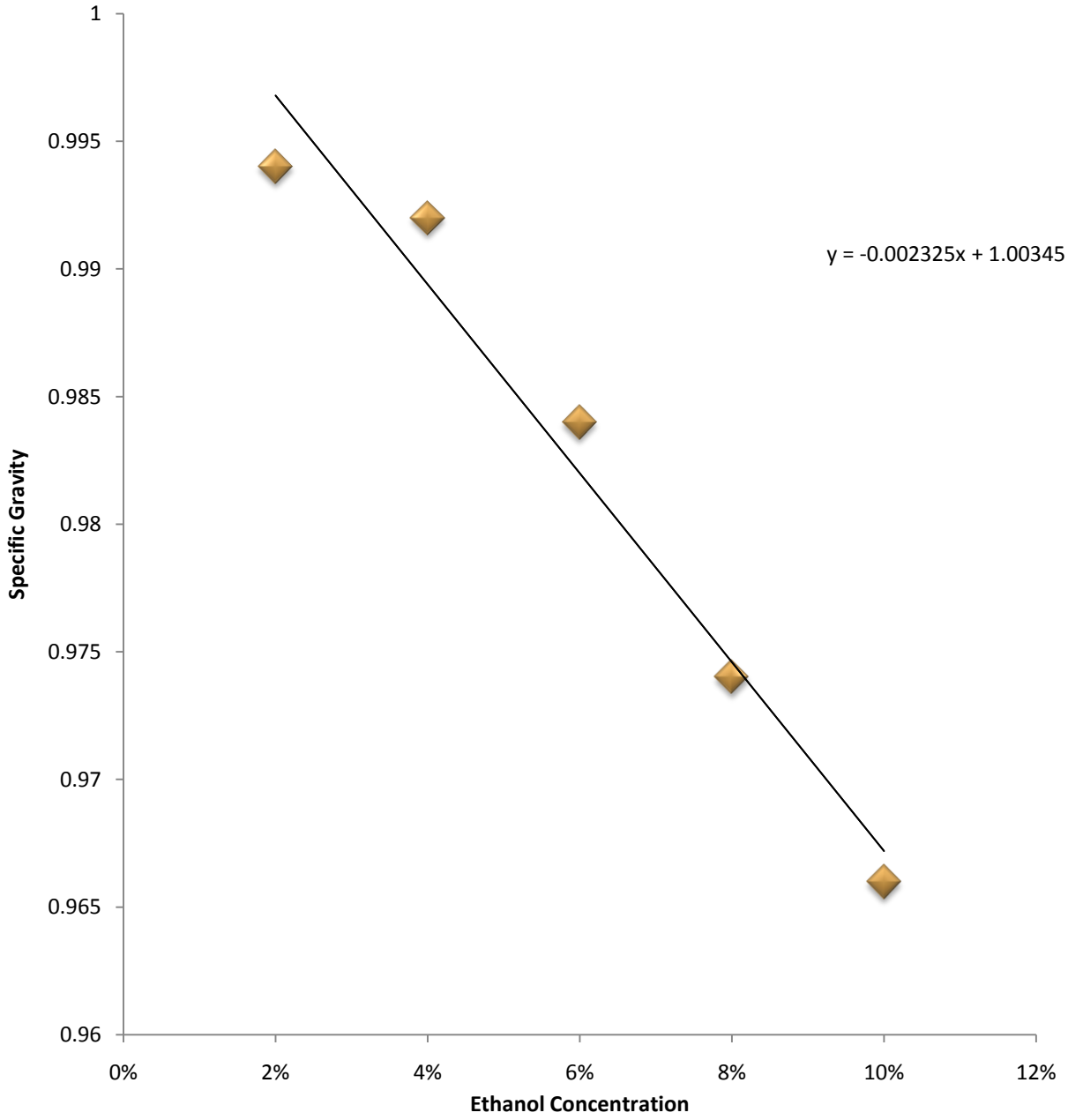
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Appendix I: Ethanol Standard Curve



