BIOETHANOL PRODUCTION FROM CORN COB USING CO-CULTURE OF *ZYMOMONAS MOBILIS* AND *BACILLUS LICHENIFORMIS*

BY

HELEN HOOMSUK MIGAP

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

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BY

HELEN HOOMSUK MIGAP
B.Sc (UNIJOS, 2006), M.Sc. (ABU, 2014)
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DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

JUNE, 2023

DECLARATION

I declare that the work in the thesis entitled‘‘**BIOETHANOL PRODUCTION FROM CORN COB USING CO-CULTURE OF *Zymomonas mobilis* AND *Bacillus licheniformis*** has been carried out by me in the Department of Microbiology, under the supervision of Prof. I. O. Abdullahi, Prof. C.M.Z. Whong and Prof. J.B. Ameh
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 any university.

Helen Hoomsuk Migap _____

Signature/Date

CERTIFICATION

This thesis entitled ‘‘**BIOETHANOL PRODUCTION FROM CORN COB USING CO-CULTURE OF *Zymomonas mobilis* AND *Bacillus licheniformis*** ’’by Helen Hoomsuk MIGAP meets the regulations governing the award of the Doctor of Phylosophy degree of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literacy presentation.

Prof. I. O. Abdullahi _____ _____
Chairman, Supervisory committee, Signature Date
Department of Microbiology,
Faculty of Life Sciences
Ahmadu Bello University, Zaria

Prof. C. M.Z. Whong _____ _____
Member, Supervisory committee, Signature Date
Department of Microbiology,
Faculty of Life Sciences
Ahmadu Bello University, Zaria

Prof. J.B Ameh _____ _____
Member, Supervisory committee Signature Date
Department of Microbiology,
Faculty of Life Sciences
Ahmadu Bello University, Zaria

Prof. M. Aminu _____ _____
Head Department of Microbiology Signature Date
Faculty of Life Sciences
Ahmadu Bello University, Zaria

Prof. M. N. Shuaibu _____ _____
Dean, School of Postgraduate Studies Signature Date
Ahmadu Bello University, Zaria

DEDICATION

This thesis is dedicated to God Almighty, my husband Mr Aloysius T. Raplong and my beautiful daughters Kelly, Thelma and Tehila Raplong.

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ABSTRACT

There have been growing interest about biosynthesis of fuels from renewable biomass resources due to depleting fossil resources and associated environmental issues in the past few decades. Bioethanol is considered to be a good choice for an alternative liquid fuel because it can be produced from a variety of agricultural renewable materials. Proximate analysis of corn cob was carried out and the results revealed that the substrate had the following nutritional composition: moisture (13.50%), ash (14.90%), crude protein (3.50%), lipid (1.00%) and carbohydrate (67.10%). The corn cob used in this research work had lipid and protein contents that can serve as sources of energy for microbial growth and replication. The substrate was rich in carbohydrates that have great potential for bio-ethanol production. In the present research work, *Zymomonas mobilis* an ethanologenic bacterium was isolated from palm sap and characterized molecularly by amplification of the alcohol dehydrogenase I gene and the 16SrRNA gene. A total of 10 Xylose fermenting *Bacillus species* were also isolated from the soil and characterized biochemically and through sequencing of the 16SrRNA genes. The sequences with their accession numbers have been deposited in the gene bank. The following *Bacillus* species were characterized: *Bacillus amyloliquifaciens* (20%), *Bacillus licheniformis* (20%), *Bacillus thuringiensis* (30%), *Bacillus cereus* (10%) and *Bacillus subtilis* (10%). *Zymomonas mobilis* isolates ZM2 and ZM3 were screened for alcohol tolerance and glucose fermentation to select the best to be used for bioethanol production. Growth of the two isolates were measured as optical densities at 540nm; *Zymomonas mobilis* isolate 3 (ZM3) had optical densities that ranged from 0.66-1.40 while ZM2 had optical densities that ranged from 0.34-1.36. ZM2 and ZM3 were also screened for glucose fermentation; ZM3 had ethanol yields that ranged from 30–80g/l while ZM2 had ethanol yields that ranged from 24-60g/l. ZM3 had higher optical densities in the presence of varying alcohol concentrations compared to ZM2 although it was not significantly different ($P \geq 0.05$). Similarly, ZM3 had higher ethanol yields compared to ZM2 which were significantly different ($P \leq 0.05$).The *Bacillus* isolates were screened for ethanol production from fermentation of xylose and cellulase production

to select the best isolate for bioethanol production. The isolates had ethanol yields from the fermentation of xylose that ranged from 18.50-35.5g/l. *Bacillus licheniformis* (SCFB22) had the highest ethanol yield of 35.5g/l while *Bacillus thuringiensis* (SBX8) had the lowest ethanol yield of 18.50g/l. The cellulase activities of all the isolates ranged from 1.35-2.98FPU/ml. *Bacillus licheniformis* (SCFB22) also had the highest cellulase activity of 2.98FPU/ml while *Bacillus thuringiensis* (SBX3) had the lowest cellulase activity of 1.38FPU/ml. Acetic acid adaptation of *Zymomonas mobilis* isolate ZM3 was carried out by treating the isolate with varying amounts of acetic acid that ranged from 0.2-1.6 % to enhance the isolates' ability to withstand high concentrations of inhibitors like acetic acid during fermentation. *Zymomonas mobilis* isolate ZMA with a slightly enhanced tolerance to relatively high acetic acid concentrations was obtained. Submerged fermentation of corn cob hydrolysate was carried out using acetate adapted ZMA isolate, the parent *Zymomonas mobilis* isolate (ZM3) and xylose fermenting *Bacillus licheniformis* in set ups that contained co-culture of the two bacteria and those containing the single strains. High bioethanol yields of 38.6g/l and 35.3g/l were obtained from the co-culture submerged fermentation of corn cob hydrolysate using acetate adapted *Zymomonas mobilis* (ZMA) and xylose fermenting *Bacillus licheniformis* (SCFB22) as well as co-culture submerged fermentation using the parent *Zymomonas mobilis* isolate (ZM3) and *Bacillus licheniformis* (SCFB22). Submerged fermentation using single isolates yielded relatively lower bioethanol contents. Isolates ZMA, *Zymomonas mobilis* ATCC 29191, ZM3, SCFB22 and *Bacillus licheniformis* ATCC 14580 had bioethanol yields of 32.1, 31.8, 31.9, 12.8 and 14.5g/l respectively. The acetate adapted isolate (ZMA) also had a higher ethanol yield of 32.1g/l than the parent isolate (ZM3) which had ethanol yield of 31.8g/ml. The results of the one way ANOVA that was carried out showed that the ethanol yields obtained from the co-culture fermentation using different isolates were significantly different ($P \leq 0.05$). Co-culture fermentation using *Zymomonas mobilis* isolate ZMA, ZM3 and *Bacillus licheniformis* isolate SCFB22 have great potential for industrial bioethanol production using agricultural waste (corn cob). The physicochemical parameters of the

bioethanol produced were all similar to that of the commercial 98% ethanol used in this study but with some variation in some of the parameters. All the bioethanol produced were colourless, the relative densities of the bioethanol produced ranged from 0.77-0.84g/cm^3 while that of the commercial ethanol was 0.76 g/cm^3.The variation in the relative densities of the bioethanol produced could be due to the different types of bacteria used in the set ups or the different bioethanol yields. The boiling points of the bioethanol produced were similar to that of the commercial ethanol. The boiling points of the bioethanol produced ranged from 78.6-78.9ºC while that of the commercial ethanol was 78.6ºC. The flash points of the bioethanol produced ranged from 14-16ºC while that of the commercial ethanol was 13ºC. The variation observed in the flash point of the bioethanol produced could be due to the fact that the bioethanol produced contained more water that the commercial ethanol. All the bioethanol produced as well as the commercial ethanol burned with a blue flame. The result of the physicochemical parameters of the bioethanol produced suggests that the bioethanol produced are of good quality and could serve as substitutes to conventional ethanol.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

There have been growing interest about biosynthesis of fuels, desired chemicals and materials from renewable biomass resources due to limited fossil resources and associated environmental issues in the past few decades (Lee *et al*., 2012 and Peralta-Yahya *et al*., 2012).Also increase in prices of petroleum products along with depletion of global oil reserves have made scientists to search for alternatives to conventional fuels. Methane, hydrogen and ethanol are considered as potential substitutes for fossil fuels (Lee *et al*., 2012). Among these three biofuels, ethanol is considered to be a good choice for an alternative liquid fuel for the following reasons; First, it can be produced from a variety of agriculture-based renewable materials, such as sugarcane juice, molasses, potatoes, corn and barley (Peralta-Yahya *et al*., 2012).

Maize has traditionally been utilized as the substrate for the production of ethanol. It has however been reported that the cost of raw material accounted for 70% of the total cost of producing the ethanol (Ramesh *et al.,* 2004). Thus, if the biofuel industry is to expand in future, researchers have recognized that non-food resources should be used so that the cost of production will be low and the biofuel industries will not compete with the food market in terms of land for cultivation (Doucha *et al*., 2008). Research into alternative substrates such as agricultural wastes like corn cob, corn stalk, rice straw, and molasses among others is on going to find low cost as well as efficient substrate for ethanol production (Ramesh *et al.,* 2004). About 180kg of cobs are obtained from each ton of maize shelled. However the

problem is the technology for conversion of the cellulose and lignocellulosic part of these materials to bioethanol.

Cellulose is a high molecular weight crystalline polymer which is highly stable and recalcitrant to enzymatic hydrolysis (Taherzadeand Karimi, 2007). Therefore, a pre-treatment process is necessary for efficient conversion of cellulose to sugars. Dilute-acid treatment has been successfully developed for pre-treatment of cellulosic materials. The pre-treatment can enhance the carbohydrates available for enzymatic hydrolysis and fermentation (Taherzadeand Karimi, 2007).

As an organism for bio-ethanol production, *Zymomonas mobilis* has shown some advantages over *S. cerevisiae*, for example, higher specific rate of sugar uptake, high ethanol yield (up to 2.5 times higher), lower biomass production, higher ethanol tolerance up to 16% (v/v), non-requirement of controlled addition of oxygen during fermentation and amenability to genetic manipulations (Paneser *et al*., 2006, Sahm*et al*., 2006 and Rogers *et al*., 2007). Extensive fundamental studies on *Zymomonas mobilis* over the last 30 years had also made this strain a promising ethanologenic organism for large-scale bio-ethanol production.

Zymomonas mobilis is a bacterium belonging to the genus *Zymomonas*. It is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. It was originally isolated from alcoholic beverages like the African palm wine, the Mexican pulque, and also as a contaminant of cider and beer in European countries (Shiva *et al*., 2012).

Zymomonas mobilis degrades sugars to pyruvate using the Entner-Doudoroff pathway. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (analogous to yeast) (Paneser *et al*., 2006). However, in spite of these attractive advantages,

several factors prevent the commercial usage of *Zymomonas mobilis* in cellulosic ethanol production. The foremost hurdle is that its substrate range is limited to glucose, fructose and sucrose. Wild-type *Z. mobilis* cannot ferment pentose sugars like xylose and arabinose which are important components of lignocellulosic hydrolysates (Pronk and Scheffers, 2006).Xylose is the major product of the hydrolysis of hemicellulose from many plant materials. It is abundant in the hydrolysates of hemicellulose, for about 10-40% of the total carbohydrates (Ladisch *et al*., 1993).

Cellulose is nature's most abundant polysaccharide consisting of β-1, 4-linked glucose units. As the major constituent of plant cell walls, the polymer represents the most important resource for production of bioethanol and other fine chemicals (Lynd *et al*. 2002). The use of cellulolytic enzymes rather than acid hydrolysis ensures environmentally friendly glucose formation; a prerequisite of a number of biotechnological applications including the above mentioned ones (Lynd *et al*. 2002). The demand for cellulases has increased due to their usefulness for food processing, in the textile and pulp and paper industry, as feed additives, and for lignocellulose based bioethanol production (Gaurav *et al*., 2017). Endo-1,4-β-glucanases randomly hydrolyse internal β-1, 4-D-glycosidic bonds, they decrease the polymer length, concomitantly increasing the concentration of reducing sugars. Although currently commercialized cellulases are predominantly produced by fungi, studies about bacterial cellulases had been performed on *Bacillus subtilis,Paenibacillus, Clostridium cellulolyticum, Thermobifida fusca, and Clostridium thermocellum*(Gaurav *et al*., 2017).

The hydrolysates of corn cob are composed of xylose, hexose, and other hydrolysis byproducts. Hexose is easily fermented to ethanol but xylose is more difficult to ferment.

Xylose is abundant in lignocellulosic biomass such as corn cob and forest industry waste. The fermentation of xylose is still a key problem in cellulose ethanol fermentation (Zhang *et al*., 2014). Successful utilization of xylose could improve the efficiency of the biomass-ethanol fermentation process. A number of attempts have been made in obtaining a stable microorganism capable of utilizing xylose. It was reported that several xylose fermentation strains had been isolated, such as *Pichia stipitis, Candida shehatae, Pachysolen tannophilus,* and some genetically engineered xylose-fermenting yeast and so on. However, these fermentation strains are relatively poor fermentative microorganisms (Zhang *et al*., 2014). The xylose utilization genes Xyl A (encoding xylose isomerase) and XylB (encoding xylulokinase) are clustered in *Escherichia coli, Bacillus subtilis, Bacillus megaterium* and *Bacillus licheniformis* (Schimieder and Hillen., 1996).

A co-culture is a mimic of natural environment and has been used for biodegradation of aromatic compounds or for biological reduction of sulfate (Enoch *et al*., 2012). Thus the co-culture is a potential bioprocess if there are no cross-interactions among microorganisms and each microorganism metabolizing its substrate is unaffected by the presence of other microorganisms. Bansal and Singh (2003) reported a comparative study of ethanol production from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. However, in Nigeria no study has been reported on the co-culture of Z*ymomonas mobilis* and *Bacillus licheniformis* on ethanol production from cellulose substrates.

Unlike *Escherichia coli* and yeast, *Zymomonas mobilis* cannot tolerate toxic inhibitors present in lignocellulosic hydrolysates such as acetic acid and various phenolic compounds(Joachimsthal and Rogers, 2000). Concentration of acetic acid in lignocellulosic

hydrolysates can be as high as 1.5% (w/v), which is well above the tolerance threshold of *Zymomonas mobilis*. Acetic acid resistant strains of *Zymomonas mobilis* can be developed by rational metabolic engineering efforts (Joachimsthal and Rogers, 2000) or adaptive evolution.

1.2 Statement of Research Problem

Fossil fuels are non-renewable resources because they take millions of years to form, and reserves are being depleted much faster than new ones are being made (IPCC (2014). In Nigeria, since the discovery of commercial oil researves, there has been overdependence on the fossil fuel which has dominated the agriculture and solid minerals exploitation for energy and revenue generation (Galadima *et al*., 2011).

The rising cost of fuelhas encouraged the search for products from biomass as alternatives to fossil fuel. Bioethanol and biodiesel are the two biofuels that are commonly been produced; Many countries have turned to bioethanol and biodiesel as the alternative and many researches are focused on finding efficient as well as cost-effective ways of producing biofuels (Lee *et al*., 2012).

Some of the major producers and consumers of bioethanol are the United State of America, Brazil,Japan and Germany (Goldemberg,2007). There is little information on the production and use of bioethanol in Nigeria. In 2007, Nigerian Government came up with a biofuel policywhich was drafted by the Nigarian national petroleum corperation (NNPC) to help reduce the overdependence on fossil fuel.The country was looking at the prospect of using food stuff like cassava, sugarcane and maize (Agba *et al*., 2010 and Agbula *et al*., 2011) for bioethanol production but later realized that it would cause competition between

the food market and the bioethanol industry in the era of food scarcity. The Nigerain Government realized that for the bioethanol industry to developthere was need for the utilization of a non-food source as substrate for bioethanol production, the policy was therefore abandoned for a while (Galadima *et al*., 2011)

Microalgae were being promoted as an ideal biofuel feedstock because of their rapid growth rate, low requirements forland use, high CO_2 fixation ability, lack of competition with food crops, and production possibilities on non-arable land (Demirbes.,2011).Despite the benefits of producing biofuel from algae, algal biofuel production is also associated with some disadvantages which include requirement of large amounts of energy and fertilizer; Algal biofuel degrades faster than other biofuels and does not flow well in cold temperatures and the overall cost of producing algal biofuel is relatively high compared to other biofuels. By 2017, due to economic considerations, most efforts to produce biofuel from algae have been abandoned (Wesoff, 2017 and Biofuel from Algae, 2020)

Traditionally, the microorganism used in the production of bioethanol has been *Saccharomyces cerevisiae*. This organism is however associated with low productivity and low alcohol tolerance. *Zymomonas mobilis* has several advantages over *S. cerevisiae* but only uses glucose, fructose and sucrose as their substrates. Since xylose is a major component of hemicellulose in most biomass feedstock (Pronk and Scheffers, 2006), it is essential for a fermenting microorganism to use this sugar in ethanol production for a good product yield from biomass.

Zymomonas mobilis is sensitive to acetic acid, especially at low pH (Lawford and Rousseau, 1993). The toxicity of acetic acid has been shown to intensify during xylose fermentation. The pretreated biomass by dilute-acid usually contains up to 1.5% acetic acid

(w/v) due to the hydrolysis of the acetylated pentoses in hemicelluloses (Lawford and Rousseau, 1993). Before using *Zymomonas mobilis* in industry, this inhibition problem has to be addressed.

1.3 Justification

Bioethanol is not only cheap and environmentally friendly, but can also help the country (Nigeria) to improve its economic fortunes by producing her own fuel from renewable source considering the high cost of petroleum products.

Traditional ethanol industries utilize crops such as maize and sugar cane as substrates in the production of ethanol; however this raises the cost of production and renders the resulting bioethanol uncompetitive. The use of these crops will lead to competition with their use as food. Utilization of a cheap substrate like corn cob may reduce the cost of production and solve the problem of competition with food crops.

Corn cob is one of the cheap agricultural wastes that are common in Nigeria. After removal of the corn grain the cob is disposed off most of the time, for natural degradation in farms. However, this waste can be utilized for the production of bioethanol. It may in the long run lead to a decrease in the overdependence on fossil fuels, reduce envinmental pollution due to fossil fuels and possibly improve agricultural benefits by advancing farming techniques and agricultural research.

Even though many microorganisms, including *Saccharomyces cerevisiae*, *Clostridium* sp., have been considered as ethanologenic microbes, *Zymomonas mobilis* is considered the better candidate for industrial alcohol production. *Zymomonas mobilis* possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance but has

limited substrate utilization. *Zymomonas mobilis* cannot ferment pentose sugars like xylose. The organism cannot also tolerate high acetate concentrations. Therefore co-culture fermentation with a xylose fermenting organism as well as modification of the organism using adaptation may improve acetate tolerance and further increase ethanol yield.

1.4 Scope of the Study

This research work involves isolation and characterization of *Zymomonas mobilis* and xylose fermenting *Bacillus* species, adaptation of *Zymomonas mobilis* isolates to acetic acid as well as fermentation of corn cob biomass to bioethanol.

1.5 Aim of the Study

The aim of this research work was to produce bio-ethanol from corn cob using co-culture of *Zymomonas mobilis* and*Bacilluslicheniformis*.

1.6 Objectives

The specific objectives of this research work were to:

1. determine the proximate composition of corn cob ;
2. isolate and characterize *Zymomonas mobilis* and *Bacilluslicheniformis* from palm sap and soil respectively;
3. characterize *Zymomonas mobilis* molecularly by amplification of the 16srRNA gene and alcohol dehydrogenase gene using PCR;
4. characterize *Bacilluslicheniformis* molecularly by sequencing of the 16srRNA gene
5. screen*Bacilluslicheniformis* for xylose fermentation and cellulose hydrolyzing ability and*Zymomonas mobilis* isolate for glucose fermentation and alcohol tolerance;
6. conduct acetic acid adaptation of Z*ymomonas mobilis*;

7. ferment corn cob hydrolysate using co-culture of *Zymomonas mobilis* and *Bacilluslicheniformis* in submerged fermentation;
8. determine the physicochemical parameters of the bioethanol produced.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biofuels: General knowledge

Biofuels are liquid fuels made from plant materials that can be used as a substitute for petroleum-derived fuel. Unlike fossil fuels that take millions of years to form, biofuels are produced over a short time frame and are regarded as renewable energy (Letcher, 2020 and Renewables Report, 2023). Biofuels can include relatively familiar ones, such as ethanol made from sugar cane, methane, hydrogen, bioelectricity, algal diesel, butanol and other forms of biofuel (Shweta *et al*., 2021).Among these biofuels the most commonly used renewable fuel in the transport sector is bioethanol (Asmamaw and Fassil, 2014). Biofuels are drawing increasing attention worldwide as substitutes for petroleum-derived transportation fuels to help address energy cost, energy security and global warming concerns associated with liquid fossil fuels (Goldemberg *et al*., 2004). In the United States of America which is the largest biofuel producer in the world, biofuels generated 4% of the energy used for transportation in 2011. In Brazil which is the second largest biofuel producer in the world, a total of 23% of the energy for road transport came from biofuels (Carlos *et al*., 2014).The two most common types of biofuel are bioethanol and biodiesel; The United state of America is the largest producer of bioethanol while the European Union is the largest producer of biodiesel (Biofuel Explained, 2023).

2.2 Biofuel Classification

2.2.1 First Generation Biofuels

First generation biofuels are made from food crops grown on arable land (Letcher, 2020). The most well-known first-generation biofuel is ethanol made by fermenting sugar extracted from sugar cane or sugar beets, or sugar extracted from starch contained in maize kernels or other starch-laden crops. Similar processing, but with different fermentation organisms, can yield another alcohol, butanol. Commercialization efforts for butanol are ongoing (Dupont, 2007), while the bioethanol industry is already well-established.

Global production of first-generation bio-ethanol in 2006 was about 51 billion litres , with Brazil (from sugar cane) and the United States (from maize) each contributing about 18 billion litres, or 35 per cent of the total. China and India contributed 11% to global ethanol production in 2006, and production levels were much lower in other countries with feedstock that include cane, corn, and several other sugar or starch crops such as sugar beets, wheat, potatoes (Luciano *et al*., 2007)

Many countries are expanding or contemplating expanding their first-generation ethanol production, with Brazil and the United States having by far the largest expansion plans (Goldemberg, 2007)

2.2.2 Second Generation Biofuels

Second-generation biofuels share the feature of being produced from lignocellulosic biomass, enabling the use of lower-cost, non-edible feedstock, thereby limiting direct food versus fuel competition. Second-generation biofuels can be further classified in terms of the

process used to convert the biomass to fuel: biochemical or thermochemical. Second-generation ethanol or butanol is made via biochemical processing, while all other second-generation fuels are made via thermo-chemical processing (Collins, 2007).

Second-generation thermochemical biofuels may be less familiar to most readers than second-generation ethanol, because there are no first-generation analogs. On the other hand, many second-generation thermochemical fuels are fuels that are already being made commercially from fossil fuels using processing steps that in some cases are identical to those that would be used for biofuel production. These fuels include methanol, refined Fischer-Tropsch liquids (FTL), and dimethyl ether (DME) (Coelho, 2006).

Mixed alcohols can also be made from fossil fuels, but there is no commercial production today due to the immature state of some components of systems for producing these. The other thermochemical biofuel is green diesel, for which there is no obvious fossil fuel analog. Unrefined fuels, such as pyrolysis oils, are also produced thermochemically, but these require considerable refining before they can be used in Engines.

2.2.3 Second Generation Biochemical Biofuels

The fuel properties of second-generation ethanol or butanol are identical to those of the first generation equivalents, but because the starting feedstock is lignocelluose, fundamentally different processing steps are involved in producing them. Second-generation biochemically-produced alcohol fuels are often referred to as “cellulosic ethanol” and “cellulosic biobutanol”. The basic steps for producing these include pre-treatment, saccharification, fermentation, and distillation. Pretreatment is designed to help separate cellulose, hemicelluloses and lignin so that the complex carbohydrate molecules

constituting the cellulose and hemicelluloses can be broken down by enzyme catalyzed hydrolysis (water addition) into their constituent simple sugars (Jeffries, 2006).

Cellulose is a crystalline lattice of long chains of glucose (6-carbon) sugar molecules. Its crystallinity makes it difficult to unbundle into simple sugars, but once unbundled, the sugar molecules are easily fermented to ethanol using well-known micro-organisms, and some micro-organisms for fermentation to butanol are also known. Hemicelluloses consist of polymers of 5-carbon sugars and is relatively easily broken down into its constituent sugars such as xylose and pentose. However, fermentation of 5-carbon sugars is more challenging than that of 6-carbon sugars. Some relatively recently developed micro-organisms are able to ferment 5-carbon sugars to ethanol (Zhang *et al.*, 2005).

2.2.4 Second Generation Thermochemical Biofuels

Thermo-chemical biomass conversion involves processes at much higher temperatures and generally higher pressures than those found in biochemical conversion systems. The key intrinsic characteristics distinguishing thermochemical from biochemical biofuels are the flexibility in feed stocks that can be accommodated with thermochemical processing and the diversity of finished fuels that can be produced (Stricklen, 2006).

Thermochemical production of biofuels begins with gasification or pyrolysis. The former is generally more capital-intensive and requires larger scale for best economics, but the final product is a clean finished fuel that can be used directly in engines. This focuses on gasification based processing, by which a variety of different biofuels can be produced, including Fisher-Tropsch liquids (FTL), dimethyl ether (DME), and various alcohols.

During gasification, biomass (with 10–20 % moisture content) is heated (typically by combusting a portion of the biomass in oxygen) to cause it to be converted into a mixture of combustible and non-combustible gases. Contaminants in the gas are removed, followed in some cases by adjustments (using the "water-gas shift" reaction) of the composition of the gas (also called synthesis gas, or syngas) to prepare it for further downstream processing. Carbon dioxide (CO_2) is a diluent in the syngas and so is then removed to facilitate subsequent reactions downstream. The major components of the now-clean and concentrated syngas are carbon monoxide (CO) and hydrogen (H_2), usually with a small amount of methane (CH_4). The CO and H_2 react when passed over a catalyst (the CH_4 is inert) to produce liquid fuel. The design of the catalyst determines what biofuel is produced (Devi *et al*., 2003).

In most plant designs, not all of the syngas passing over the catalyst will be converted to liquid fuel. The unconverted syngas typically would be burned to make electricity to provide some or all of the power needed to run the facility and in some cases to export electricity to the grid.

In 2008, fossil fuels accounted for 88% of the global primary energy consumption (Brennan and Owende., 2010). However, the use of fossil fuels is now widely accepted as unsustainable due to depleting resources and the accumulation of greenhouse gases in the environment.

It has become increasingly important to develop abatement techniques and adopt policies to promote those renewable energy sources to minimize the dependency on fossil reserves and also to maintain environmental and economic sustainability (Brennan and Owende, 2010 and Singh and Nigan, 2010).

Biofuels are expected to reduce dependence on imported petroleum with associated political and economic vulnerability, reduce greenhouse gas emissions and other pollutants, and revitalize the economy by increasing demand and prices for agricultural products (Balat, 2010). Biofuels are an attractive alternative to current petroleum based fuels as they can be utilized as transportation fuels with little change to current technologies and have significant potential to improve sustainability (Carere *et al.*, 2008). There are two global liquid transportation biofuels: bioethanol and biodiesel, respectively (Demirbas, 2008). Bioethanol is a good alternate fuel that is produced almost entirely from food crops (Hammond *et al*., 2008, Tan *et al*., 2009, Börjesson, 2009).

All automobile manufacturers produce vehicles that can readily use 10% ethanol or 85% ethanol (E85) blends for fuel, and ethanol can replace diesel in heavy vehicles as well. Nearly all fuel ethanol is produced by fermentation of corn glucose in the United States or sucrose in Brazil (Rosillo-Calos and Cortes., 1998).

Generally, ethanol can be derived from any material that contains sugar. Today, raw materials used in the manufacture of ethanol by fermentation are classified as sugars, starches and cellulosic materials (Bailey and Ollis, 1986). Sugars can be converted to ethanol directly, while starches first have to be hydrolyzed to fermentable sugars by the action of enzymes. Cellulose must likewise be converted to sugars before further fermentation, generally by the action of mineral acids (Bashir *et al.*, 1994).

2.2.5 Third Generation Biofuels

Third generation biofuels, also termed advanced biofuels, are produced from fast growing microalgae and are potential replacements for conventional fuels. Microalgae are being

promoted as an ideal third generation biofuel feedstock because of their rapid growth rate, low requirements for fertilizer and land use, high CO_2 fixation ability, lack of competition with food crops, and production possibilities on non-arable land (Demirbes.,2011). Microalgae have broad bio-energy potential as they can be used to produce biodiesel and bio-ethanol. For example, *Chlorella vulgaris* has been considered as a potential raw material for bio-ethanol production because it can accumulate high levels of starch (Hirano *et al*., 1997). Production of bioethanol by using microalgae can also be performed via self-fermentation. As reported by Ueno *et al*., (1998), dark fermentation of marine green algae *Chlorococcum littorale* at 30ºC could produce 450 μmol of ethanol g/1 biomass.

Marine microalgae, or phytoplankton, are mostly represented in ocean populations: the best known are the diatoms (Bacillariophyta), the dinoflagellates (Dinophyta), the green algae (Chlorophyta) and the blue-green algae (Cyanophyta). The unicellular marine microalgae wereconsidered to be an abounding resource for carotenoids, lipids, and polysaccharides, and were widely investigated in the fields of food supplements and bio-fuel production (Liau *et al*., 2010). While terrestrial plants in temperate climates can achieve a photoconversion efficiency of onlybelow 1%, microalgae can convert up to 5% of the solar energy into chemical energy (Rosch *et al*., 2012).

Microalgae can be converted to biodiesel, bioethanol, bio-oil, biohydrogen and biomethane via thermochemical and biochemical methods **(**Demirbas, 2011). Using microalgae as a source of biofuels is not a new idea (Chisti, 1980), but it is now being taken seriously because of the increasing cost of petroleum and, more significantly, the emerging concern about global warming arising from burning fossil fuels (Sawayama *et al*., 1995).

Despite the benefits of producing biofuel from algae, algal biofuel production is also associated with some disadvantages which include requirement of large amounts of energy and fertilizer; Algal biofuel degrades faster than other biofuels and does not flow well in cold temperatures and the overall cost of producing algal biofuel is relatively high compared to other biofuels (Biofuel Frm Algae, 2020). By 2017,due to economic considerations, most efforts to produce biofuel from algae have been abandoned (Wesoff, 2017)

2.2.6 Fourth Generation Biofuels

Fourth generation biofuels are a step ahead from third generation biofuels. In fourth generation systems, the feedstock is tailored to improve the processing efficiency and designed to capture more carbon dioxide than normal. The processing methods are also coupled to "carbon capture and storage (CCS)" technologies which funnel the generated carbon dioxide into geological formations or mineral storage systems. In this way, fourth generation biofuels are better at reducing greenhouse gas emissions than other generation biofuels (Chandrashekhar*et al*., 2012).

Fourth generation biofuel crops include high biomass crops, trees with increased carbon storage capacity, drought-tolerant energy crops, grass species that are able to grow on acidic soils, and new plants with particular properties catering to a specific bioconversion process (for example, low lignin trees, maize with embedded enzymes for rapid conversion). Recent examples include designed *Eucalyptus* trees with a low-lignin content which allows for easier conversion into cellulosic ethanol.

2.3 Bioethanol: In perspective

Bioethanol isa distilled colorless liquid fuel obtained from fermentation of numerous potential feedstock varieties sugar or starch rich crops such as wheat, corn, cassava, fruits, bagasse, barley, molasses, skim milk (whey),potatoes, sorghum, switch grass and cellulose biomass such as wood, paper, straw and other cellulose wastes such as grasses, others includes municipal solid wastes (Bayetoro *et al*., 2022). These various waste streams for Ethanol production have their peculiar properties and generally differ. Ethanol as an alternative fuel, offers a sustainable economy by reducing the use of imported petroleum, emitting neutral CO_2, boost economy providing value added market opportunities for the Agricultural sector (Shell Global, 2001).

2.3.1 Utilization of Bioethanol and Biodiesel as Transportation Fuels

Plant and algae-derived ethanol can be used as a pure fuel or as a mixture with gasoline in certain proportions to replace conventional fuels, without affecting the performance of the machine. Being an oxygenated fuel (contains 35% of oxygen), use of bioethanol reduces exhaust emissions such as CO and unburned hydrocarbons in to atmosphere (Balat, 2009). The most common blend of bioethanol with petrol is gasohol, also called E10 containing 10% bioethanol and 90% gasoline. E10 could be burned in the internal combustion engines (ICEs) of most modern cars (Demirbas, 2008).

Bioethanol could also be used in blended fuels such as E85 (containing 85% ethanol and 15% gasoline). The biologically produced ethanol contains about 5% water and by using an appropriate emulsifier this hydrated ethanol could be mixed with diesel. A mixture of

hydrated ethanol with diesel with the emulsifier is called diesohol. This contains 84.5% diesel, 15% hydrated ethanol and 0.5% of emulsifier.

In Brazil, ethanol is used as clean or as gasohol (24% ethanol and 76% gasoline) (Balat, 2009). While in the European Union, according to the EN228 standard, bioethanol could be used as a 5% blend with gasoline. On the other hand, biodiesel is the most common biofuel in Europe. Pure biodiesel (B100) is the lowest emission fuel. Biodiesel can be used in any diesel engine when mixed with diesel.Biodiesel is produced from oils or fats using transesterification and it can be used in its pure form (E100), it is safe to handle and transport as it is biodegradable and has a high flash point compared to diesel (Bayetoro *et al*., 2022). Bioethanol can be blended with normal gasoline in various forms: low-level blends (E10), high-level blends (E85 or E95) (Lynd, 1996). E10 (10% ethanol and 90% gasoline) is the most common ethanol blend in USA, and this can be used in new vehicle engines with non- engines. Most new cars sold in Brazil are flexible-fuel vehicles (FFV) that can run on pure 100% anhydrous ethanol as well as blends with up to 80% of gasoline. In Europe, a large volume of bioethanol is used in blends with gasoline (5% ethanol and 95% gasoline) (Wheals et al., 1999).

2.3.2 Other Uses of Bioethanol

Ethanol or ethyl alcohol, has been identified as one of the most interesting synthetic oxygen-containing organic chemical because of its unique combination of properties as a solvent, a beverage, an antifreeze, and more especially due to its versatility as a chemical intermediate for other chemicals. Ethanol is an industrial chemical which has high significant utilization. It can be used in the transportation sectors as well as in production of

pharmaceutical products, dyestuffs, perfumes and numerous products. Ethanol under ordinary condition is a volatile, flammable, clear, colorless chemical compound. The largest bioethanol producers in the world are the USA and Brazil, though they utilize cornstarch and sugarcane juice as the main substrate for bioethanol production, which is globally seen as unsustainable because of energy, food and feed controversy (Pimental *et al*., 2009; Wheals *et al*., 1999).

However, the market potential for bioethanol is not just limited to transport fuel or energy production but has a great potential to supply the existing chemical industry. Ethanol is also used as an oxygenate additive for conventional gasoline, as a replacement for methyl tertiary buthyl ether (MTBE), which is normally mixed with gasoline as additive to improve the octane number. Due to toxic properties associated with MTBE, which is also responsible for groundwater contamination, it is therefore more frequently replaced by ethyl tertiary butyl ether (ETBE) that is normally produced from bioethanol (Rutz and Janssen, 2007; Twidell and Weir, 2006). Ethanol is therefore an excellent additive for preventing engine knock and overheating of the engine valves (Wyman, 1996).

Ethanol has higher octane number (96- 113) than conventional gasoline (86-87) and thus, when blended the octane number increases, thereby reducing the need for toxic, octane enhancing additives (Mielenz, 2001). It enables combustion engines to run at a higher compression ratio and therefore provides a net performance gain of nearly 15% w/w (Wheals *et al*., 1999; Knapp *et al*., 1998). As earlier mentioned, the main chemical industries that patronize ethanol industry are: solvents and alcoholic for

beverages.According to Fukuda *et al*. (2009), about 73% of produced ethanol worldwide is used as fuel ethanol, while the rest goes to the beverage and industrial sectors.

2.3.3 World Market of Ethanol

Today, bio-ethanol is the most dominant bio-fuel and its global production showed an upward trend over the last 25 years with a sharp increase from 2000. As of 2005, worldwide production capacity for bio-ethanol fuel was about 45 billion liters per year, with approximately 15% annual growth between 2000 and 2005. This value increased to 49 billion liters in 2006, when the Americans produced 75% of the total world ethanol output, followed by Asia/Pacific and Europe/Africa with respective values of 15 and 10% (Licht, 2006). The industrial alcohol market showed a rather modest rate of growth similar to the increase in Gross Domestic Product in many countries. The market for beverage alcohol in most developed countries is stagnating, due to increased health awareness. In 2009, production of fuel ethanol reached an estimated 76 billion liters, an increase of 10 percent over 2008. The United States and Brazil accounted for 88 percent of global ethanol production in 2009. Most of the increased production occurred in the United States (Licht, 2006).

After a significant downturn in the U.S. fuel ethanol market in 2008, U.S. production rose 16 percent to about 41 billion liters in 2009, accounting for approximately 54 percent of global ethanol production. According to one estimate, U.S. ethanol (which is mostly corn-based) displaced more than 360 million barrels of imported oil for gasoline production. The highest sugar prices in years, combined with adverse weather conditions in a major producing region, resulted in a drop in Brazil's ethanol production from 27.1 billion liters

in 2008 to 26.3 billion liters in 2009. All ethanol produced in Brazil is from sugar cane (Goldemberg,2007)

All fueling stations in Brazil sell both pure ethanol and gasohol, a 25 percent ethanol/75 percent gasoline blend. Flex-fuel cars, which can use pure ethanol, gasoline, or any blend of the two, provide the flexibility to choose fuel based on price at the pump. They have been widely embraced by drivers and represent more than 95 percent of all new cars sold in Brazil. In recent years, significant global trade in fuel ethanol has emerged, with Brazil being the leading exporter. However, Brazilian ethanol export declined by almost 31 percent in 2009. International demands declined in great part because of the global economic crisis (Licht, 2006).

2.3.4 Feedstock for Bioethanol Production

Application of agricultural wastes in bio-fuel industry is the focus of many researches aimed at achieving an effective and efficient waste management scheme.Fossil fuel is a non renewable source of energy and the concern generated by its sustainability, economic and environmental impact led to the use of renewable sources for fuel (Schneider, 1989).

Energy from biomass is renewable and can contribute to sustainable development. In addition, biomass resources are often available locally and processing is feasible without high capital investment. Furthermore, dependence on biomass energy can help reduce green house gas emission (Lynd, 1996) thereby impacting on public health.The various raw materials used in the manufacture of ethanol via fermentation are classified into three main types: sugars, starchand cellulose materials (Linand Tanaka, 2006)

2.3.4.1 Sugars

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethanol and other byproducts. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to ethanol. However, since sugar materials are in the human food chain, these materials are usually too expensive to use for ethanol production. One example of a sugar feedstock is sugarcane. Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*Saccharomyces cerevisiae* also known as Bakers' yeast, since it is commonly used in the baking industry) is frequently used to ferment glucose to ethanol.

Theoretically, 100 grams of glucose will produce 51.4 g of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100%. Otherbiomass feedstocks rich in sugars include sugar beet, sweet sorghum, and various fruits. However, these materials are all in the human food chain and,except for some processing residues are generally too expensive to use for fuel ethanol production (Janick and Badger, 2002).

2.4 Lignocellulosic Biomass

Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited, and inexpensive. Such resources include: leaves, stems, and stalks from sources like corn cob, corn stover, sugarcane bagasse, rice hulls, woody crops, and forest residues. Also, other multiple sources of lignocellulosic waste from industrial and

agricultural processes include citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste, and paper mill sludge. In addition, dedicated energy crops for biofuels could include perennial grasses such as switchgrass and other forage feedstocks such as *Miscanthus giganteus*, *M. sinensis*, Bermuda grass, elephant grass, poplar etc. (Maki *et al*., 2009).

Cellulose materials represent the most abundant global source of biomass and have been largely unutilized. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about 200×10^9 tons per year, where about 8 to 20×10^9 tons of the primary biomass remains potentially accessible (Lin *et al*., 2006). Cellulose is not used for food and the biofuels industries that use lignocellulosic materials do not compete for raw materials. Cellulosic biomass such as switch grass and agricultural wastes are cheaper to produce and requires fewer inputs in form of energy.

Cultivation of such plants improves soil fertility and is accompanied by less soil erosion. Moreover, the process also represents a means of effective and efficient waste management as a large proportion of agricultural and municipal wastes are lignocellulosic. Another benefit of cellulosic ethanol is the reduction in greenhouse gas emission. Compared to gasoline, ethanol burns cleaner with greater efficiency thereby releasing up to 85% less carbon dioxide (Demain *et al*., 2005).

In contrast, ethanol from corn which frequently involve the use of natural gas as energy source for processing may not reduce green house gas emission at all (Maki *et al*., 2009).Lignocelluloses are complex substrates composed of a mixture of carbohydrate polymers i.e. cellulose and hemicelluloses tightly bound to lignin, a complex aromatic

polymer, mainly by hydrogen bonds but also by some covalent bonds. Lignin interferes with cellulose hydrolysis because it acts as a physical barrier that prevents the contact of cellulase to cellulose. The first step in biofuels production from lignocelluloses therefore is delignification to liberate cellulose and hemicelluloses from their complex with lignin. It is a very crucial, rate limiting and difficult task (Demain *et al*., 2005).

Cellulose from wood, agricultural residues, etc must be converted to sugars, either by acid or alkali hydrolysis, or by action of cellulase enzymes and the sugar can be fermented to ethanol.The chemical compositions (cellulose, hemicellulose and lignin content) of various feedstocks for bioethanol production in Table2.1show that, on average, agricultural wastes with the highest cellulose content are corn cob, softwood stems, cotton seed hairs and saw dust. Forest residues comprise about 80% of the world's biomass (Demirbas, 2005) and in the United States alone 33.5-44.6 million metric tons of corn cob are available for harvest each year.

2.4.1 Composition of Lignocellulosic Materials

Lignocellulosic materials do not contain monosaccharides that are readily available for bioconversion but polysaccharides such as cellulose and hemicellulose, lignin, extractives, and ashes. The polysaccharides need to be hydrolysed by means of either enzymes or acids to fermentable sugars (Kalman, 2006).