Appendix II: ethanol yield obtained from substrate 4%, 30^{o}C and pH 6.0

	Ethanol yield % (v/v)
Day 1	1.48 ± 0.10^{a}
Day 2	1.91 ± 0.50^{a}
Day3	4.49 ± 0.41^{b}
Day 4	6.22 ± 0.40^{d}
Day 5	5.78 ± 0.30^{c}

Values are Mean±SD: Values with different superscript across the rows are statistically different ($P<0.05$)

Appendix III: Ethanol Yield Obtained at Different Substrate Concentration

	2%	4%	6%	8%	10%
Day 1	0.19 ± 0.05^{a}	0.62 ± 0.02^{b}	2.34 ± 0.05^{c}	2.77 ± 0.05^{d}	2.77 ± 0.05^{d}
Day 2	0.62 ± 0.03^{a}	1.48 ± 0.004^{b}	4.93 ± 0.01^{c}	5.79 ± 0.01^{e}	5.36 ± 0.01^{d}
Day3	1.48 ± 0.03^{a}	4.07 ± 0.01^{b}	6.22 ± 0.03^{c}	7.94 ± 0.04^{e}	7.08 ± 0.05^{d}
Day 4	4.93 ± 0.08^{a}	5.79 ± 0.08^{b}	7.51 ± 0.05^{c}	9.66 ± 0.06^{e}	8.37 ± 0.08^{d}
Day 5	4.50 ± 0.05^{a}	4.93 ± 0.04^{b}	7.51 ± 0.06^{c}	8.80 ± 0.05^{d}	7.51 ± 0.06^{c}

Values are Mean±SD: Values with different superscript across the rows are statistically different ($P<0.05$)

Appendix IV: Ethanol Yield Obtained from 8% substrate at different pH values

	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0
Day 1	3.20 ± 0.20^{c}	3.63 ± 0.20^{d}	3.20 ± 0.10^{c}	2.34 ± 0.10^{b}	1.48 ± 0.10^{a}
Day 2	7.08 ± 0.30^{d}	7.94 ± 0.40^{e}	4.92 ± 0.10^{c}	3.20 ± 0.40^{b}	1.91 ± 0.10^{a}
Day3	7.94 ± 0.30^{d}	9.66 ± 0.40^{e}	6.22 ± 0.50^{c}	4.92 ± 0.40^{b}	4.49 ± 0.50^{a}
Day 4	10.09 ± 0.80^{d}	10.52 ± 0.80^{e}	7.51 ± 0.50^{c}	6.65 ± 0.60^{b}	5.48 ± 0.40^{a}
Day 5	10.09 ± 0.50^{d}	10.52 ± 0.40^{e}	7.95 ± 0.60^{c}	6.22 ± 0.70^{b}	5.35 ± 0.60^{a}

Values are Mean±SD: Values with different superscript across the rows are statistically different ($P<0.05$)

Appendix V: ethanol yield obtained from 8% substrate and pH 5.5 at different temperatures

Temperature	25°C	30°C	35°C	40°C
Day 1	3.20±0.20[b]	4.07±0.20[c]	4.07±0.10[c]	2.34±0.10[a]
Day 2	7.94±0.30[b]	8.80±0.40[e]	9.23±0.10[d]	6.06±0.40[a]
Day3	8.80±0.30[b]	10.81±0.40[c]	12.63±0.50[d]	8.37±0.40[a]
Day 4	10.95±0.80[b]	11.88±0.80[c]	11.81±0.50[d]	10.52±0.60[a]
Day 5	10.52±0.50[b]	11.38±0.40[c]	11.81±0.60[d]	10.09±0.70[a]

Values are Mean±SD: Values with different superscript across the rows are statistically different ($P<0.05$)

Appendix VI: Ethanol Yield Obtained from Optimum Parameters for 10 Days

	Ethanol yield % (v/v)
Day 1	4.07 ± 0.50^{a}
Day 2	9.66 ± 0.28^{b}
Day3	12.67 ± 0.40^{g}
Day 4	11.81 ± 0.60^{f}
Day 5	11.81 ± 0.70^{f}
Day 6	10.95 ± 0.45^{d}
Day 7	11.38 ± 0.40^{e}
Day 8	11.38 ± 0.40^{e}
Day 9	10.95 ± 0.60^{d}
Day 10	10.92 ± 0.70^{c}