Table 2.1: Contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40 – 55	24 – 40	18 – 25
Softwood stems	45 – 50	5 – 35	25 – 35
Nut shells	25 – 30	25 – 30	30 – 40
Corn cobs	45	35	15
Grasses	25 –40	35 – 50	10 – 30
Paper	85 – 99	0	0 – 15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15 – 20	80 – 85	0
Cotton seed hair	80 – 95	5 – 20	0
News paper	40 – 55	25 – 40	18 – 30
waste paper from chemical pulps	60 – 70	10 – 20	5 – 10
Solid cattle manure	1.6 – 4.7	1.4 – 3.3	2.7 – 5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.5	12

Source: Sun and Cheng (2002).

2.5 Corncobs as a Feedstock of Ethanol

The maize plant comprise of the stalks, husks, shanks, silks, leaf blades, leaf sheaths, tassels and cobs. The corn cob carries the grain and together with associating husks, shanks and silks are harvested from the farm. The other parts are left on the farm to rot. Corn cobs form about 30% of maize agro-wastes (Rangkuti and Djajanegara, 1983). The agricultural residues from maize production are potential sources of sugar for ethanol production, in addition to starch and by-products. When maize is harvested in the field, the corn grain is separated from the cobs, stalks, and leaves. While the grain is transported for storing and processing, the stover is currently not widely collected. However, this biomass could be used for lignocellulosic ethanol production. Corn stover includes stalks, leaves, and corn cobs. As a renewable raw material, the corncobs from grain maize are a potential feedstock for the production of biogas, biodiesel and bioethanol to fulfill the increasing demand for biofuels (Ioannidou *et al*., 2009).

Before its use as a substrate for fermentation processes, the raw material has to be pretreated. Pretreatment is one of the many steps in the cellulose-to-ethanol process, but represents a currently critical step for hydrolysis. An effective pretreatment is performed at conditions that avoid degradation of pentose from hemicellulose, or glucose from cellulose, and limit formation of degradation products that inhibit the growth of fermentative microorganisms (Mosier *et al*. 2005). Corncobs are a lignocellulosic material composed of cellulose, hemicellulose and lignin. These polymeric fibres consist of monomeric molecules. Cellulose is built of C6 sugars; hemicellulose mainly of the C5 sugars xylose and arabinose. Lignin consists of phenolic macromolecules. Corn cobs contain 32.3-45.6%

cellulose, 39.8% hemicelluloses - mostly composed of pentosan and 6.7-13.9% lignin (Sun and Cheng, 2002) and can be converted to fermentable sugar for ethanol production.

2.6 Overview of Ethanol Process

Ethanol can be produced in two different ways. Either chemically, by hydration of ethylene, which is derived from crude oil or natural gas, or by fermentation of sugar containing feeds, starchy feed materials or lignocellulosic materials. About 5 - 10% of the ethanol produced in the world is a petroleum product. Petroleum ethanol product is made by the catalytic hydration of ethylene with sulfuric acid as the catalyst. It can also be obtained via ethylene or acetylene, from calcium carbide, coal, oil gas, and other sources. The two primary ways of producing fuel ethanol from cellulosic feedstock are: Biochemical conversion process and Thermo chemical conversion process (Wondale, 2014).

2.7 Pretreatment

Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields (Sun and Cheng, 2002; Mosier *et al*., 2005).

The effect of pretreatment of lignocellulosic materials has been recognized for a long time. The purpose of the pretreatment is to remove lignin, reduce cellulose crystalline and increase the porosity of the materials. Pretreatment must meet the following requirements: Improve the formation of sugars or the ability to subsequently form sugars by acidic or enzymatic hydrolysis; avoid the degradation or loss of carbohydrate; avoid the formation of

byproducts inhibitory to the subsequent hydrolysis and fermentation processes. Physical, chemical, physico-chemical, and biological processes have been used for pretreatment of lignocellulosic materials (Sun and Cheng,2002).

2.7.1 Physical Pretreatment

2.7.1.1 Mechanical Comminution

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling. The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Sun and Cheng, 2002).

2.7.1.2 Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300ºC, cellulose rapidly decomposes to produce gaseous products and residual char. The decomposition is much slower and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1 normal H_2SO_4, 97ºC, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80–85% conversion of cellulose to reducing sugars with more than 50% glucose .The process can be enhanced with the presence of oxygen. When zinc chloride or sodium carbonate is added as a catalyst, the decomposition of pure cellulose can occur at a lower temperature (Sun and Cheng, 2002).

2.7.2 Physico-chemical pretreatment

2.7.2.1 Steam explosion (autohydrolysis)

Steam explosion (uncatalysed or catalysed) is one of the most applied pretreatment processes owing to its low use of chemicals and limited energy consumption. With this method high-pressure saturated steam is injected into a batch or continuous reactor filled with biomass. During the steam injection, the temperature rises to 160-260ºC. Subsequently, pressure is suddenly reduced and the biomass under-goes an explosive decompression with hemicellulose degradation and lignin matrix disruption as result.

Results of steam-explosion pretreatment depend on residence time, temperature, particle size and moisture content (Sun and Cheng, 2002). Studies have been carried out to try to improve the results of steam explosion by addition of chemicals such as acid or alkali (Cara *et al.*, 2008). Limitations of steam explosion include the formation of degradation products that may inhibit downstream processes.

Steam explosion is recognized as one of the most cost effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods (Sun and Cheng, 2002).

2.7.2.2 Ammonia fiber explosion (AFEX)

In the AFEX process, biomass is treated with liquid ammonia at high temperature and pressure. After a few seconds, pressure is swiftly reduced. A typical AFEX process is carried out with 1-2 kg ammonia/kg dry biomass at 90ºC during 30 min. It reduces the lignin content and removes some hemicellulose while decrystallising cellulose. The cost of

ammonia and especially of ammonia recovery drives the cost of the pretreatment (Holtzapple *et al.*, 1991 & 1994), although ammonia is easily recovered due to its volatility.

To reduce the cost and protect the environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with a temperature up to 200ºC was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery .The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash is not necessary .AFEX pretreatment does not require small particle size for efficacy (Sun and Cheng, 2002).

2.7.2.3 Carbondioxide Explosion

Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Compared CO_2 explosion with steam and ammonia explosion, found that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion (Sun and Cheng,2002).

2.7.3 Chemical pretreatment

2.7.3.1 Alkaline Pretreatment

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials. The mechanism of

alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks. Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. The digestibility of NaOH-treated hardwood increased from 14-55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Sun and Cheng,2002)

2.7.3.2 Dilute-sulfuric acid pre-hydrolysis (DAPH)

Acid pretreatment firstly developed in Germany in 1898. In this method concentrated or dilute mineral acids like sulfuric acid are used in order to break down hemicelluloses into monomeric sugars and simultaneously removing part of the lignin. This method needs a small amount of water since a small amount of energy is required to get an optimum temperature.

Some advantages of this method are: (1) High yield of hemicelluloses sugar, (2) Remove of lignin and hemicelluloses in this method increases exposing of cellulose to enzyme, (3) Remove of heavy metals in the raw materials.

Some disadvantages of this method are: (1) Neutralization of acids is necessary, (2) Degradation of hemicelluloses sugar produce some inhibitors like acetic acid and furfural,

(3) High cost of reactor due to high pressure and temperature and resistance to low PH (Pilcher *et al*. 2004).

2.7.3.4 Ozonolysis

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw, and poplar sawdust. The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust .Ozonolysis pretreatment has the following advantages: (1) it effectively removes lignin; (2) it does not produce toxic residues for the downstream processes; and (3) the reactions are carried out at room temperature and pressure. However, a large amount of ozone is required, making the process expensive (Sun and Cheng, 2002).

2.7.3.5 Oxidative delignification

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2. The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% lignin and most hemicellulose were solubilized by 2% H_2O_2 at 30ºC within 8 h, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45ºC for 24 h used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170ºC, 5–10 min), and achieved 85% conversion yield of cellulose to glucose (Sun and Cheng,2002).

2.7.3.6 Organosolv process

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, and triethylene glycol and tetrahydrofurfuryl alcohol. Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process. At high temperatures, the addition of catalyst was unnecessary for satisfactory delignification. Usually, a high yield of xylose can be obtained with the addition of acid. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system is necessary because the solvents may be inhibitory to the growth of organisms, hydrolysis, and fermentation (Sun and Cheng, 2002).

2.7.4 Biological pretreatment

In biological pretreatment processes, microorganisms such as brown, white and soft-rot fungi are used to degrade lignin and some hemicellulose in waste materials. Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials.

The white-rot fungus P. chrysosporium produces lignin-degrading enzymes, lignin peroxidases and manganese- dependent peroxidases, during secondary metabolism in response to carbon or nitrogen. Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell walls. Other enzymes including

polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin. The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low (Sun and Cheng, 2002).

2.8 Hydrolysis

After the pretreatment process, there are two types of processes to hydrolyze the feedstocks for fermentation into ethanol, most commonly used are acid (dilute and concentrated) and enzymatic hydrolysis. In addition, there are some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocellulose may be hydrolyzed by gamma-ray or electron-beam irradiation, or microwave irradiation. However, those processes are commercially unimportant.

Both enzymatic and chemical hydrolyses require a pretreatment to increase the susceptibility of cellulosic materials (Demirbas, 2005).

2.8.1 Acid hydrolysis

2.8.1.1 Dilute Acid Hydrolysis

The dilute acid process is conducted under high temperature and pressure, and has a reaction time in the range of seconds or minutes, which facilitates continuous processing. The combination of acid and high temperature and pressure dictate special reactor materials, which can make the reactor expensive. The first reaction converts the cellulosic materials to sugar and the second reaction converts the sugars to other chemicals.

Unfortunately, the conditions that cause the first reaction to occur also are the right conditions for the second to occur.

The biggest advantage of dilute acid processes is their fast rate of reaction, which facilitates continuous processing. Since 5-carbon sugars degrade more rapidly than 6-carbon sugars, one way to decrease sugar degradation is to have a two-stage process. The first stage is conducted under mild conditions to recover the 5-carbon sugars while the second stage is conducted under harsher conditions to recover the 6-carbon sugars (Demirbas, 2005)). A range of temperatures between 170-190ºC in the first stage and 200-230ºC in the second stage is commonly used.

2.8.1.2 Concentrated Acid Hydrolysis

Hydrolysis of cellulosic materials by concentrated sulfuric or hydrochloric acids is a relatively old process. The concentrated acid process uses relatively mild temperatures, and the only pressures involved are those created by pumping materials from vessel to vessel. Reaction times are typically much longer than for dilute acid (Demirbas, 2005)). This method generally uses concentrated sulfuric acid followed by a dilution with water to dissolve and hydrolyze or convert the substrate into sugar. This process provides a complete and rapid conversion of cellulose to glucose and hemicelluloses to 5-carbon sugars with little degradation. The critical factors needed to make this process economically viable are to optimize sugar recovery and cost effectively recovers the acid for recycling. The solid residue from the first stage is dewatered and soaked in a 30 to 40% concentration of sulfuric acid for one to four hours as a pre-cellulose hydrolysis step. The solution is again dewatered and dried, increasing the acid concentration to about 70%.

After reacting in another vessel for 1 to 4 h at low temperatures, the contents are separated to recover the sugar and acid. The sugar/acid solution from the second stage is recycled to the first stage to provide the acid for the first stage hydrolysis.

The primary advantage of the concentrated acid process is the potential for high sugar recovery efficiency. The acid and sugar are separated via ion exchange and then acid is re-concentrated via multiple effect evaporators. The low temperatures and pressures employed allow the use of relatively low cost materials such as fiberglass tanks and piping. The low temperatures and pressures also minimize the degradation of sugars (Demirbas, 2005)).

Unfortunately, it is a relatively slow process and cost effective acid recovery systems have been difficult to develop. Without acid recovery, large quantities of lime must be used to neutralize the acid in the sugar solution. This neutralization forms large quantities of calcium sulfate, which requires disposal and creates additional expense (Demirbas, 2005).

2.8.2 Enzymatic hydrolysis

The hydrolysis of cellulose by cellulolytic enzymes has been investigated intensively since the early 1970s, with the objective of developing a process for the production of ethanol. Enzymatic hydrolysis methods have shown distinct advantages over acid based hydrolysis. The very mild process conditions (pH 4.8 and temperature 45– 50ºC) give potentially higher yields, the utility cost is low (no corrosion problems). Therefore, this is the method of choice for future cellulosic-to-ethanol processes (Duff and Murray, 1996). Enzymatic hydrolysis involves soluble enzymes working on insoluble substrates, so a better understanding of the action of cellulase enzyme systems and their substrates is required as

this complex reaction involves multiple cellulose-hydrolyzing activities and substrate features.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials.

These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

Due to the tough crystalline structure, the enzymes currently available require several days to achieve good results. Since long process times tie up reactor vessels for long periods, these vessels have to either be quite large or many of them must be used. Either option is expensive. Currently the cost of enzymes is also too high and research is continuing to bring down the cost of enzymes.

However, if less expensive enzymes can be developed enzymatic processes hold several advantages: (1) their efficiency is quite high and their byproduct production can be controlled; (2) their mild process conditions do not require expensive materials of construction; and (3) their process energy requirements are relatively low (Janick and Badger, 2002).

2.9 Fermentation

Lignocellulose is often hydrolyzed by acid treatment. The hydrolysate obtained is then used for bioethanol fermentation by microorganisms such as yeast. Because such lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of bioethanol (Katahira *et al.*, 2006). In general, the conversion of lignocellulosic material to sugar and then ethanol isgoverned by equation (3) below:

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

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

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

$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethyl alcohol and other by products. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, cellulosic biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to bioethanol.

To get an efficient fermentation severe inhibition should be avoided. There are four different strategies to do this. These are modifying the hydrolysis process, detoxification, i*n-situ* detoxification and using less sensitive microorganisms to inhibitors (Taherzadeh *et al*., 2007). Microorganisms, termed ethanologens, presently convert an inadequate portion of the sugars from biomass to bioethanol (Demirbas, 2005). There are a number of microorganisms that produce significant quantities of bioethanol (Stewart and Russell, 2007).

Xylose-fermenting microorganisms are found among bacteria, yeast and filamentous fungi. Today, xylose fermenting bacteria include both native and genetically engineered organisms, and many have characteristics useful for simultaneous saccharification and fermentation (Jeffries and Jin, 2000).

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

The microorganism is capable of growing at a pH as low as 5.0 and temperatures as warm as 308 K. Natural xylose-fermenting yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *C.parapsilosis,* can metabolize xylose via the action of xylose reductase (XR) to convert

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

Microorganisms for bioethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas, 2004). All the recombinant strains are mesophilic organisms and function best between 303 and 311 K.

An organism must maintain fairly constant balance of pH to survive. Most bacteria grow best in a narrow range of pH from 6.5 to 7.5.

The ability to lower pH below 4.0 offers a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants. The majority of organisms cannot tolerate bioethanol concentrations above 10–15 % (w/v) (Hettenhaus, 1998).

2.10 Distillation

Distillation is one of the steps of the purifications. Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. This is because all materials have intermolecular interactions with each other, and two materials will co-distill during distillation. This means that proportion between two materials, in this case ethanol and water can be changed, and still, there are two materials in layers, the liquid and the vapor layers (Onuki, 2005).

Whatever method of preparation is used, the ethanol is initially obtained in a mixture with water. The ethanol is then extracted from this solution by fractional distillation. Although the boiling point of ethanol, 78.3ºC, is significantly lower than the boiling point of water, 100ºC, these materials cannot be separated completely by distillation. Instead, an azeotrope mixture (i.e. a mixture of 95% ethanol and 5% water) is obtained, and the boiling point of the azeotrope is 78.15^{o}C. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill from the distillation flask, and this material is the azeotrope of 95% ethanol which has the lowest boiling point. If an efficient fractionating column is used, 95% alcohol could be obtained first and then a small intermediate fraction of lower concentration, and then water. But no matter how efficient the fractionating column used, 95% alcohol cannot be further concentrated by distillation because the vapor has exactly the same composition as the liquid; towards distillation, then, 95% alcohol behaves exactly like a pure compound (Jackman, 1987)

2.11*Zymomonas mobilis*

2.11.1 Cell Morphology.

Zymomonas mobilis is a gram-negative, facultative anaerobic, non-sporulating rod shaped bacterium, 2 to 6 um long and 1 to 1.5 um wide, occurring singly but mostly in pairs (Swings and De Ley, 1977).The large cell-width of *Zymomonas mobilis* is very striking upon microscopic observation; most other bacteria are only 0.5 to 0.75m wide. In solid standard medium, deep colonies are lenticular, regular, entire edged, butyrous, white or cream colored, and 1 to 2 mm in diameter after 2 days. Anaerobic surface colonies are spreading, entire edged,convex or umbonate, and 3 to 4 mm in diameter. It is the only species found in the genus *Zymomonas*.

2.11.2 Occurrence of *Zymomonas mobilis*

Zymomonas mobilis was originally isolated from alcoholic beverages like the African palm wine, the Mexican pulque and in Europe, these bacteria occasionally grow in and spoil beer, fermented apple juice (cider), and pear juice (perry) (Obire, 2005)

2.11.3 Central Metabolic Pathways

Zymomonas mobilis produces ethanol and carbon dioxide in equimolar, near-theoretical amounts from the sugars glucose, fructose, and sucrose using the Entner-Doudoroff (ED) pathway, in combination with the pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (ADH) enzymes (Dawes et al. 1966). *Zymomonas mobilis* uses the ED pathway anaerobically to produce only one mole of ATP per mole of glucose (Kalnenieks, 2006). *Z. mobilis* is restricted to the ED pathway for the metabolism of sugars because its genome

lacks sequences for the 6-phosphofructokinase enzyme of the EMP pathway and the 2-oxoglutarate dehydrogenase complex and malate dehydrogenase enzymes of the tricarboxylic acid (TCA) cycle (Seo *et al.* 2005). Genes for most pentose phosphate pathway (PPP) enzymes are also not present on the *Z. mobilis* chromosome (Seo *et al.* 2005). Similar to glucose and fructose, sucrose metabolism also proceeds through the ED pathway in *Z. mobilis*, with the sugar first being degraded into glucose and fructose monomers by extracellular levansucrases, frequently with the formation of a levan byproduct (Goldman *et al*., 2008)

2.11.4 *Zymomonas mobilis* Growth Response to Different Conditions of Growth in some ordinary media.

Zymomonas mobilis grows on fermentable sugars like glucose, fructose, or (for some strains) sucrose as carbon sources and cannot utilize pentoses like xylose, which is the second most abundant sugar in pretreated and saccharified biomass slurries (Kerr *et al*.,2011 and Yang *et al*,2012). In the liquid standard medium good growth and gas production is observed after 24 hr. In peptone broth plus 2% glucose, in beer plus 2% glucose, in nutrient broth or nutrient agar plus 2% glucose or in palm juice, good growth occurs. *Zymomonas* does not grow in 0.5% yeast extract, beer with 0.5% yeast extract and peptone broth with 0.5% yeast extract, nutrient broth or agar, or nutrient agar with 0.5% yeast extract.

2.11.5 Growth at different pH values.

Richard and Corbey (1976) found that *Zymomonas mobilis* grows within the pH range 3.4 to 7.5. They determined that two *Zymomonas* isolates from sick cider grew in the pH range 3.5 to 7.9; in the pH range 2.5 to 3.4, there was no growth; at pH 3.5 and 3.6, growth occurred sometimes. The wide pH range for growth, from 3.5 to 7.5, and the acid tolerance are both quite typical for *Zymomonas*. This is not surprising, since in nature they live in acid palm wines and ciders below pH 4.

2.11.6 Growth at different temperatures.

Z. mobilis grows best at room temperature,above 30ºC and growth at 40ºC is rarely observed.

2.11.7 Growth in the presence of ethanol.

Zymomonas mobilis is alcohol tolerant, as it is isolated from alcoholic beverages containing 2 to 10% ethanol. A concentration of 5.5% ethanol in liquid standard medium is quite harmless to *Zymomonas*, since most strains grow at 7.7 and 10% ethanol content (Swings and De Ley, 1977).

2.11.8 Growth in high glucose concentrations and Hydrogen sulphide production

Swings and De Ley (1977) observed that the *Z. mobilis* was able to grow in and ferment media with 25% glucose. H_2S Formation and Sulfur Metabolism "Sulfury" aromas are of great technological importance in beer and cider. They are due to H_2S and other unknown compounds. The formation of H_2S by *Zymomonas* has been described by Richards and Corbey, 1976.

2.11.9*Zymomonas mobilis* as an Organism Used In Fermentation

Traditionally, ethanol has been produced in batch fermentation with fungal strains such as *Aspergillus niger*, *Mucor mucedo* and *Saccharomyces cerevisiae*, which cannot tolerate high concentrations of ethanol. Therefore, improvement programmes are required in order to obtain alcohol-tolerant strains for fermentation (Gunasekaran and Chandra, 2007). *Zymomonas mobilis*, a Gram negative bacterium, is considered an alternative organismin for large scale ethanol production. Its advantages over yeasts include higher sugar uptake and ethanol yield, lower biomass production and higher ethanol tolerance. This organism can be isolated from palm wine or rotten oranges (Gunasekaran and Chandra, 2007). *Zymomonas mobilis* is a bacterium that is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects (Shiva *et al*., 2012). *Z. mobilis* degrades sugars to pyruvate using the Entner-Doudoroff pathway. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (analogous to yeast) (Paneser *et al*., 2006). However, in spite of these attractive advantages, several factors prevent the commercial usage of *Z. mobilis* in cellulosic ethanol production. The foremost hurdle is that its substrate range is limited to glucose, fructose and sucrose. Wild-type *Z. mobilis* cannot ferment pentose sugars like xylose and arabinose which are important components of lignocellulosic hydrolysates (Pronk and Scheffers, 2006).

2.11.10 *Zymomonas mobilis*for ethanol production from lignocellulosic biomass

Bioethanol used to be produced by the yeast *S. cerevisiae* from the six-carbon sugars found in food crops such as corn and sugarcane (Wheals *et al*., 1999). However recently, focus has shifted to producing biofuels from lignocellulosic feedstocks, such as agricultural and

municipal wastes (Schubert 2006). Cellulosic feedstocks are cheap, abundant, and do not compete with the nation's food supply. They are made up of three primary components: cellulose, hemicellulose, and lignin (Demirbas, 2009). After mechanical degradation and an acid pretreatment, cellulose and hemicellulose can be broken down enzymatically into a mixture primarily composed of the six-carbon sugar glucose and the five-carbon sugars xylose and arabinose, which can then be fermented into ethanol or other biofuels by microorganisms (Carroll and Somerville 2009; Lawford and Rousseau, 2002). The sugar mixture may also contain a variety of growth inhibitors, including acetic acid, formic acid, furfural, 5-hydroxymethylfurfural and levulinic acid (Shuai *et al*., 2010). In order for the cellulosic ethanol production process to be economical, it is important that the microorganism used have several important characteristics. It must be able to ferment both five and six-carbon sugars rapidly to ethanol, in the process tolerating high concentrations of both the sugars and the ethanol, and it must also have a high tolerance to the inhibitors in the hydrolysate (Dien *et al*., 2003). Further, the organism should be safe, resistant to contamination, and amenable to genetic manipulation (Nevoigt, 2008).

2.12*Bacillus* species: Basic understanding

Bacillus refers to a specific genus of bacteria. All members of the family Bacillaceae are Gram-positive, rod-shaped bacteria which form endospores, with two main divisions: the anaerobic spore-forming bacteria of the genus *Clostridium* and the aerobic or facultatively anaerobic spore-forming bacteria of the genus *Bacillus(*Vilian *et al*., 2006)

Characteristically, *Bacillus* cultures are gram-positive when young, but may become Gram-negative as they age. *Bacillus* species are aerobic, sporulating, rod-shaped bacteria which are ubiquitous in nature. Gram-stained cells, 1 µm wide, 5-10 µm long, arranged singly or in short chains.

The genus *Bacillus* consists of the best known species such as *Bacillus subtilis, Bacillus anthracis, Bacillus cereus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus sphaericus* and *Bacillus thuringiensis* with other 50 valid species. Studies with 16SrRNA have now allowed a start to be made on breaking the genus down into more manageable and better-defined groups (Rossler *et al*., 1991). Many of these species now fall into several apparently distinct rRNA sequence groups such as *Bacillus subtilis* group, *Bacillus cereus* group, and *Bacillus sphaericus* group.

2.12.1 *Bacillus licheniformis*

2.12.2 Structure

Bacillus licheniformis is a Gram positive, spore forming, facultative anaerobic,rod shaped bacterium (Clement *et al*.,2002). *B. lichenifromis* has a variety of colony morphologies with the rough 'licheniform' cream coloured colonies giving the organism its name. It is found in a wide variety of environments especially in the soil and feathers of birds where it is degrading β-keratin. Its optimum growth temperature is 40-50ºC but its optimal temperature for enzyme secretion is 37ºC (Logan and Paul, 2015).

2.12.3 *Uses of Bacillus licheniformis*

B. licheniformis secretes serine protease which is used in laundry detergent formulation due to its ability to perform at high ph levels. Amylases are also produced by *B. licheniformis* and used for industrial purposes. They are used in the production of peptide antibiotics such as bacitracin. It has been used as a probiotic in animal feed where it has been shown to prevent disease and promote growth (Muras *et al*.,2021)

2.12.4 Pathogenicity

Although *B. licheniformis* are generally regarded as safe, some strains can cause disease in immunocompromised patients. It can also contaminate food especially dairy and can cause ropiness in bread (Dhakal *et al*.,2013)

2.13 Adaptative Laboratory Evolution

Adaptive laboratory evolution is a method used in biological science to gain insight into the basic mechanisms of molecular evolution and adaptive changes that accumulate in microbial populations during long term selection under specified growth conditions. Adaptive laboratory evolution (ALE) as a scientific approach is very important towards the analysis of evolutionary phenomena in a controlled laboratory setting. ALE dates back to scientists such as Antony Van Leeuwenhoek,Louis Pasteur, Robert kock and Charles Darwin with their discovery of microbes,the importance of natural and artificial selection for biological evolution and breeding. Hence ALE was already performed about a hundred years ago (Benneth and Hughes, 2009). However,in the last 25 years,there has been an increasing number of such experiments with *E. coli* and *Sacharomyces cerevisiae* being the

most prominent organisms under investigation (Benneth *et al*.,1990,Pacquin and Adams ,1983). During ALE, a microbe is cultivated under clearly defined conditions for prolonged periods of time,in the range of weeks to years which allows selection of improved phenotypes. ALE allows phenotypic changes to be clearly associated with a certain growth environment that leads to the selection of traits.

2.13.1 Adaptative Laboratory Evolution Selection method

Genarally,ALE experiments involve batch cultivation in shake flasks which can be perfomed to propergate microbial cells in parallel serial cultures. At regular intervals, an aliquot of the culture is transferred to a new flask with a fresh medium for an additional range of growth (Gonzalez and Bell, 2013). In a batch culture, several environmental conditions can be controlled including temperature, pH, varying nutrient, oxygen, stress factors among others. During ALE,certain trait values change and are associated with improved fitness such as an improved phenotype or property (Dettman *et al*.,2012)

2.13.2 Adaptation towards nutrient sources and environmental stresses

In ALE, microbes are being challenged with changing environmental conditions where they experience fluctuations in temperature, pH, oxygen availability, nutrient availability or deprivation and stress factors. Microbial cells usually evolve mechanisms to successfully cope with the stressful conditions in order to maintain cellular homeostasis. Microbes with improved Industrial traits are being evolved during ALE (Nam *et al*.,2011). During acid pretreatment of lignocelluloses biomass, several inhibitory compounds like acetate, hydroxymethylfurfural (HMF), and furfural are produced from the biomass pretreatment

process leading to hydrolysate toxicity which is a major problem for microorganisms to achieve complete sugar utilization. Therefore, development of more robust microorganisms using ALE that are capable of utilizing the sugars released from biomass under toxic environment is critical.

2.14 Co-culture fermentation

Co-culture fermentation is an approach that involves the use of two or more microorganisms simultaneously in the same medium for fermentation. Utilization of co-cultures for ethanol production appears to have an advantage over single culture since there is potential for synergistic action of metabolic pathways of all the strains involved (Bader *et al*., 2010). According to US Department energy (DOE) study, co-culture bioconversion is a very plausible and potentially high payoff opportunity for ethanol production (Department of energy,2005). Research has been conducted to study co-culture fermentations for ethanol production and have shown promising results. These include co-culture of immobilized *Z. mobilis* and *Pichia stipitis*, co-culture of ethanologenic *E. coli* strain KO11 and *S. cerevisiae* (Fu *et al*., 2009).

2.15 Bioethanol Production Using Biomass (Cellulose Waste)

Bioethanol is a renewable energy source produced mainly by the sugar fermentation process (Anuj *et al*., 2007). Biomass is seen as an alternative energy source for bioethanol production for several reasons. The main reason is that biomass can contribute to sustainable development. Resources are often locally available and conversion into secondary energy carriers is feasible without high capital investments. Furthermore, energy

plantations may also create new employment opportunities in rural areas; it also contributes to the social aspect of sustainability. However, the use of food crops such as corn and sugarcane to produce biofuels (bioethanol) is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of biowastes into a biomass economy (Mahro and Timm, 2007).

The long-term benefits of using waste residues as lignocellulosic feedstocks will be to introduce a sustainable solid waste management strategy for a number of lignocellulosic waste materials, contribute to the mitigation in greenhouse gases through sustained carbon and nutrient recycling, reduce the potential for water, air, and soil contamination associated with the land application of organic waste materials, and to broaden the feedstock source of raw materials for the bioethanol production industry. Agricultural wastes, including wood, herbaceous plants, crops and forest residues, as well as animal wastes are potentially huge source of energy. In Nigeria, large quantities of these wastes are generated annually and are vastly underutilized. The practice is usually to burn them or leave them to decompose. However, studies have shown that these residues could be processed into liquid fuel such as biogas and bioethanol, or combusted to produce electricity and heat (Soltes, 2000). Ethanol production processes only use energy from renewable sources and there is no net CO2 emission to the atmosphere, thus making ethanol an environmentally beneficial energy source. In addition, ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the green house gas effect. This reduction of green house gas emission is the main advantage of utilizing biomass conversion into ethanol (Anuj *et al*., 2007).

Research into alternative cellulose substrates such as biomass like corn cob, corn stalk, rice straw, and molasses among others is on going to find low cost as well as efficient substrate for bioethanol production (Ramesh *et al.,* 2004). Production of bioethanol will go a long way to reduce green house gas effect and global warming.

2.16 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a method widely used to make millions to billion copies of a specific DNA sample. PCR allows scientist to take a very small sample of DNA to amplify it to a large amount to study it in detail (The Nobel Prize in Chemistry, 1993).PCR is fundamental to many of the procedures used in genetic testing and research including the analysis of ancient samples of DNA and identification of infectious agents (Sarki *et al*., 1985). PCR is the most sensitive of the existing rapid methods of detecting microbial pathogens (Yoshimasa, 2002). The PCR in diagnosis involves several critical steps such as DNA extraction from specimens, PCR amplification and detection of amplicons. In PCR specific genes are been targeted,the choice of the target gene and the design of oligonucleotide primers are critical elements in determining the specificity of PCR (He *et al*., 1994). The reaction is commonly carried out in volumes of 10-20 micrometre in a thermal cycler. The individual steps common to PCR include; Initialization, denaturation, annealing and extension which are been carried out at different temperatures (Sharkey *et al*., 1994). To check whether the PCR has successfully generated the target DNA region (amplicon), agarose gel electrophoresis may be done to separate the PCR products based on their sizes. The size of the PCR products is been determined by

comparism with a DNA ladder, a molecular weight marker which contains DNA fragments of known sizes which runs on the gel alongside the PCR products (Berker *et al*., 2000).

2.17 16srRNA Gene Sequencing

The 16srRNA gene sequencing is commonly used for identification, classification and quantification of microbes within complex biological mixtures (Schmidt *et al*.,1991). The 16srRNA gene is a highly conserved component of the transcriptional machinery of all DNA-based life forms and thus is highly suited for a target gene for sequencing DNA in samples containing thousands of different species (Cox *et al*., 2013). The 16srRNA gene is about 1550bp long and is composed of both variable and conserved regions. The 16srRNA gene sequence is universal in bacteria and so relationships can be measured among all bacteria (Jill, 2004). There are several methods to determine gene sequences. Bacterial genomic DNA is first extracted using standard protocol; the DNA is then used as a template for PCR to amplify a segment of about 1500bp of the 16srRNA gene sequence. Universal primers complimentary to conserved regions are used so that the region can be amplified from any bacteria. The PCR products are purified to remove excess primers and nucleotides. The next step is called cycle sequencing which is similar to PCR in that it uses DNA as a template and the product is a mixture of DNA of different lengths (Jill, 2004).

2.18 Molecular Phylogenetics

Molecular phylogenetics predates DNA sequencing by several decades. It originates from the traditional methods of classifying organisms according to their similarities and differences (Brown,2002).The sequences of proteins and DNA molecules provide the most detailed and unambiguous data for phylogenetics. The objective of most phylogenetic

studies is to reconstruct a tree-like pattern that describes evolutionary relationships between the sequences of the organism been studied with those available in the gene bank data base. The term phylogenetic comes from two ancient Greek words “phulon” meaning lineage and “genesis” meaning origin (Baily, 1981). A rooted phylogenetic tree (also refered to as a dendogram) is a tree with a unique node (root) corresponding to the most recent common ancestor of all the entities at the leaves. The most common method for rooting a tree is the use of an outgroup close enough to allow inference from trait data or molecular sequencing but far enough to be an outgroup (Dang *et al*., 2022)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

Fresh palm sap samples were obtained from palm wine tappers at Bokkos Local Government area Plateau State, Manchok Kaduna State and Vom, Plateau State. The samples were stored in sterile universal bottles and placed in a flask containing ice packs and transported to the Microbiology laboratory (Central diagnostic laboratory) of National Veterinary Research Institute (NVRI) Vom.Soil samples were collected from the top 2cm profile of the soil into universal bottles using a sterile spatula from NVRI refuse dump sites in Vom.Corn cob samples were obtained from different farms in Vom, Jos South LGA Nigeria, Plateau state after corn harvesting had taken place.

3.2 Proximate Analysis of corn cob

3.2.1 Pulverization of corn cob

Dried corn cobs were pound mechanically using a laboratory pestle and mortar. The pound material was then milled using a grinding machine. The powdered corn cob was stored in a polythene bag at room temperature prior to the commencement of analysis.

The Pulverized corn cob sample was examined for moisture content,ash content ,crude protein, crude lipid, and carbohydrate content using the methods described by AOAC (1990),Kirk and Sawyer (1980) and James (1995).

3.2.2 Moisture content

Moisture content was determined according to the method of Der-Jiun*et al*. (2012). A clean crucible was dried to constant weight in a hot air oven at 105ºC and then cooled in

desiccators and was weighed (W1). Two grams of the corn cob sample was weighed into the previously labeled crucible and reweighed (W2). The container was dried in hot air oven at 105ºC to constant weight (W3). The percentage moisture content was calculated as:

$$\text{Percentage moisture content} = \frac{W_3-W_1\times 100}{W_2-W_1}$$

3.2.3 Ash content

Ash content was determined according to the method reported by Association of Analytical Chemists (AOAC) (2010) and Adeniyi *et al*. (2011). A porcelain crucible was dried in an oven at 100ºC for 10 mins; it was cooled in a desiccator and weighed (W1). Two grams of the corn cob sample was placed into the weighed crucible and was reweighed (W2). The sample was heated in the furnace at 500ºC for eight hours to ensure proper ashing. The crucible containing the ash was removed and cooled in desiccators and weighed (W_3). The percentage ash content was calculated

as:

$$\text{Percentage ash content} = \frac{W_3-W_1\times 100}{W_2-W_1}$$

3.2.4 Crude protein

3.2.4.1 Protein Digestion

The protein content was determined using the method adopted by Babalola and Akinsoyinu (2011). Corn cob sample (1.5g) in an ashless filter paper was dropped into 300 ml Kjeldahl flask. Twenty-five millilitres (25ml) of H_2SO_4 and 3g of digesting mix catalyst (which was weighed separately into an ashless filter paper) were dropped into Kjeldahl flask. The flask was then transferred to Kjeldahl digestion apparatus. The sample was digested until a clear green colour was obtained. The digest was cooled and diluted to 100ml with distilled water.

3.2.4.2 Distillation of the digest

Twenty milliliters of the diluted digest was measured into a 500 ml Kjeldahl flask containing anti-bumping chips and 40 ml of 40% NaOH was slowly added by the side of the flask. A 250 ml conical flask containing a mixture of 50 ml of 2% of Boric acid and 4 drops of mixed indicator was used to trap the ammonia liberated. The conical flask and the Kjeldahl flask was then placed in the Kjeldahl distillation apparatus, with the tubes inserted into the conical flask and the Kjeldahlflask. The flask was then heated to distill out the ammonia.The distillate was collected into boric acid solution. From the point when the boric acid turned green, 10 min was allowed for complete distillation of the ammonia present in the digest. The distillate was titrated with 0.1M HCl.

Calculation:

$$\text{Percentage N} = \frac{14 \times M14 \times Vt \times Tv \times 100}{\text{Weight of sample (mg)} \times V_s}$$

Where M = Actual molarity of acid, Tv = titre volume of acid used, Vt = Total volume diluted digest, Vs = Aliquot volume distilled.

The protein percentage was determined by conversion of nitrogen percentage of protein by using conversion factor (6.25)

Percentage crude protein = % Nitrogen (N_2) x 6.25

3.2.5 Crude lipid content

A clean, dry 500ml round bottom flask, containing few anti-bumping granules was weighed (W_1) and 300ml of petroleum ether (40 – 60ºC) for extraction was poured into the flask fit with soxhlet extraction unit (AOAC, 2010). The extractor thimble containing twenty grams

of the sample was fixed into the soxhlet extraction unit. The round bottom flask and a condenser were connected to the soxhlet extractor and cold water circulation was put on. The heating mantle was switched on and the heating rate was adjusted until the solvent was refluxed at a steady rate. Extraction was carried out for 6 h. The solvent was recovered and the oil was dried in the oven at 70ºC for one hour (1 h) (AOAC, 2010). The round bottom flask containing the oil was cooled in the desiccators and then weighed (W_2).

The lipid content was calculated thus:

$$\text{Percentage crude Lipid Content} = \frac{W_2 - W_1 \times 100}{\text{Weight of sample}}$$

3.2.6 Carbohydrate content (by difference)

The total carbohydrate content was determined by difference. The sum of the percentage moisture, ash, crude lipid and crude protein was subtracted from 100 (Der-Jiun*et al.*, 2012).

Calculation:

Total carbohydrate (%) = 100 - (%Moisture + %Ash + %Fat + %Protein).

3.3 Isolation of *Zymomonas mobilis* from palm sap

The method described by Obire (2005) was adopted for isolation of *Zymomonas mobilis*.Malt yeast peptone glucose (MYPG) medium with the following composition was used: malt extract (3.0g), yeast extract (5.0g), peptone (5.0g), glucose (20g), distilled water (1litre),the medium pH was adjusted to 4.8. The medium was supplemented with 10mg cycloheximide (actidion) per litre to inhibit fungal growth and sterilized by autoclaving at 121ºC, 15 psi for 15min.An aliquot (1ml) of the palm sap sample was taken and serially diluted in several test tubes containing 9ml sterile distilled water to form 10^{-1}-10^{-6} dilutions. An aliquot (0.1ml) of the diluents were obtained with a sterile pipette and inoculated onto

the surface of the MYPG agar. A sterile bent glass rod was used to evenly spread the inoculum. Inoculated plates were incubated for 2 days at 30ºC in an anaerobic jar containing anaerobic gas packs to exhaust the oxygen and produce CO_2 saturated atmosphere. Presumptive colonies of *Zymomonasmobilis* were sub-cultured on fresh MYPG medium and incubated for 2 days at 30ºC. Isolates were preserved by refrigeratingon MYPG slantsuntil required.

3.4 Identification and Characterization of Presumptive *Zymomonasmobilis*

Presumptive *Z. mobilis* isolates were Gram stained and subjected to some biochemical tests for characterization. The biochemical tests include: catalase test, motility, oxidase, urease, nitrate reduction, indole, carbohydrate fermentation tests as well as alcohol tolerance test. The isolates were further confirmed by amplification of the alcohol dehydrogenase I gene and 16SrRNA gene.

3.4.1 Gram staining

A drop of normal saline was placed on a clean grease free slide. Wire loop was heated until red hot, cooled and used to pick a part of the colony of the test organism and placed on the slide and a smear was made. The smear was heat fixed by passing the slide four times over a flame of a Bunsen burner. The heat fixed smear was covered with crystal violet for one minute and washed with distilled water. This was followed by covering the smear with lugol's iodine solution for one minute. The smear was washed with distilled water and then decolorized with acetone for 30 sec. It was further washed off with distilled water and flooded with saffranin for 30 sec after which it was washed with distilled water and blot

dried. A drop of immersion oil was placed on the dried stained slides and the slides were viewed under the microscope using 100Xoil immersion objectives (Beveridge, 2001).

3.4.2 Indole test

A loopful of the bacterial suspension was inoculated in a bijou bottle containing 3 ml of sterile tryptone water. It was then incubated at 30ºC for up to 48 h followed by addition of 0.5 ml of Kovac's reagent and shaken gently. A red colour in the surface layer within 10 min indicated a positive result (Cheesebrough, 2010).

3.4.3 Oxidase test

A piece of filter paper was placed in a clean petri dish followed by addition of 3 drops of freshly prepared oxidase reagent. With the aid of a piece of an applicator stick, a colony of the test organism was smeared on the filter paper. The development of a blue-purple colour within ten (10) sec indicated a positive result (Cheesebrough, 2010).

3.4.4 Urease test

A loopful of the bacterial suspension was inoculated in a bijou bottle containing 3 ml sterile Christensen's modified urea agar and Incubated at 30ºC for 12 h. The appearance of a pink colour in the medium indicated a positive result (Brenner *et al*., 2004).