Values are Mean±SD; Values with different superscript within a column are statistically different ($P<0.05$)

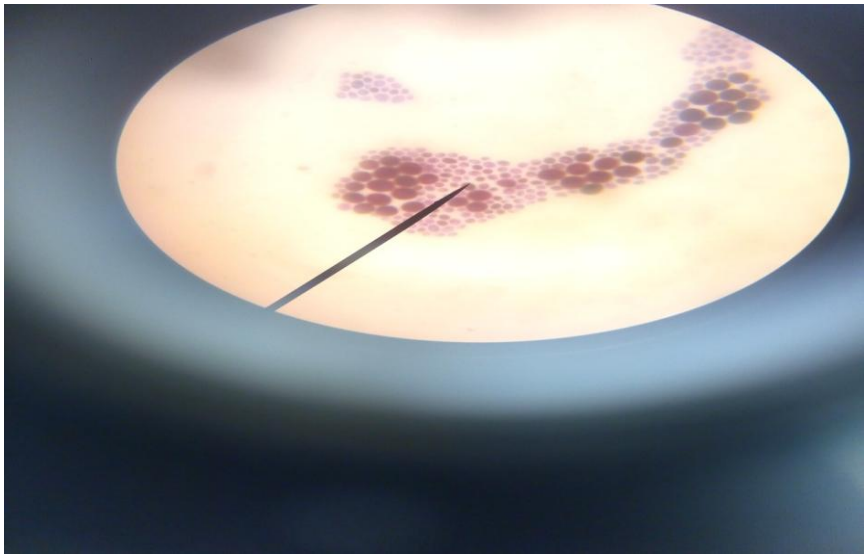


Plate V: A portion of stained yeast isolate viewed under the microscope using 100× objectives

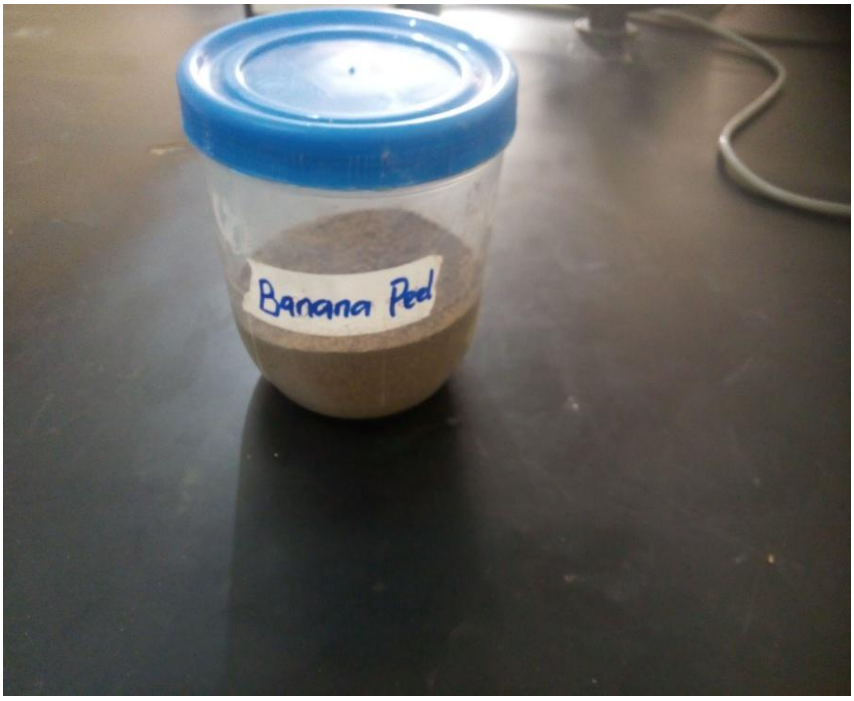


Plate VI: Banana peel substrate in air tight container



Plate VII: Left over fermentation broth after fermentation time



Plate VIII: Improvised distillation setup used for the study



Plate IX: An adjustable shaker-incubator with fermentation flasks