3.4.5 Carbohydrate fermentation

The ability of the bacteria to ferment various sugars using glucose, fructose, sucrose, maltose, lactose, arabinose and xylose was determined by growing the isolates in ZSM liquid media with the following composition: yeast extract (3g/l), glucose (5g/l) and 1% (w/v) of the particular sugar. Durham-tubes were inverted into the culture tubes for gas

collection, an aliquot (10ml) of the media containing each of the sugars were dispensed into different test tubes. A loopful of the test organism was inoculated into each of the test tubes. Incubation was done at 30ºC for 24h and un-inoculated broths were used as controls. Collection of gas in the Durham tubes indicated positive sugar fermentation (Obire, 2005).

3.4.6 Determination of alcohol tolerance

The alcohol tolerance of the isolates was determined by culturing a loopfull of the isolates in *Zymomonas* selective media (ZSM) containing 2.5%, 5%, 7.5%, 10%, 12% and 15% ethanol. The alcohol was aseptically added to the sterile media. The cultures were incubated at 30ºC for 24 h. The absorbance of the culture were recorded at 600nm using a Busch and Lomb Spectrophotometer. Un-inoculated broths were used as the control (Obire, 2005).

3.4.7 PCR Amplification of 16srRNA Gene from *Zymomonas mobilis*

3.4.7.1 DNA extraction

This was carried outby using DNA extraction kit from Thermo Scientific Products (batch number). A single colony of *Z. mobilis* was inoculated into 10ml ZSM (*Zymomonas* selective media) broth and incubated overnight at 30ºC.The bacterial cells were harvested by centrifugation for 10 minutes at 500 × g and the supernatant was discarded. The pellet was resuspended in 180 µL of digestion solution with addition of 20µL of proteinase K solution and mixed thoroughly by vortexing to obtain a uniform solution. The sample was incubated at 56ºC by shaking in a water bath until the cells were completely lysed for 30min. An aliquot (20uL) RNase A solution was added and mixed by vortexing and incubated for 10 min at room temperature. An aliquot (200ml) of lysis solution was added

and mixed by votexing for about 15 sec until a homogeneous mixture was obtained. To the mixture 400 µL of 50% ethanol was added and mixed by vortexing. The DNA was purified using GeneJET Genomic DNA Purification column with the aid of wash and elution buffers. The purified DNAwasstored at -20ºC until required (Mojtaba *et al*., 2017).

3.4.7.2 Polymerase Chain Reaction

The target primers for detecting alcohol dehydrogenase I gene were*Zad HIF* and *Zad HIR* while the target primers for detecting 16SrRNA were Z16P3-F and Z16P5-R. The PCR reaction mixture of 50µl contained 2.5 mM dNTPs, 5 µg genomic DNA template and 20 µmol of each primer. The PCR mixture was subjected to the following thermal conditions using DNA thermal cycler: The Products were isolated after 40 cycles of amplification with initial denaturation at 94ºC for 30 sec, 30 seconds at 58ºC and 2 minute at 72ºC, final extension for 3 min at 72ºC. Purification to obtain PCR products was done with the QIA quick PCR purification kit (Qiagen)(Sambrook *et al*., 1989)

3.4.7.3 Agarose Gel Electrophoreses

Amplification products were separated on 1% agarose gel which was stained with ethidium bromide.One percent agarose gel was prepared bymixing 1.5g agarose with 150ml 1 X TBE buffer. The mixture was mixed in a microwave until all agarose had dissolved. This was allowed to cool down under cold running tap water. An aliquot (6µl) of Ethidium Bromide was added and swirled to mix. The gel was poured into the tray and allowed to stand fro 30 min before removing the combs. The gel was placed in an electrophoresis tank. Five microlitres of loading die was spotted for 10µl of each DNA sample on a parafilm

paper.Using a pipptte, 15µl of each sample was loaded into each well of the prepared 1 % agarose gel; also 0.5 microgram of 250 bp ladder(Lambda DNA HindIII) was loaded into the marker well. It was run for 45 min at 110 milli Amps. The PCR products were observed under an ultraviolet (UV) light transilluminator (Sambrook *et al*., 1989).

Table 3.1: **Oligonucleotide primer sequences for amplification of Alcohol dehydrogenase I (AdhI) and 16SrRNA genes of *Zymomonas mobilis***

Target gene	Primer sequence	DNA fragment size (bp)	Gene amplified	Reference
ZadhIF	5'CCCTCGAGGTAATCGGCTGGCAAT CGTTTTCC 3'	1200	Alcoholdehydrogenase I gene	Zhang*et al*.,2014
ZadhIR	5'GTTCTAGAGATAGCGGCTTATAGCAACGAGTG 3'			
Z16P3-F	5'-CAAGCCTGCAAAGGTTAG-3'	900	16SrRNA	Coton *et al*.,2005
Z16P5-R	5'-ATGCTCTTACCTCACGCT-3'		16SrRNA	

3.5 Isolation and Identificationof *Bacillus*species from Soil Samples

3.5.1 Isolation of *Bacillus* species from soil samples

Ten (10) g of soil sample was dispensed in 90ml of sterile distilled water and heated to 80ºC. A serial dilution of 10^{-1}-10^{-6} of the soil sample was carried out after which 0.1ml of the 10^{-4}-10^{-6}dilutions were pipetted onto plates of nutrient agar. The inoculated agar plates were incubated at 37ºC for 24h (Amika *et al*., 2018). Colonies were picked and sub-cultured on plates of nutrient agar. The isolates were further transferred to nutrient agar slants, incubated for 24h and stored in the refrigerator.

3.5.2 Identification and Characterization of Presumptive *Bacillus* species

Bacillus species were identified based on the cultural, microscopic and biochemical characteristics and confirmed using the *Bacillus* microgen identification kit (Microgen Ltd, UK) (Sneath, 1984).

3.5.2.1 Cultural Characterization

The colonial morphologies were noted with respect to colour, shape, size and nature of the colonies by visual examination.

3.5.2.2 Microscopic examination

Gram staining

The bacterial isolates were Gram stained and observed under 100X oil immersion objective using the light microscope (as described in 3.4.1).

Endospore staining

Endospore staining was performed to observe the morphology and position of the spores as described by Sneath (1984). A loopful of the suspension of each of the isolates was smeared on a clean slide. The smeared slide was heat fixed by placing it over a flame of a Bunsen burner. An adsorbent paper was placed on the smeared heat fixed slide and flooded with malachite green stain. It was flamed until steam was observed from the slide.The slide was washed with water and counter stained with safranin; air dried and viewed 100X objective of the light microscope. Cells with endospore will appear pink-red with the greenish coloured endospore resting centrally,subterminally or terminally.

Motility test

A drop of the bacterial culture from a 24h broth culture was placed in the middle of a cover slip. A thin line of petroleum jelly was placed at the edges of the cover slip so that the depression was over the drop of culture. The slide was inverted so that the drop hung free from the cover slip into the space of the depression. The slides were observed using 100X objective of the light microscope (Cheesebrough, 2010). The presence of motile rod shaped cells is indicative of a positive motility test.

3.5.2.3 Biochemical tests

Catalase

Fresh colonies of the isolates were immersed in test tubes containing 3ml of hydrogen peroxide solution using a wooding applicator and observed for gas bubbling which indicated a positive result (Brenner *et al*., 2004).

Voges-Proskauer

Fresh colonies of the isolates were inoculated into methyl red Voges-Proskauer broth (MR-VP) and incubated for 24hr.An aliquot(0.6ml) of alpha naphthol and 0.2ml of 40% potassium hydroxide was added to 2.5ml of the culture broth and shaken well. A positive reaction was indicated by the formation of a pink–red product within 5min (Brenner *et al*., 2004).

Nitrate reduction

The culture of the isolate was grown overnight in nitrate broth. Ten (10) drops of N, N-dimethyl-naphthalene and sufanilic acid in 30% acetic acid was added to the overnight culture. The development of red colour within 2min was an indication of a positive result (Cheesebrough, 2010).

3.5.2.4 Confirmation of presumptive Bacillus species using the Microgen Bacillus identification kit

A 24hr suspension of all the presumptive *Bacillus* isolates were prepared in the suspending medium that was provided. It was carried out by picking fresh single colonies of the isolates with a sterile inoculating loop into the suspending medium. The adhesive sealing tape from each strip of the micro wells were peeled and the wells were inoculated with 3 drops of inoculum. The Arginine well was overlaid with Mineral oil and the entire wells sealed and incubated at 30ºC for 24 h. The results (coloured reactions) of the sugar fermentations including Citrate and Urease tests were read and recorded and were further Incubated at 30ºC for 24 h. The results were read and recorded. The Octal Codes for all the

isolates were calculated using the results of the coloured reactions. All tests which showed a positive reaction after either 24 h incubation or 48 h incubation were recorded as positive. Only reactions negative at 24-48h incubation were recorded as negative. Results were recorded on the report form provided and all the accumulated positive test results from both the 24 and 48 h readings were summed up. Results were analyzed using the Microgen Identification System Software.

3.6 Molecular characterization of *Bacillus* isolates

Molecular characterization of the *Bacillus* isolates was carried out by sequencing of the 16SrRNA gene.

3.6.1 DNA Extraction

This was done by usimg Thermo Scientific GeneJet Genomic DNA extraction Kit (K0721, MAN0012663). Single colonies of the isolates were seperately inoculated into 10ml nutrient broth and incubated for 24h at 37ºC.The bacterial cells were harvested by centrifugation for 10 min at 500 × g and the supernatant was discarded. The pellet was resuspended in 180 µL of digestion solution with addition of 20µL of proteinase K solution and mixed thoroughly by votexing to obtain a uniform solution. The sample was incubated at 56ºC for 30min by shaking in a water bath until the cells were completely lysed. An aliquot(20uL) of RNase A solution was added and mixed by votexing and incubated for 10 min at room temperature. An aliquot(200ml) of lysis solution was added and mixed by votexing for about 15 sec until a homogeneous mixture was obtained. To the mixture 400 µL of 50% ethanol was added and mixed by vortexing. The DNA was purified using

GeneJET Genomic DNA Purification column with the aid of wash and elution buffers. The purified DNA was stored at -20ºC until required (Russel, 2010).

3.6.2 PCR Amplification of the 16SrRNA gene of the *Bacillus* isolates

The target primers were for 16SrRNA. Bacterial universal primers 27F 5'(AGA GTT TGA TCM TGG CTC AG)3' and 1492R 5' (TAC GGT TAC CTT GTT ACG ACT T) 3' of about 1500bp size were used (Mojtaba and Hoda 2017). The PCR reaction mixture of 50µl contained 2.5 mM dNTPs, 5 µg genomic DNA template and 20 µmol of each primer. The PCR mixture was subjected to the following thermal conditions using DNA thermal cycler: The Products were isolated after 40 cycles of amplification with initial denaturation at 94ºC for 30 sec, 30 sec at 58ºC and 2 min at 72ºC, final extension for 3 min at 72ºC. Purification to obtain PCR products was done with the QIA quick PCR purification kit (Qiagen)(Sambrook *et al*., 1989).

3.6.3Agarose Gel Electrophoreses

Amplified products were separated on 1% agarose gel which was stained with ethidium bromide.One percent agarose gel was prepared bymixing 1.5g agarose with 150ml 1 X TBE buffer. The mixture was mixed in a microwave until all agarose had dissolved. This was allowed to cool down under cold running tap water. An aliquot (6µl) of Ethidium Bromide was added and swirled to mix. The gel was poured into the tray and allowed to stand fro 30 min before removing the combs. The gel was placed in an electrophoresis tank. Five microlitres of loading die was spotted for 10µl of each DNA sample on a parafilm paper.Using a pipptte, 15µl of each sample was loaded into each well of the prepared 1 %

agarose gel; also 0.5 microgram of 500 bp ladder (Lambda DNA HindIII) was loaded into the marker well. It was run for 45 min at 110 milli Amps. The PCR products were observed under an ultraviolet (UV) light transilluminator (Sambrook *et al*., 1989).

3.6.4 Sequencing of PCR product

The PCR products were sequenced using the BigDye terminator on an ABI3730 sequencer by Macrogen biotech laboratory (Europe).The sequencing was carried out by electrophoresis on a DNA analyzer 3730xl-titania (Applied Biosystems) using the same primers as those used for the amplification by PCR for the gene coding for the 16srRNA. The 16SrRNA sequences were analyzed against those available from the National centre for biotechnology information (NCBI) database using the BLAST search system to identify the most similar sequences (Fally *et al*., 2020)

3.6.5 Phylogenetic analysis of the 16SrRNA gene sequences

The 16SrRNA sequences were analyzed for anomalies using Chromas lite (2.01) software package, then assembled using the CAP contig program in bioedit (7.1.3.0). FASTA and BLAST subroutine were used to determine the closest relatives in the Genbank database (Altschul *et al*., 1997). The sequences were compared with other closely related bacterial sequences from the Genbank using the FASTA Algorithm. Sequences were aligned and analysed with CLUSTALX program (Thompson *et al*., 1997). The phylogenetic package MEGA6 was used to carry out the nucleotide substitution model of Jukes and Cantor in order to obtain distance matrices (Tamura *et al*., 2013). Six Phylogenetic trees wereconstructed using the neighbour-joining method (Saitu and Nei, 1987) for the different

Bacillus species sequenced; the trees were all rooted to an *E. coli* strain from Nigeria. A tree was constructed for all the ten Bacillus species, the tree was also rooted to an *E.coli* strain from Nigeria.

3.6.6 16SrRNA gene sequence accession numbers

The 16SrRNA gene sequences of *Bacillus* species isolates were deposited in the Genbank with their accession numbers.

3.7 Screening of *Bacillus* isolates for Cellulase production

*Bacillus*isolates were screened on selective carboxymethyl cellulose agar containing (g/l): $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$.$7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose (1%), sodium salt 10.0, peptone 0.2, agar 17.0. Plates were inoculated with suspension of pure *Bacillussp* culture and incubated at 30ºC. After 24h, plates were flooded with 1% Congo red solution for 15 min then de-stained with 1M NaCl solution for 15 min. The zones of decolorization around each colony were noted (Kanmani *et al*., 2011).

3.7.1 Crude Cellulase Production

The cellulose production media consisted of four 10g carboxymethyl cellulose, 50 ml of Mandel and Sternberg's mineral media (as described by Singh *et al*., 2009) containing: 1.4g/l $(NH_4)_2$ SO_4, 2.0 g/l KH_2PO_4, 0.3 g/l $CaCl_2$, 0.3 mg/l $MgSO_4$. $7H_2O$, 5.0 mg/l $FeSO_4$.7 H_2O, 1.6 mg/l $MnSO_4$. H_2O, 1.4 mg/l $ZnSO_4$. $7H_2O$, 2.0 mg/l $CoCl_2$. The initial pH of the medium was adjusted to 5 before 100ml were dispensed into different 250ml conical flasks and autoclaved at 121ºC for 15min. After cooling the flasks were inoculated

with 5ml of each bacterial culture that is equivalent to 1.0 McFarland ($3.0x10^8$ CFU/ml) and incubated at 30^oC on an orbital shaker at 120 rpm for 7 days. Samples were withdrawn after 1,2,3,4,5,6,7 days for cellulase activity using the endoglucanase activity method.

3.7.2 Enzyme Assays

The endoglucanase (CMCase) activity assay was used to determine the cellulase activity. Half milliliter of 1 % carboxymethyl cellulose was dissolved in 0.05M Na–citrate buffer, pH 4.8 and temperated for 10 min at 50ºC. After that, 0.5 ml of an appropriately diluted enzyme was added to the tube and incubated at 50ºC for 30 min. appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath;reaction was stopped by addition of 3 ml of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for colour development and cooled. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a UV-VIS spectrophotometer (Spectrumlab721S, 90-250V, 40W, No. 721S20072). The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity is defined as the amount of enzyme required for liberating 1mg of glucose per milliliter per minute and was expressed as U/ml (Zakpaa *et al*., 2009)

3.8 Pretreatment and Hydrolysis of Corn Cob Biomass

3.8.1 Dilute Acid Pretreatment of Pulverized corn cob Biomass

This was carried out according to the method described by Gupta *et al.,* 2009. One hundred grams (100g) of driedpowdered corn cob was weighed into eight different 500ml conical

flasks. Five hundred millilitres (500ml) each of 5% dilute H_2SO_4 was added intothe eight conical flasks containing the dried samples. The Conical flasks wascovered with cotton wool, wrapped in aluminium foil, then heated at 50ºC for30min.The conical flasks were allowed to cool and then filtered through no.1 whatman filter paper, the pH was adjusted to 4.5 using 40M NaOH to neutralize acidity. The pretreated substrate was then washed with distilled water to remove residual alkali and dried at 50ºC for 24hrs.

3.8.2 Dilute Acid Hydrolysis

Dilute acid hydrolysis was carried out according to the method described by Ademiluyi and Mepba, 2012. One hundred grams (100g) of pretreated corn cob was weighed into eight different conical flasks. Five hundred mililitres (500mls) of 0.2 molar dilute sulphuric acid was dispensed into each of the conical flasks. The samples were allowed to stand for 90min and heated to 70ºC with stirring. The hydrolysates were allowed to cool to 30ºC and neutralized to a pH 0f 6 with 0.5 Molar sodium hydroxide.

3.9 Standardization of Inocula

The concentration of the bacterial suspension was standardized using 1.0 McFarland nephelometry by adding 0.1ml of 1% barium chloride to 9.9ml of H_2SO_4 in a test tube. The tube was stopped tightly and sealed. Into a tube of the same diameter as the standard tube containing 10ml of 0.85% normal saline, a measured aliquot of the bacterial suspension was added until the turbidity produced matched the turbidity of the standard tube corresponding to the concentration of $3.0x10^8$cfu/ml (Campbell *et al*., 1964)

3.10 Screening of *Z. mobilis* Isolates for Glucose Fermentationand*Bacillus* isolates for xylose fermentation ability

To evaluate glucose fermentation by different isolates of Z. *mobilis*, 100 ml of rich medium (RM) containing 20 g/L glucose, 10 g/L yeast extract, 2 g/L monobasic Potassium phosphate were used. The medium was inoculatedwith 5% (v/v) *Z. mobilis* culture; the cultures were incubated at 30^{o}C without agitation for 24hours. Similarly,to evaluate xylose fermentation by different*Bacillus* isolates, 100 ml of seed medium (SM) containing 20 g/L xylose, 10 g/L yeast extract, 2 g/L monobasic Potassium phosphate were used. The medium was inoculatedwith 5% (v/v) of overnight *Bacillus* isolatesculture; the cultures were incubated at 37ºC with agitation for 24h. The ethanol yields were used to assess the fermentation performance. The ethanolyields from glucose fermentation by *Z. mobilis* isolates and *Bacillus* isolates were determined by potassium dichromate method using UV visible spectrophotometer while the residual sugars were analyzed by the DNS method of Zambere, 2011. The isolates with the highest ethanol yields were used for further studies (Zhang *et al*., 2014).

The glucose and xylose fermentation efficiencies were represented by sugar consumption percentages and sugar to ethanol conversion yields. The isolates with the highest glucose and xylose fermentation efficiencies and ethanol conversion yields were used for further studies. The sugar consumption percentage in the glucose and xylose fermentation experiments were calculated as:

$SP = (S1 - S2)/S1$

Where; SP is sugar consumption %, S_1 is sugar concentration (g/l) before fermentation, S_2 is sugar concentration (g/l) after fermentation.

The sugar to ethanol conversion yield in the glucose and xylose fermentation experiments was calculated as:

$E_r = C_e / (S_1 - S_2)$

Where; $S1$ is sugar concentration (g/l) before fermentation, $S2$ is sugar concentration (g/l) after fermentation, and Ce is ethanol concentration (g/l) after fermentation (Zhang *et al*., 2014).

3.11 Acetic Acid Adaptation

Different media were used for different experiments. Seed medium was used for adaptation of the isolates and solid medium was used for single colony screening.

3.11.1 Seed Medium for acetic acid adaptation

Seed medium contained 20 g/L glucose, 10 g/L yeast extract, 2 g/L monobasic Potassium phosphate. This medium is also known as rich medium (RM). Acetic acid concentrations of 0.2% to 1.6% w/v were added to different conical flasks containing 100ml of the seed medium. The medium was sterilized by autoclaving at 121ºC for 15min.The flask containing 0.2% acetic acid was inoculated with 5ml of a 24h culture of 1 Mac Farland *Z. mobilis*. It was incubated at 30ºC and monitored for growth (optical density) at 600nm using a spectrophotometer for 72 h.After 72h, 5ml of cells from the 0.2% conical flask was transferred to the conical flask containing 0.4% acetic acid and incubated for 72h.Cell growth was monitored and the transfers were subsequently done from the 0.4% flask to the 1.6% acetate conical flask

3.11.2 Solid medium for single colony screening

The solid medium was a mixture of 100ml seed medium containing 1.5% agar and 100 ml seed medium with varied amounts of acetic acid (1.2-1.6%). The media were inoculated with a loop ful 1.6% acetate treated isolates using the spread plat technique and incubated at 30ºC for 48h in an anaerobic jar containing gas packs. The acetate adapted isolates were used for further studies (Zhang *et al*., 2014).

3.12Preparation of Bacterial Inoculum

Zymomonas mobilis and *Bacillus licheniformis* inocula were prepared by introducing cultures from slant cultures into 150ml of sterile liquid standard medium for *Z. mobilis* and nutrient agar for *B. licheniformis* in a 500ml Erlenmeyer flask. The flasks were incubated at 30ºC for 72hrs. (Ado *et al*., 2009)

3.13Fermentation of corn cob hydrolysate with co-culture of *Zymomonas mobilis*and xylose fermenting *Bacilluslicheniformis*

Fermentation of corn cob hydrolysate by co-culture of acetate adapted *Zymomonas mobilis*(ZMA) and xylose fermenting *Bacillus* isolate (SCFB22) was carried out in submerged fermentation. Fermentation studies consisted of eight set ups containing 100g hydrolyzed substrates per litre of distilled water and 5% inoculum concentrations of the two strains;*Zymomonas mobilis* without acetate adaptation (ZM3) alone,*Zymomonas mobilis* with acetate adaptation(ZMA) alone, *Bacillus* isolateSCFB22alone, co-culture of acetate adapted*Zymomonas mobilis*(ZMA) with *Bacillus* isolate(SCFB22), co-culture of *Zymomonas mobilis* without acetate adaptation (ZM3) with *Bacillus*

isolate(SCFB22),*Zymomonas mobilis* standard strain alone, *Bacillus* standard strain alone and a set up containing substrate without inoculum served as control.Samples were withdrawn daily for nine (9) days for determination of residual sugars, total bacterial count and ethanol yield. The samples were distilled using simple distillation at 78ºC. Experiments were carried out in triplicate for all the runs. Residual sugars were determined using the DNS method while the ethanol content was assayed by dichromatemethod of Oyeleke and Jibrin (2009).

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

$$Efficiency\ (\%)\ =\ Ethanol\ produced\ (g/l)\ X\ 100/TRSi\ -\ TRSf$$

Where, TRSi is the initial sugar content (before fermentation) and TRSf is the final sugar content after fermentation (Alvarenga *et al*., 2011).

3.14 Determination of Total bacterial counts during fermentation

Total bacteria counts were carried out to monitor the growth of the bacteria during fermentation. This was carried out by transferring 1ml of the fermentation broth to 9ml sterile distilled water, after which it was serially diluted from 10^{-1}-10^{-8} dilutions. Zero point one (0.1) ml of the diluents were transferred to the surface of solid standard media and nutrient agar for *Zymomonas mobilis* and *Bacillus licheniformis* respectively. A sterile bent glass rod was used to evenly spread the inocula. The inoculated plates were then incubated

for 24h at 30ºC and 37ºC for *Zymomonas mobilis* and *Bacillus licheniformis* respectively. Visible colonies were counted and the total bacterial counts were determined by multiplying the number of colonies counted by the inverse of the dilution factor and expressed as CFU/ml (Ado *et al*., 2009).

3.15Distillation

This was carried out using distillationapparatus (set up). The fermented liquid was transferred into a round bottom flask and placed on aheating mantle fixed to a distillation column enclosedin running tap water. Another flask was fixed to theother end of distillation column to collect thedistillate at 78ºC (standard temperature for ethanol production). This was done for each of the fermentedbroth (Ado *et al*., 2009).

3.16UV-Visible Spectroscopy

Determination of concentration of bioethanol produced was carried outusing the method described by Oyeleke and Jibrin (2009). 1ml of standard ethanol was diluted with 100 ml of distilled water to give a concentration of 1%. From the stock solution 0, 0.2, 0.4 and 0.6% of the ethanol was prepared by diluting withdistilled water. To 5ml of each of the varying ethanol concentrations 2mls of chromium reagent was added and allowed to stand for an hour for colour development.One millilitre of sodium potassium tartrate (40%) (Rochelle salt) was added for stabilization of the produced color. Theabsorbance of each concentration was measured at 579.7 nm using UV-VISspectrophotometer and the readings used to develope standard ethanol curve.Then 5mls of each bio-ethanol samples were put in

test tubes and treated with 2mlsof the chromium reagent. The mixtures were allowed to stand for an hour and the absorbances were measured as for standard curve.

3.17 Preparation of Jone's (chromium) reagent.

Five grams (5g) of $K_2Cr_2O_7$were weighed and dissolved in 50% H_2SO_4 (Luzzio, 1998)

3.18 Determination of Reducing Sugar Yield

The reducing suger content of the fermentation medium was determined before and after fermentation using the DNS method of Zambere, 2011.

3.19 Determination of Bioethanol Quality through Analysis of the Physicochemical Parameters of the samples

The methods of Ado *et al*.,2009 and Ademuliyi and Mepba.,2013 were used to determine the pH, density, boiling points and flash point of the bioethanol produced as described below:

3.19.1 pH Test

The pH meter was standardizedby inserting into a buffered solution pH 7.0 and then placed in the sample (bioethanol) and the readings were obtained(Ado *et al*.,2009).

3.19.2 Density and specific gravity

An empty pycnometer was weighed, the pycnometer was filled with the sample (bioethanol), the excess was wiped off and the weight was recorded and the density was calculated using the formular:

Density (g/ml) = Mass/volume

Distilled water was then filled into the pycnometer, weighed and recorded. The specific gravity was calculated using the formular below:

Specific gravity (spg) = Density of ethanol / Density of water (Ademuliyi and Mepba.,2013)

3.19.3 Boilng point

A known volume of bioethanol was turned into a distillation flask, fixed at its position, a thermometer was inserted at the top and the heat was turned on. A receiver cylinder was fixed at the distillate recovery point. The temperature reading was taken at the first drop of the distillate and recorded as the boiling point (Ademuliyi and Mepba.,2013)

3.19.4 Flash point Determination

This was carried out using Pensky Martins flash point apparatus. The cup in the apparatus was dried; 50ml of each sample (bioethanol) was transferred into the flash point cup. The cup was fixed into the position of the apparatus assembled with the thermometer and the apparatus was switched on; the heat was controlled by a steady stirrer to maintain the temperature while passing a small flame across the material every five seconds. The temperature at which the vapour first flashes with a blue flame was recorded as the flash point. After each test, the cup was dried before subsequent test. This was carried out twice for each sample (Ademuliyi and Mepba.,2013).

3.20 Statistical analysis

Results were presented as mean ± standard error of mean (SEM).Two sample T-test and One way ANOVA were used to test the differences between the means of variables using Statistical Package for the Social Sciences (SPSS) version 20 software at 0.05 level of significance ($P \leq 0.05$) and 95% confidence level.

CHAPTER FOUR

4.0 RESULTS

Proximate analysis of corn cob was carried out to know the nutritional composition of the substrate before fermentation and to ascertain if the substrate was a suitable substrate for bioethanol production. The result (Table 4.1) showed the mean proximate composition of corn cob. The moisture content was 13.50%,ash content was 14.90%, crude protein was 3.50%,lipid was 1.00% and carbohydrate content was 67.1%.

The results of the cultural and biochemical properties of the presumptive *Zymomonas mobilis* isolates that were obtained from palm sap revealed that all the ten (10) isolates were rod shaped, Gram negative, had the ability to ferment glucose, fructose and sucrose. Only isolates ZM2, ZM3, ZM4 and ZM 5 could not ferment maltose, lactose and xylose. All the isolates were urease negative, indole negative and oxidase negative. Isolates ZM2, ZM3, ZM4 and ZM5 were further characterized using molecular techniques (PCR Amplification of the 16SrRNA gene and Alcohol dehydrogenase (ADH I)gene of *Zymomonas mobilis*)

The results of the gel electrophoreses (Plate IA ana Plate IB) for the PCR Amplification of the Alcohol dehydrogenasegene (1200bp DNA fragment) and 16SrRNA gene (900bp DNA fragment) of *Zymomonas mobilis* ATCC 29191, bacterialisolates ZM2, ZM3, ZM4 and ZM5 showed that *Zymomonas mobilis* ATCC 29191(positive control), isolates ZM2 and ZM3 had bands at 1200bp position of the ladder, there were no bands for ZM4, ZM5 and the negative control. Similarly, *Zymomonas mobilis* ATCC 29191,isolates ZM2 and ZM3 had bands at 900bp position of the ladder but there were no bands for ZM4,ZM5 and the negative control.

Table 4.1: Mean proximate composition of corn cob used in the study

Parameter	Percentage composition (% ± SEM)
Moisture	13.50 ± 0.01
Ash	14.9 ± 0.03
Crude protein	3.50 ± 0.02
Lipids	1.0 ± 0.01
Carbohydrate	67.1± 0.01



ML IML II

Plate I: Amplification of Alcohol dehydrogenase gene I (ADH I, 1200bp) of *Zymomonas mobilis*

Key: Lane 1(ML): Molecular ladder (250bp) for ADH 1 gene, lane 2: Positive control*Z. mobilis* (ATCC 29191),

Lane 3:ZM2, Lane 4: ZM3, Lane 5:ZM4, Lane 6:ZM5, Lane 7: Negative control.

Plate II: Amplification of 16SrRNA gene (900bp) of *Zymomonas mobilis*

Key: Lane 12 (ML): Molecular ladder (250bp) for 16SrRNA gene,Lane 13: *Z. mobilis* (ATCC 29191), Lane 14: ZM2,Lane 15: ZM3, Lane 16: ZM ,Lane 17:ZM5 and Lane 18: Negative control.

A total of forty soil samples were collected from soil samples containing decaying cellulosic materials and from refuse dump sites. From the soil samples a total of thirty (30) Isolates were obtained from plates of nutrient agar and characterised culturally and physiologically. The isolates were also characterized using the *Bacillus* microgen identification kit.

The Cultural and physiological properties of the *Bacillus* species isolated from the soil (Table 4.2) showed that theisolates had colonies that were round in shape. The colonies were raised, large, flat, and rough and most of the isolates were dry but a few were moist. The isolates were also rod shaped, gram positive and spore forming indicating they belong to the genus *Bacillus*. Some of the isolates had the ability to both ferment xylose and hydrolyze cellulose, some could ferment xylose but couldn't hydrolyze cellulose while some could not ferment xylose but could hydrolyze cellulose. The isolates that could ferment xylose and hydrolyze cellulose were further characterized using the *Bacillus* Microgen identification kit.

The biochemical characterization and identification of *Bacillus* isolates using the Microgen Bacillus-ID System (Table 4.3) showed that all the isolates fermented most of the sugars differently but they were all positive to voges proskauer, arginine dihydrolase and nitrate reduction tests indicating that most of the isolate belong to the same genus but different species. The percentage identities of all the isolates ranged from 60.6-100%. A total of three (3) *Bacillus* species were identified, they include; *Bacillus cereus* (12), *Bacillus firmus* (3) and *Bacillus licheniformis* (5).

Of the twenty (20) *Bacillus* species identified using the *Bacillus* Microgen identification kit, ten (10) isolates (those that had the ability to ferment xylose and hydrolyze cellulose) were further characterized molecularly by sequencing of the 16SrRNA genes using bacterial universal primers

Table 4.2: Morphological Characteristics and Biochemical characterization of presumptive *Bacillus* spp.

ISOLATE	COLONY MORPHOLOGY	VEGETATIVE MORPHOLOGY	GRAM REACTION	STARCH HYDROLYSIS	SPORE	MOTILITY	CATALASE	XYLOSE FERMENTATION	CELLULOSE HYDROLYSIS	VOGES PROSKAUER TEST	NITRATE REDUCTION	IDENTITY
1	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
2	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
3	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
4	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
5	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
6	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
7	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
8	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
9	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
10	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
11	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
12	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
13	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
14	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
15	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
16	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
17	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
18	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
19	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp
20	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
21	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
22	Spherical,moist,creamy	Rod shape	+	+	+	+	+	+	+	+	+	*Bacillus* spp
23	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	+	-	+	+	*Bacillus* spp
24	Spherical,moist,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp
25	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp
26	Spherical,moist,creamy	Rod shape	+	+	+	+	+	-	+	+	+	*Bacillus* spp
27	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	-	+	+	+	*Bacillus* spp
28	Spherical,moist,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp
29	Spherical,Dry ,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp
30	Spherical,moist,creamy	Rod shape	+	+	+	+	+	-	-	+	+	*Bacillus* spp

Table 4.3: Biochemical characterization of *Bacillus spp* using Microgen Bacillus Identification Kit

Profile Number	Arabinose	Cellobinse	Inositol	Mannitol	Mannose	Raffinose	Rhamnose	Salicin	Sorbitol	Succrose	Trehalose	Xylose	Adonitol	Galactose	Mdm	Mdg	Inulin	Melezitose	Indole	ONGP	Nitrate	Arg	Citrate	Voges Preskauer	Octal Code	Identity	Percentage (%) Identity
1B	_	+	_	_	_	_	_	+	_	_	+	_	_	_	_	_	_	_	_	_	_	+	+	+	20220036	*B. cereus*	99.90
2B	_	+	_	_	+	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_	+	22200033	*B. cereus*	97.89
3B	_	+	_	_	_	_	+	+	_	_	+	+	+	_	_	_	_	+	_	_	+	+	_	+	20620113	*B. cereus*	99.69
4B	_	+	_	_	_	_	_	+	_	_	+	_	_	_	_	_	_	_	_	+	+	+	_	+	20220033	B. cereus	99.69
5B	_	+	_	_	+	+	_	+	_	+	+	+	+	_	_	_	_	_	_	_	+	+	+	+	23260017	*B. cereus*	97.29
7B	+	_	_	_	+	+	_	_	+	+	_	+	+	_	_	_	_	_	_	+	+	+	+	+	43140037	*B. firmus*	93.37
8B	_	_	_	_	_	_	_	_	_	_	+	+	+	_	_	_	_	_	_	_	+	+	_	+	00020016	*B. cereus*	89.45
9B	+	+	_	_	+	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	+	+	_	+	62000033	*B. firmus*	64.08
10B	_	_	_	_	_	_	_	_	_	+	+	+	_	_	_	_	_	_	_	+	+	+	_	+	00070033	*B. firmus*	94.22
12B	_	_	_	_	_	_	_	+	_	_	+	+	+	_	_	_	_	_	_	+	+	+	_	+	02220033	*B. cereus*	92.44
18B	_	+	_	_	+	_	_	_	_	+	+	+	+	_	_	_	_	_	_	_	+	+	_	+	22060013	*B. cereus*	94.11
21B	_	_	+	+	+	_	+	+	_	+	+	+	+	+	_	+	_	_	_	+	+	+	+	+	16672437	*B. licheniformis*	100
22B	_	_	+	+	+	+	+	+	+	+	+	+	+	+	_	+	_	_	_	+	+	+	+	+	17772437	*B. licheniformis*	100
23B	_	_	+	+	+	_	+	+	_	+	_	+	+	+	_	+	_	_	_	+	+	+	+	+	16652437	*B. licheniformis*	99.9
24B	+	_	_	_	+	+	_	+	+	_	_	_	_	_	_	_	_	_	_	+	+	+	_	+	43340037	*B. licheniformis*	82.2
26B	_	_	+	_	_	+	_	_	+	+	+	+	_	+	_	+	_	_	_	+	+	+	+	+	11172437	*B. licheniformis*	60.6
27B	_	+	_	_	+	_	_	_	_	_	+	+	_	_	_	_	_	_	_	+	+	+	+	+	22030037	*B. cereus*	74.89

Key: Mdm= Methyl-D-Mannoside, Mdg=Methyl-D-Glucoside, Arg= Arginine Dihydrolase,ONGP= O-nitrophenyl-β-D-galactopyranoside,SB=Soilbacillu

The gel electrophoreses picture of the PCR Amplification of the 16SrRNA gene (1500bp DNA fragment) of bacterial isolates 3B, 5B, 7B, 8B, 12B, 16B, 18B, 21B, 22B and 23B (Plate II) showed that all the isolates (3B, 5B, 7B, 8B, 12B, 16B, 18B,21B 22B and 23B) had bands at 1500bp position of the ladder. This suggests that they are all bacterial isolates.

The sequencing codes of *Bacillus* isolates, the percentage nblast identity, the identity and accession numbers of the strains identified by 16SrRNA (Table 4.4) showed that the nblast identity of all the sequenced isolates ranged from 97.77-99.93 %. All the ten (10) *Bacillus* isolates were identified to the species level by sequencing of 16SrRNA genes, their various accession numbers were obtained and submitted to the Genbank.

The Phylogenetic tree (Fig 4.1) showed evolutionary relationship of *Bacillus thuringiensis* obtained from soils in Jos, Nigeria (in red) with other strains available in the Gene Bank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al*., 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL).All positions containing gaps and missing data were eliminated. There were a total of 1408 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Felsenstein *et al*., 1969). The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that all three *Bacillus thuringiensis* isolates SXFB3, SXFB7 and SXFB8 clustered together with the strains gotten from the Genbank. This suggests that they share a similar evolutionary relationship that is different from the outdoor *E. coli* group as shown on the tree. The three *Bacillus thuringiensis* isolates were not closely clustered together which may be due to the fact that they may have evolved from different ancestors.

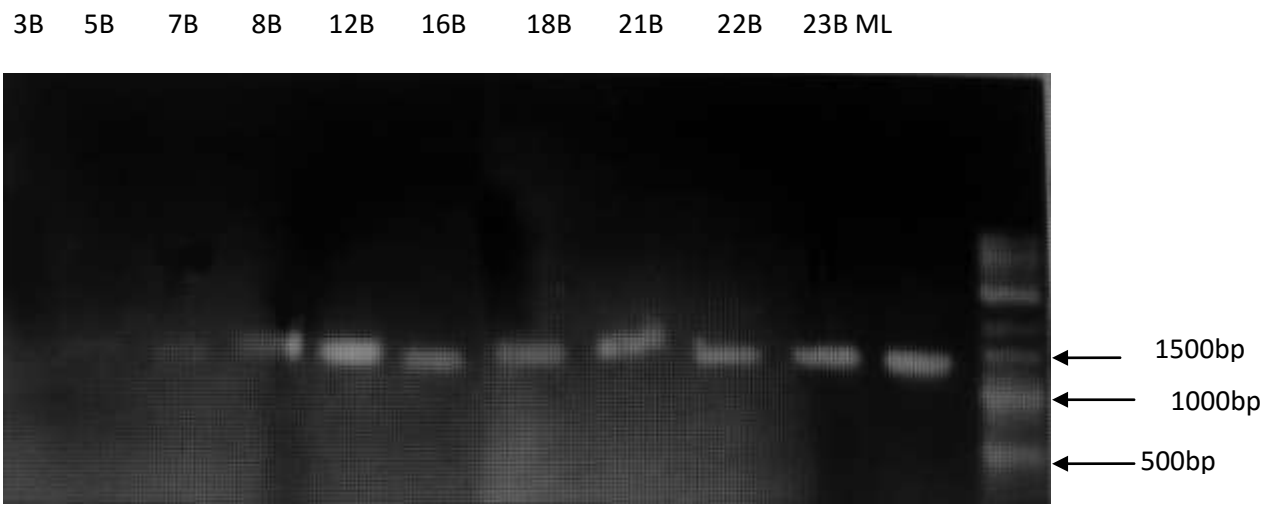


Plate III: Amplification of the 16SrRNA gene of *Bacillus* isolates 3B, 5B, 7B, 8B, 12B, 16B, 18B,21B 22B and 23B.

Key: Lane 1: 3B,Lane 2:5B,Lane 3:7B,Lane 4: 8B,Lane 5:12B,Lane 6: 16B,Lane 7:18B,Lane 8:21B,Lane 9: 22B,Lane 10:23B,Lane
11 (ML): Molecular marker ladder (500bp).

Table 4.4: Sequencing codes of *Bacillus* isolates, accession numbers,percentage nblast identity and identity and of the strains characterised by sequencing of 16SrRNA gene.

Isolate Code	Accession Number	nBLAST identity (%)	E-Value	Highest query coverage (%)	Isolate identity
SBX3	OM648225	99.79	0.0	99	*Bacillus thuringiensis*
SBX5	OM648226	99.52	0.0	99	*Bacillus cereus*
SBX7	OM648227	99.93	0.0	100	*Bacillus thuringiensis*
SBX8	OM648228	99.79	0.0	100	*Bacillus thuringiensis*
SBX12	OM648229	99.31	0.0	99	*Bacillus amyloliquefaciens*
SBX16	OM648230	99.30	0.0	98	*Bacillus amyloliquefaciens*
SBX18	OM648231	97.77	0.0	100	*Bacillus subtilis*
SCFB21	OM648232	99.65	0.0	100	*Bacillus licheniformis*
SCFB22	OM648233	99.79	0.0	100	*Bacillus licheniformis*
SCFB23	OM648234	99.72	0.0	100	*Bacillus velezensis*

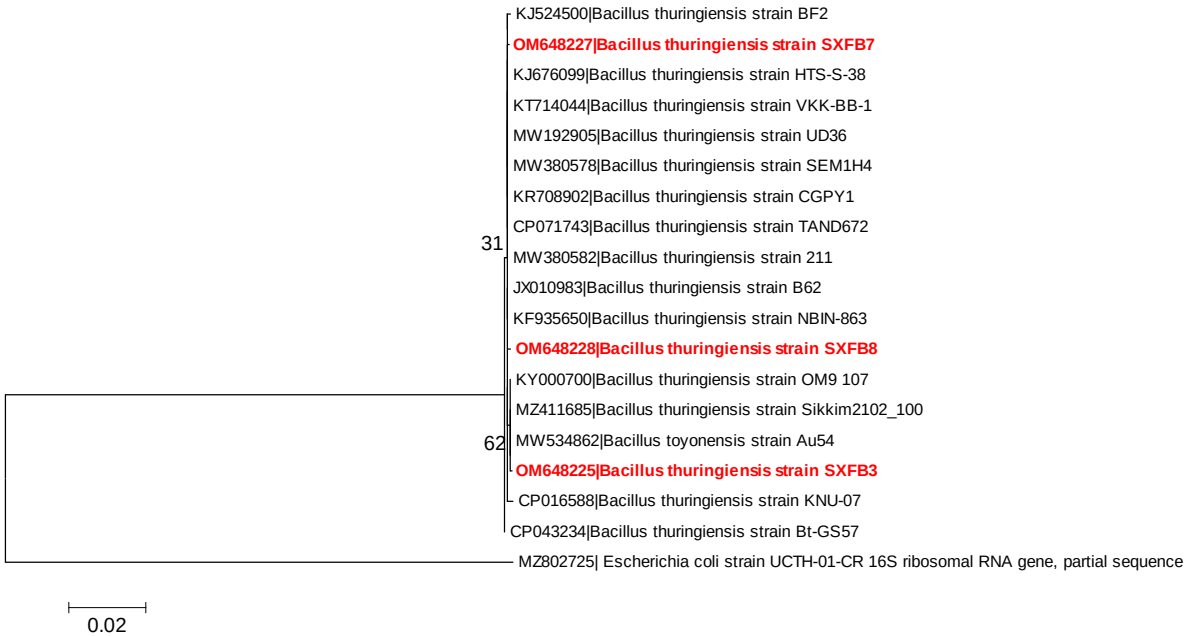


Fig 4.1: Phylogenetic tree evolutionary relationship of *Bacillus thuringiensis* obtained from soils in Jos, Nigeria with others stains available in the GenBank database.

The Phylogenetic tree (Fig 4.2) showed evolutionary relationship of *Bacillus cereus* obtained from soils in Jos, Nigeria (in red) with other strains available in the GenBank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al.*,1987). The tree with the highest log likelihood (-3075.1323) was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1413 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Felsenstein *et al.*, 1985). The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that the *Bacillus cereus* isolate SXFB5 clustered together with the strains gotten from the Gene bank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree.



Fig 4.2: Phylogenetic tree evolutionary relationship of *Bacillus cereus* obtained from soils in Jos, Nigeria with others strains available in the Gene Bank database.

The Phylogenetic tree (Fig 4.3) showed evolutionary relationship of *Bacillus amyloliquefaciens* obtained from soils in Jos, Nigeria (in red) with other strains available in the GenBank database.The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al*.,1987). The tree with the highest log likelihood (-3146.7257) was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1407 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 .The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that all two *Bacillus amyloliquifaciens* isolates SXFB12, SXB16 clustered together with the strains gotten from the Genbank. This means that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree. The two *Bacillus amyloliquifaciens* isolates were not closely clustered together which may be due to the fact that they may have evolved from different ancestors. Also, SXFB16 seems to be more recently evolved *Bacillus* specie than SXB12 which is indicated by the branching to the right as shown on the tree

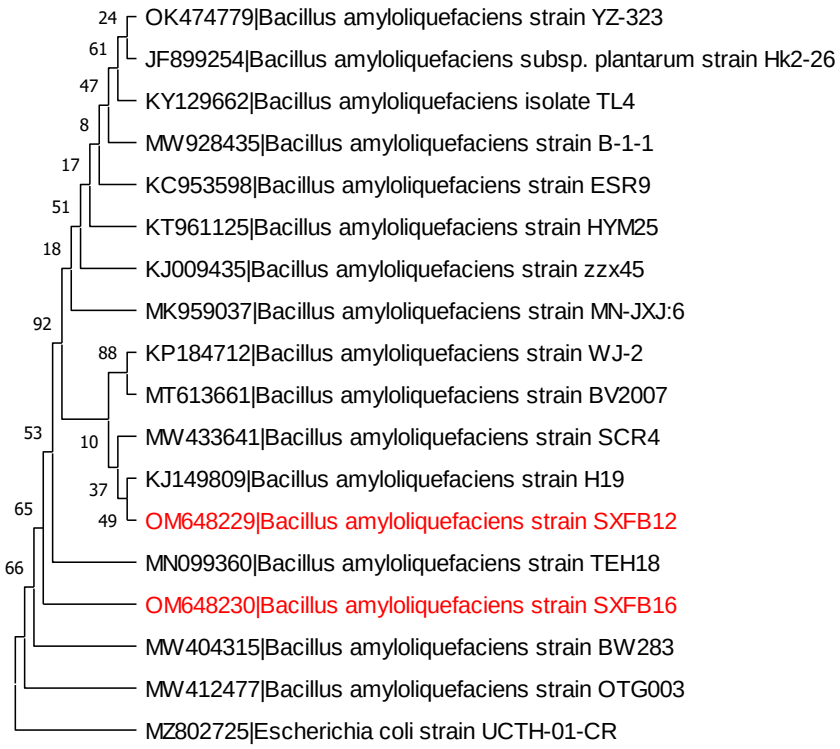


Fig 4.3:Phylogenetic tree evolutionary relationship of *Bacillus amyloliquefaciens* obtained from soils in Jos, Nigeria with others strains available in the Gene Bank database.

The Phylogenetic tree (Fig 4.4) showed evolutionary relationship of *Bacillus subtilis* obtained from soils in Jos, Nigeria (in red) with other strains available in the Gene Bank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al*.,1987). The tree with the highest log likelihood (-3249.9362) was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1413 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Felsenstein *et al*.,1985). The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that the *Bacillus subtilis* isolate SXB18 clustered together with the strains gotten from the Genbank. This means that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree. Also, SXFB16 seems to be more recently evolved *Bacillus* specie than the strains from the Genbank which was indicated by the branching to the right as shown on the tree.

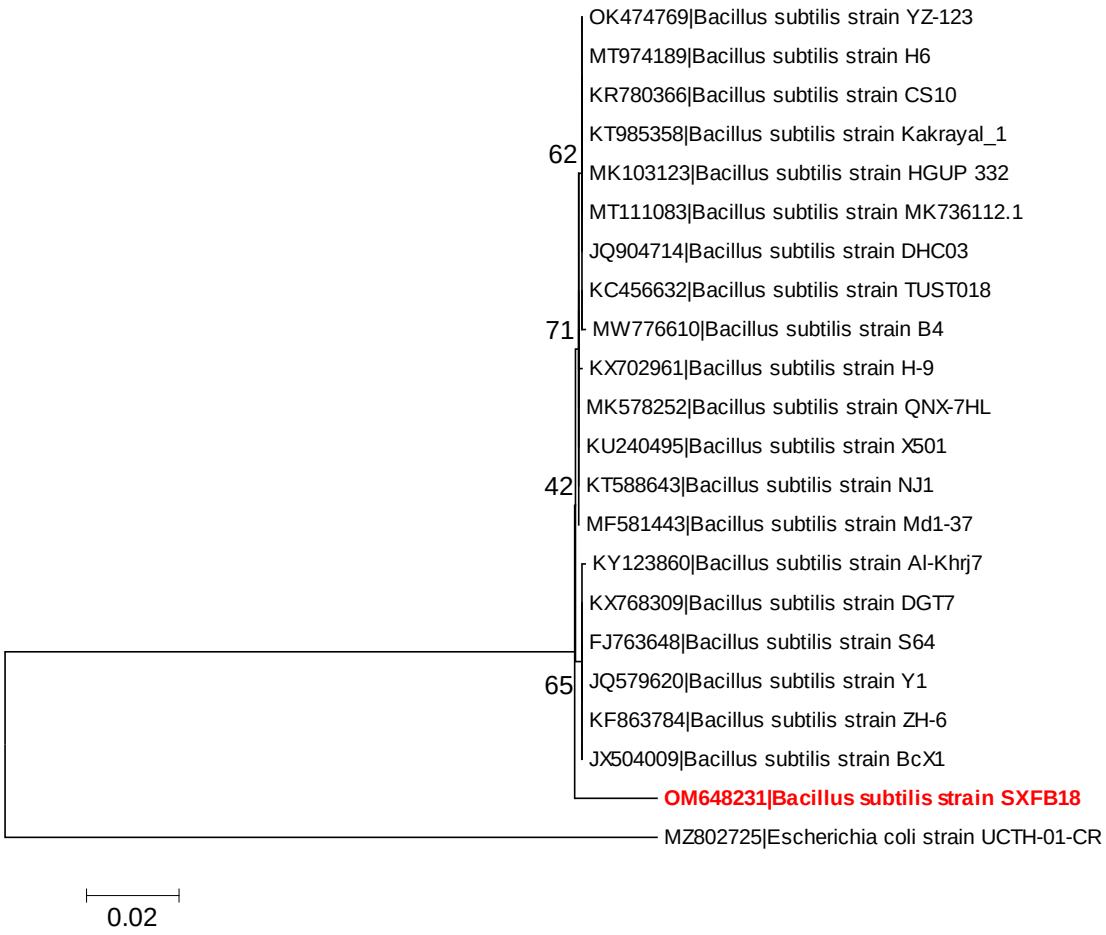


Fig 4.4: Phylogenetic tree evolutionary relationship of *Bacillus subtilis* obtained from soils in Jos, Nigeria (in red) with others strains available in the Gene Bank database.

The Phylogenetic tree (Fig 4.5) showed evolutionary relationship of *Bacillus licheniformis* obtained from soils in Jos, Nigeria (in red) with other strains available in the Gene Bank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al*., 1987). The tree with the highest log likelihood (-3110.0397) was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1396 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Felsenstein *et al*.,1985) .The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that all two *Bacillus licheniformis* isolates SCFB21, SCFB22 clustered together with the strains gotten from the Genbank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree. The two *Bacillus licheniformis* isolates were closely clustered together which may be due to the fact that they may have evolved from the same ancestor.

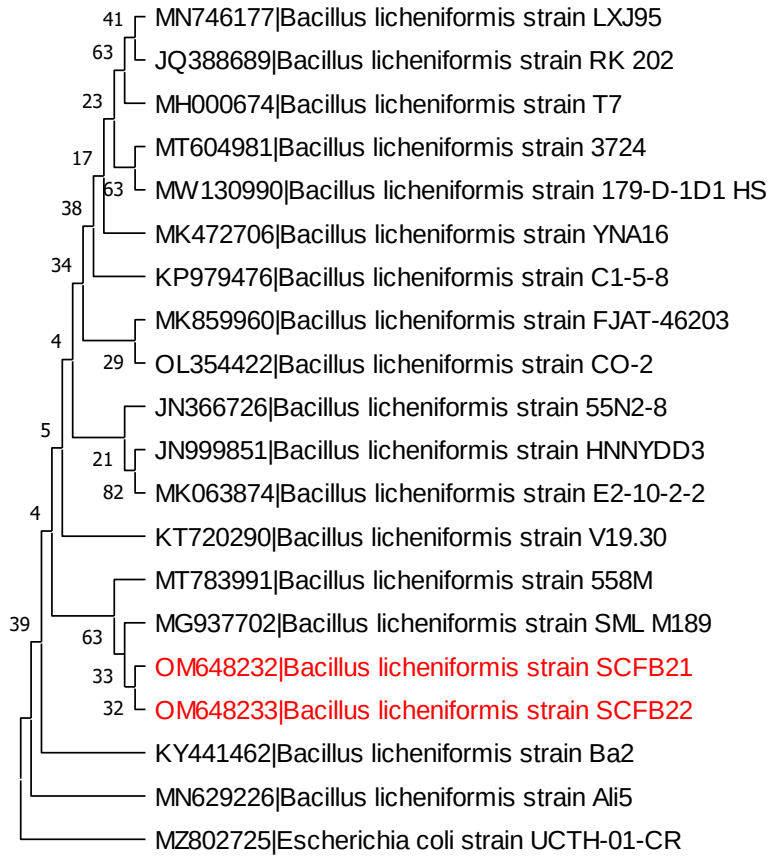


Fig 4.5: Phylogenetic tree evolutionary relationship of *Bacillus licheniformis* obtained from soils in Jos, Nigeria with others strains available in the Gene Bank database.

The Phylogenetic tree (Fig 4.6) showed evolutionary relationship of *Bacillus velezensis* obtained from soils in Jos, Nigeria (in red) with other strains available in the Gene Bank database. The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al*., 1987). The optimal tree with the sum of branch length 0.26183495 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein *et al*., 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor., 1969) and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1524 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2013).The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that the *Bacillus velezensis* isolate SCFB23 clustered together with the strains gotten from the Genbank. This means that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree.

The Phylogenetic tree (Fig 4.7) showed evolutionary relationship of all ten *Bacillus* species obtained from soils in Jos,the tree was rooted to an *E. coli* outdoor group as shown on the tree. The tree showed that all the ten *Bacillus* species clustered together as compared to the outdoor *E.coli* strain.

Sequencing of the gene encoding 16SrRNA has allowed the identification of ten (10) bacterial isolates (Fig 4.8).The figure described the proportion of bacteria isolates analyzed on the blast database. Of the bacteria sequenced 2 (20%) were *Bacillus amyloliquifaciens*, 2 (20%) were *Bacillus licheniformis*, 3 (30%) were *Bacillus thuringiensis*, 1 (10%) was *Bacillus cereus* and 1 (10%) was *Bacillus subtilis.Bacillus thuringiensis* was the predominant bacteria (30%) of the isolates sequenced.

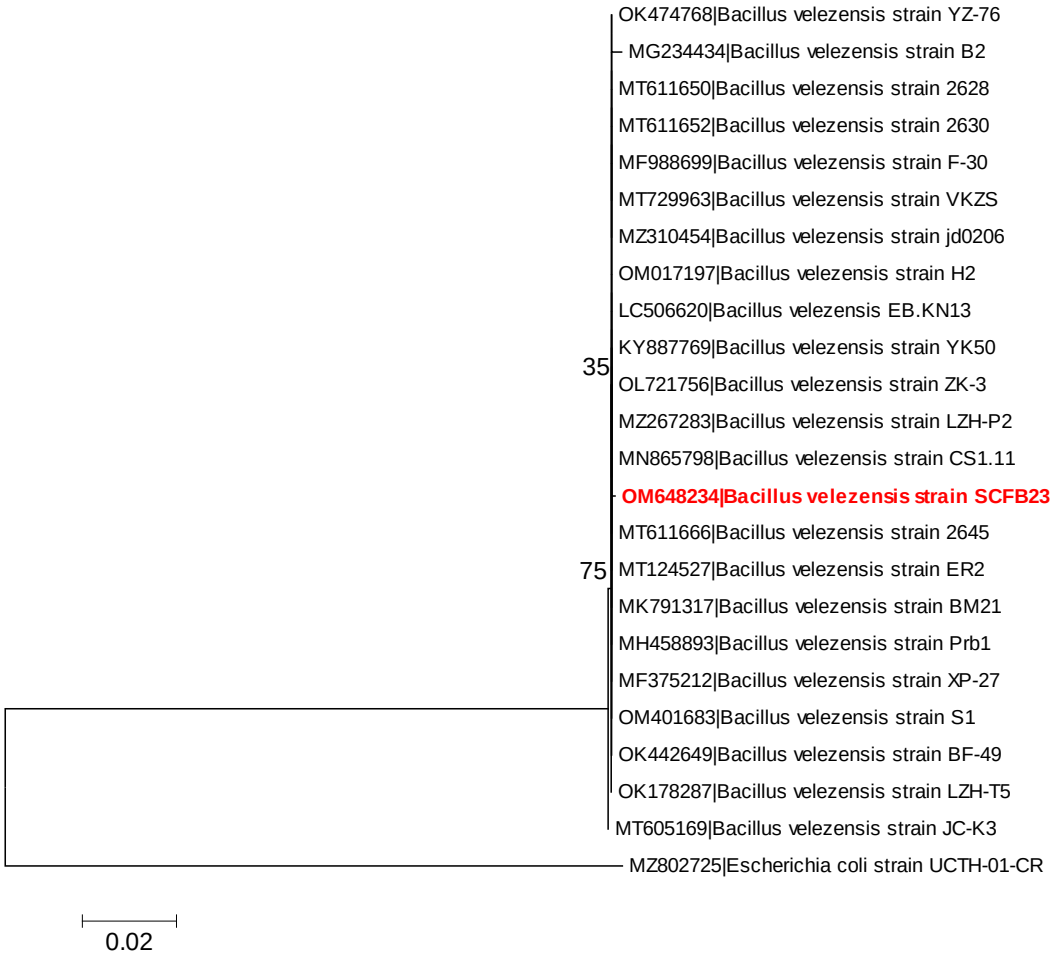


Fig 4.6: Phylogenetic tree evolutionary relationship of *Bacillus velezensis* obtained from soils in Jos, Nigeria with others strains available in the Gene Bank database.

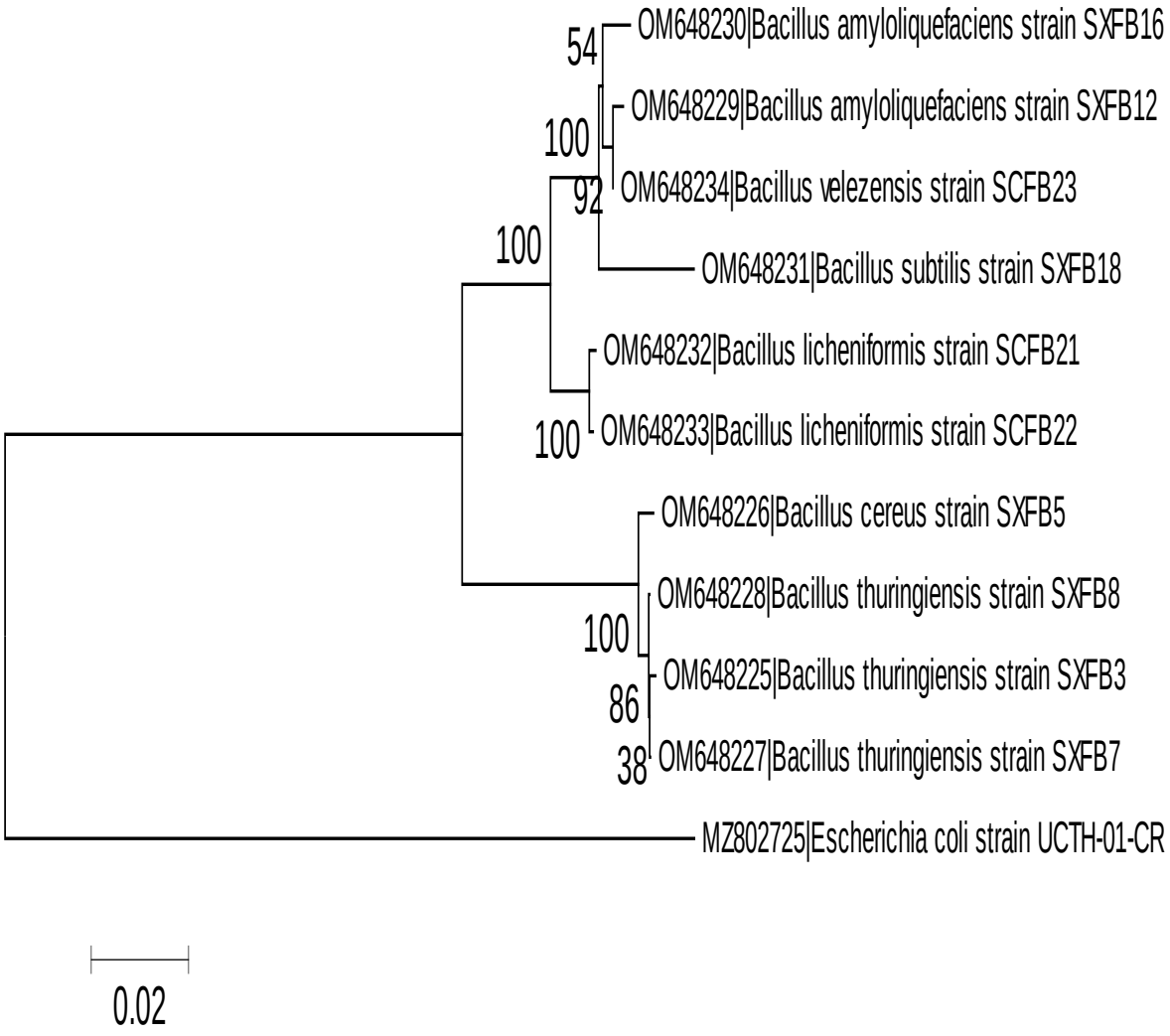


Fig 4.7:Phylogenetic treeevolutionary relationship of all ten *Bacillus* species obtained from soils in Jos.

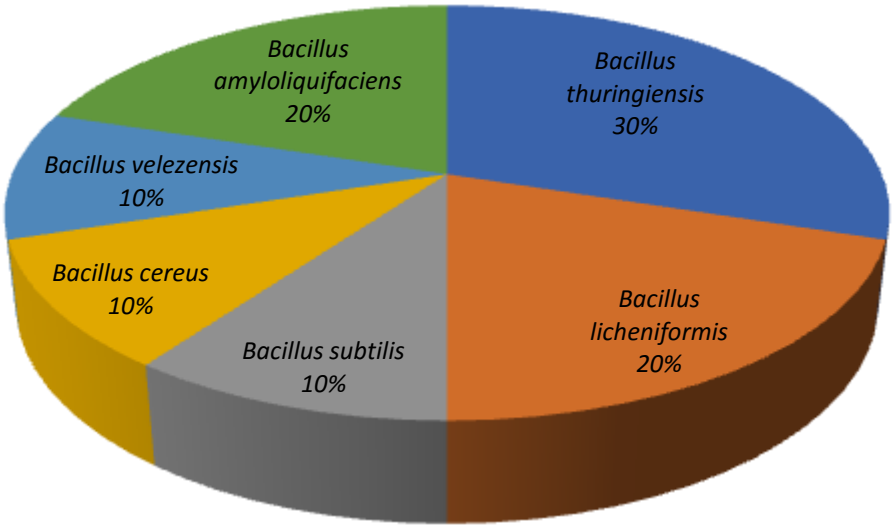


Fig 4.8: Proportion of isolated *Bacillus* species after sequencing of the 16SrRNA genes

Some parameters were checked to choose the best *Zymomonas mobilis* isolate to use for bioethanol production. They include the growth of the isolates in the presence of varying ethanol concentrations and the isolate with the highest ethanol yield.The result (Table 4.5) showed the growth of *Z. mobilis* isolates at varying ethanol concentrations while table 4.6 showed the ethanol yield in Rich medium. ZM2 had optical densities that ranged from 1.36-0.34, as the ethanol concentrations increased from 2.5-15.0% while ZM3 had optical densities that ranged from 1.40-0.66 as the ethanol concentrations increased from 2.5-15.0%. ZM3 had the highest ethanol yield of 80g/l, while ZM2 had the lowest ethanol yield of 60g/l. The result of the statistical analysis (two samples T-Test) carried out showed that statistical significant difference exists for the ethanol yields of ZM3 and ZM2 after 48h and 72h fermentation with $P \leq 0.05$

Some parameters were also checked to get the best *Bacillus* isolate to use for bioethanol production; the ethanol yields from the fermentation of xylose as well as the cellulase activities of the isolates. The result (Table 4.7) showed the ethanol yield from fermentation of xylose while table 4.9 showed the cellulase activities of *Bacillus* Isolates. The ethanol yields of all the isolates ranged from 18.5-35.5g/l. Isolate SCFB 22 had the highest ethanol yield while isolate SXFB 8 had the lowest ethanol yield. The result of the statistical analysis carried out using one way ANOVA revealed that there was no significant difference $P \geq 0.05$ in the ethanol yields of SCFB22 and SXFB23 but there was significant difference in the ethanol yields of the two strains (SCFB22 and SCFB23) $P \leq 0.05$ as compared to the other eight (8) strains. The cellulase activities of all the isolates (Table 4.8) ranged from 1.67 – 3.35 FPU/ml. Isolate SCFB22 had the highest cellulase activity while isolate SXFB 3 had the lowest cellulase activity.

Table 4.5:Mean optical densities of ZM2 and ZM3 at different alcohol concentrations (Alcohol tolerance)

	Growth (Optical Density At 540nm) at Varying Ethanol Concentrations ± SEM (%)					
Isolate	2.5	5.0	7.5	10.0	12.5	15.0
ZM2	1.36±0.02^{b}	1.23±0.01^{b}	1.22±0.01^{b}	1.09±0.01^{b}	1.00±0.02^{b}	0.34±0.01^{b}
ZM3	1.40±0.02^{ab}	1.24±0.01^{b}	1.21±0.01^{b}	1.11±0.01^{b}	1.06±0.01^{b}	0.66±0.01^{b}

Key: Means along the column that do not share at least a superscript are significantly different $P \leq 0.05$.

ZM = *Zymomonas mobilis*

Table 4.6 Ethanol yield of ZM2 and ZM3 after fermentation of glucose in Rich medium

	Ethanol yield in rich medium (g/l)/Time (hr)		
		Mean$\pm SEM$	
Isolate	24	48	72
ZM2	24.0 ± 2.83^{a}	48.0 ± 0.00^{b}	60.0 ± 8.49^{b}
ZM3	30.0 ± 2.83^{a}	69.0 ± 0.14^{a}	80.0 ± 7.07^{a}

Key: Means along the column that do not share at least a superscript are significantly different $P\leq0.05$

ZM = *Zymomonas mobilis*

Table 4.7 Mean Ethanol yields of *Bacillus* isolates after xylose fermentation

Strain	ETHANOL YIELD (g/l) Mean$\pm SEM$
SXFB3	24.0 ± 2.83^{c}
SXFB 5	22.0 ± 0.00^{cd}
SXFB 7	22.50 ± 0.42^{cd}
SXFB 8	18.50 ± 0.00^{d}
SXFB 12	21.60 ± 1.41^{cd}
SXFB 16	21.40 ± 0.57^{cd}
SXFB 18	22.50 ± 0.00^{cd}
SCFB 21	30.50 ± 0.14^{b}
SCFB 22	35.50 ± 0.14^{a}
SCFB 23	33.30 ± 0.00^{ab}

Key: Means that do not share at least a superscript are significantly different

$P \leq 0.05$

SEM = Standard error of mean

SXFB = Soil xylose fermenting *Bacillus*

SCFB = Soil cellulose fermenting *Bacillus*

Table 4.8 Mean values of cellulase Activities of *Bacillus* Isolates

Isolate	Mean Of Cellulase Activity(FPU/ml)± SEM
SXFB3	1.35 ± 0.01^{h}
SXFB 5	1.44 ± 0.02^{gh}
SXFB 7	1.38 ± 0.01^{gh}
SXFB 8	1.68 ± 0.02^{f}
SXFB 12	1.84 ± 0.02^{e}
SXFB 16	1.45 ± 0.02^{g}
SXFB 18	2.56 ± 0.01^{b}
SCFB 21	2.35 ± 0.02^{c}
SCFB 22	2.98 ± 0.02^{a}
SCFB 23B	2.06 ± 0.03^{d}

Key: Mean along the column that do not share atleast a superscript are significantly different $P\leq0.05$

SXFB = Soil xylose fermenting *Bacillus*

SCFB = Soil cellulose fermenting *Bacillus*

ZM3 was treated with different acetate concentrations in Rich medium to boost the isolates ability to withstand high acetate concentrations and adapt the ethanologenic bacterium to improve its ethanol productivity in the presence of some inhibitory substances (acetate). The results (Table 4.9) showed that ZM3 was able to grow in varying acetate concentrations which was evident by the increase in the optical density of the isolate from 1.02-1.30 after 72h incubation. As the isolate was exposed to higher acetate concentrations, the growth of the isolate decreased, although there were some observable changesin the colony sizes. The colony sizes increased with increase in acetate concentration. The Acetate adapted isolates were selected for bioethanol production.

Hydrolyzed corn cob biomass were fermented using two different bacterial isolates (in different set ups) in co-culture fermentation. The results (Table 4.10) revealed that ethanol yields for the fermentation ranged from 12.8-38.6g/l. The co-culture fermentation using acetate adapted *Zymomonas mobilis* and *Bacillus licheniformis* 22 (ZMA + SCFB 22) yielded the highest ethanol content of 38.6g/l, ZMA alone yielded ethanol content of 32.1g/l, *Zymomonas mobilis* ATCC 29191 (standard strain) yielded 31.8g/l of ethanol,co-culture fermentation with isolate ZM3 and SCFB22 yielded 32.3g/l of ehanol,ZM3 alone yielded 31.9, *Bacillus licheniformis* ATCC 14580 (standard strain) alone yielded 14.5g/l ethanol while isolate SCFB 22 yielded the lowest ethanol content of 12.8g/l. The result of the statistical analysis carried out using one way ANOVA showed that there was significant difference in the mean ethanol yields of the co-culture fermentation of corn cob hydrolysates using acetate adapted ZMA and SCFB22 as compared to co-culture fermentation using non acetate adapted ZM3 and SCFB22 with $P \leq 0.05$, statistical significant difference also exists when compared to the other set ups containing single strains. The ethanol yields were monotored using different strains in co-culture fermentation for nine (9) days to know the effect of fermentation time on the ethanol yields. The results (Fig 4.9) showed that there was no ethanol yield for the different set ups after 24h fermentation (Day 1). At 48hr fermentation (Day 2) the ethanol yields ranged from 2.2 -5.8g/l, there was a progressive increase in ethanol yields from day 2 to day 7. The highest ethanol yield was recorded (38.6g/l) after 7 days fermentation. On the 8^{th} day, there was no increase in the ethanol yields, instead there was decrease in the ethanol yields for some of the set ups from day 8-9.

Table 4.9 Acetate Adaptation of *Zymomonas mobilis* Isolate ZM3

	GROWTH (OPTICAL DENSITY AT 600nm) AT VARYING ACETATE CONCENTRATIONS / (%)							
TIME(HR)	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6
24hrs	1.02 ± 0.01^{a}	1.00 ± 0.01^{a}	0.90 ± 0.02^{b}	0.74 ± 0.01^{c}	0.72 ± 0.01^{c}	0.68 ± 0.01^{cd}	0.64 ± 0.01^{d}	0.60 ± 0.02^{d}
48hrs	1.28 ± 0.02^{a}	1.26 ± 0.01^{ab}	1.24 ± 0.01^{ab}	1.22 ± 0.02^{ab}	1.21 ± 0.01^{ab}	1.21 ± 0.00^{ab}	1.20 ± 0.01^{b}	1.20 ± 0.02^{b}
72hrs	1.30 ± 0.02^{a}	1.29 ± 0.01^{a}	1.27 ± 0.01^{ab}	1.24 ± 0.01^{ab}	1.22 ± 0.01^{b}	1.22 ± 0.00^{b}	1.21 ± 0.01^{b}	1.21 ± 0.01^{b}

Key: Means along each row that do not share at least a superscript are significantly different $P\leq0.05$

TABLE 4.10: Mean Bioethanol yields after 7 days co-culture fermentation of corn cob during the study

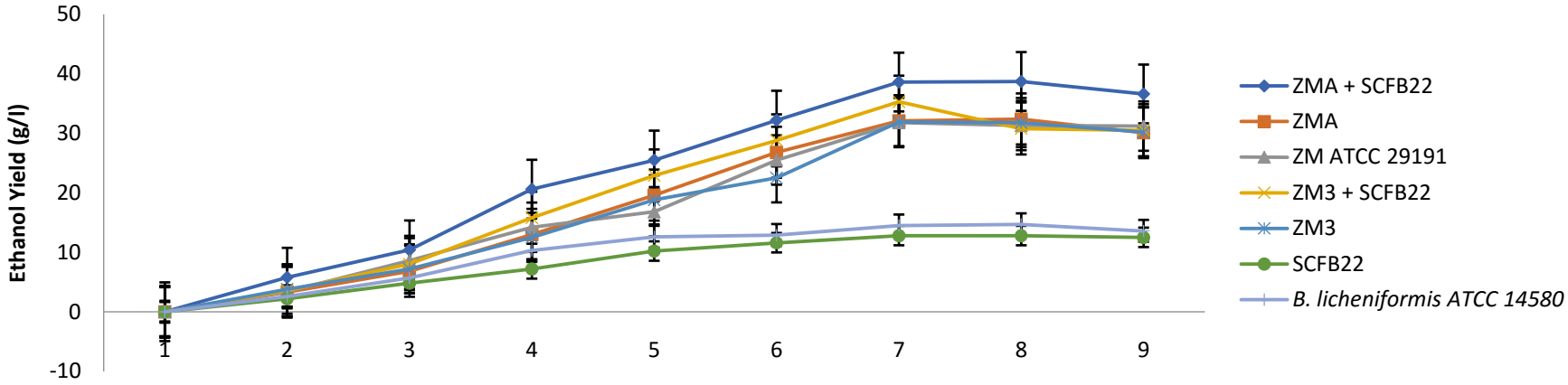
Strain (s)	Ethanol Yield (g/l) Mean$\pm SEM$
ZMA + SCFB22	38.6 ± 0.14^{a}
ZMA	32.1 ± 0.14^{c}
Zymomonas mobilis ATCC 29191	31.8 ± 0.14^{c}
ZM3 + SCFB22	35.3 ± 0.42^{b}
ZM3	31.9 ± 0.14^{c}
SCFB22	12.8 ± 0.07^{e}
Bacillus licheniformis ATCC 14580	14.5 ± 0.42^{d}

Key: Means that do not share at least a superscript are significantly different $P \leq 0.05$

ZMA = Acetate adapted *Zymomonas mobilis*

ZM3 = *Zymomonas mobilis* isolate 3

SCFB22 = Soil fermenting and cellulose hydrolysing *Bacillus licheniformis* isolate 22



Fermentation Period (Days)

Fig 4.9 Effect of varying fermentation time on the bioethanol yields using co-culture of *Zymomonas mobilis* and *Bacillus licheniformis*

ZMA = Acetate adapted *Zymomonas mobilis*

ZM3 = *Zymomonas mobilis* isolate 3

SCFB 22 = Soil xylose fermenting and cellulose hydrolyzing *Bacilluslicheniformis* isolate 22

ZM ATCC 29191 = *Zymomonas mobilis* standard strain ATCC 2919

The pH of the fermentation media were monitored during co-culture fermentation of hydrolysed corn cob. The result (Fig 4.10) showed that the fermentation media pH for the various set ups decreased from the range of 6.0 – 4.0.

The reducing sugar concentrations of the fermentation media were monitored to ascertain the sugar consumption abilities of the fermenting Bacteria (Fig 4.11). The results showed that the reducing sugar quantities of the fermentation media in gram per litre before and after fermentation. Before fermentation, the reducing sugar quantities of the fermentation media ranged from 78.7–81.0 g/l. After fermentation, the reducing sugar quantities of the fermentation media decreased to a range of 22.4-53.0 g/l.

The total bacterial count (TBC) of the fermentation media (Table 4.11) showed that the combination (co-culture) of acetate adapted *Z. mobilis* (ZMA) and *B. licheniformis* (SCFB22) had the highest initial TBC of 3.8×10^6 CFU/ml and the highest TBC of 8.6×10^6 CFU/ml at 7 days fermentation. The fermentation set up that contained SCFB22 alone had the lowest initial TBC of 1.8×10^8 CFU/ml and the lowest TBC of 2.4×10^8 CFU/ml at 7 days fermentation. The TBC of all the set ups increased with increase in fermentation time but decreased after seven days fermentation.

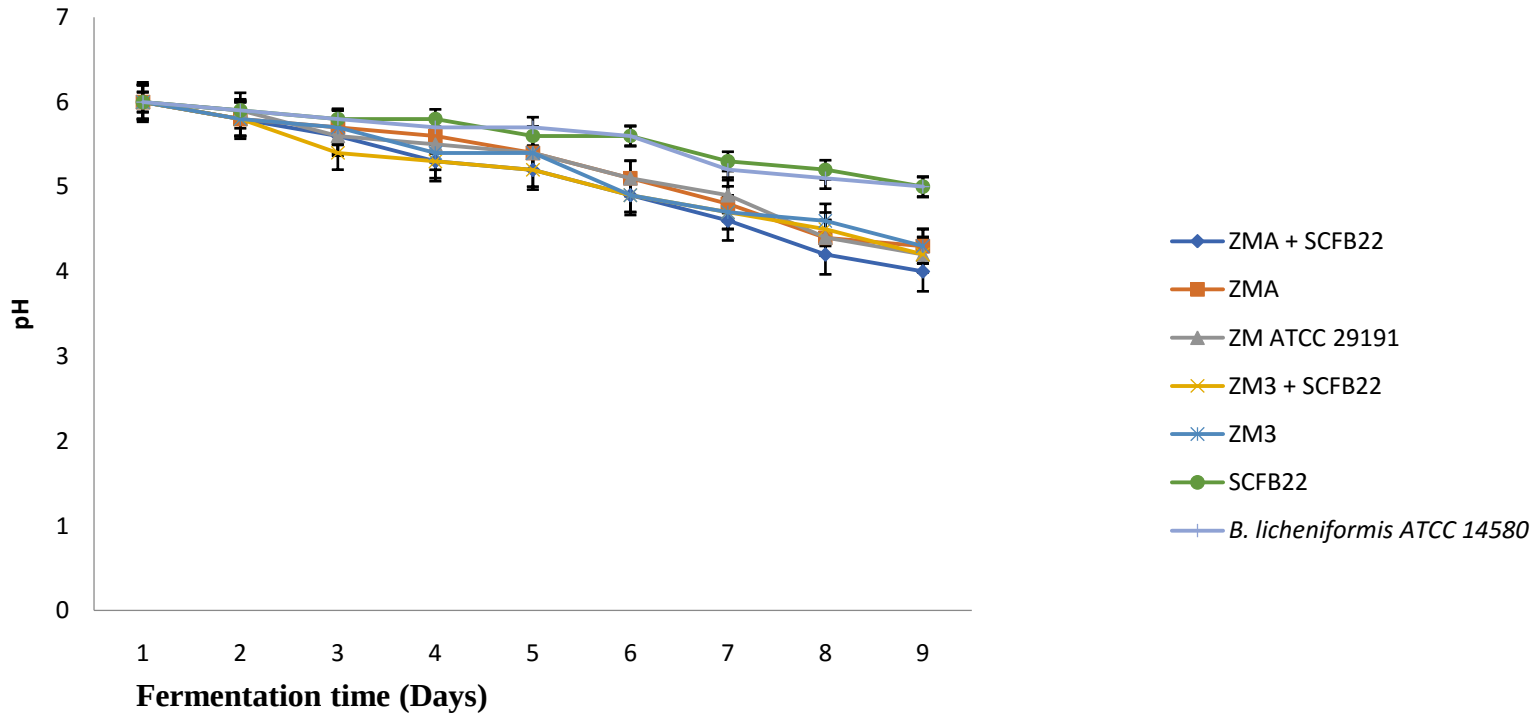


Fig 4.10 Fermentation media pH during Bio-ethanol production using co-culture of *Zymomonas mobilis* and *Bacillus licheniformis*
ZMA = Acetate adapted *Zymomonas mobilis*
ZM3 = *Zymomonas mobilis* isolate 3
SCFB = Soil cellulose fermenting Bacillus
ZM ATCC 29191 = *Zymomonas mobilis* standard strain ATCC 29191

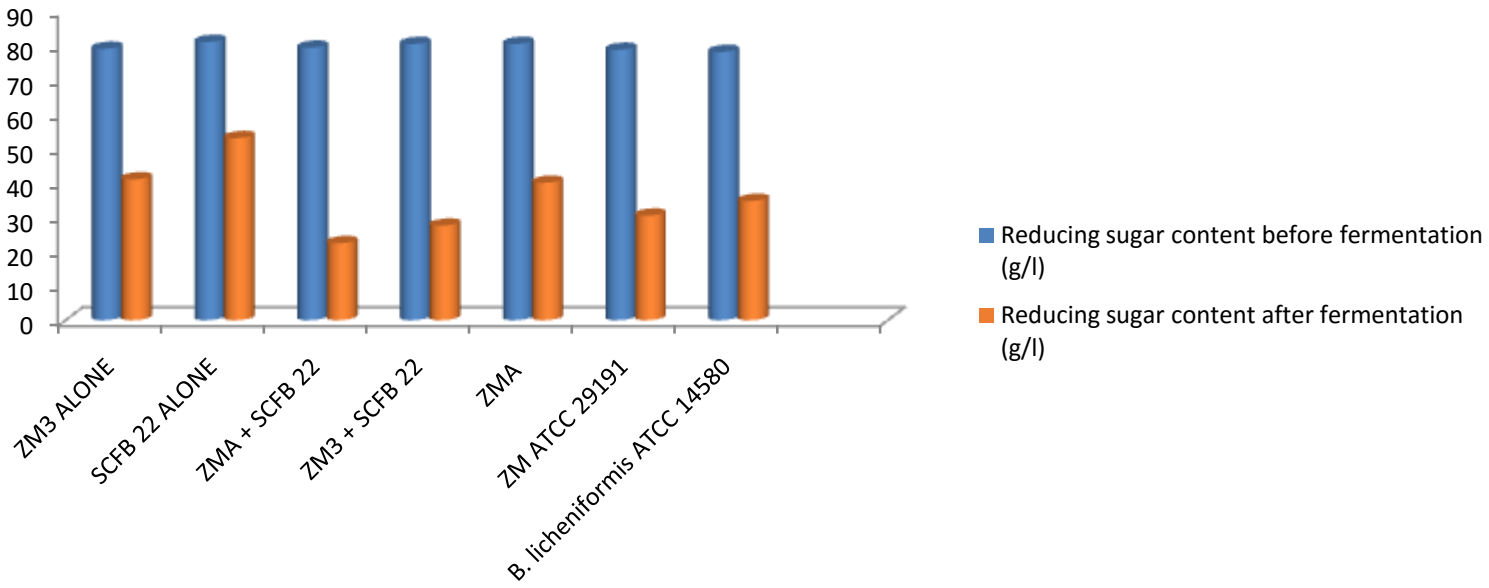


Fig 4.11 Reducing Sugar Quantity of Fermentation Media (g/l) Before and After Fermentation of Corn Cob to Bioethanol

ZMA = Acetate adapted *Zymomonas mobilis*

ZM3 = *Zymomonas mobilis* isolate 3

SCFB = Soil cellulose fermenting Bacillus

ZM ATCC 29191 =*Zymomonas mobilis* standard strain ATCC 29191

Table 4.11: Mean values of Bacterial counts during fermentation of corn cob hydrolysate

	Total bacterial counts of fermentation media (x 10^6CFU/ml ± SEM) for the different set ups						
Fermentation time (Days)	ZMA+SCFB22	ZMA	ZMSS	ZM3+SCFB22	ZM3	SCFB22	BSS
1	3.8±0.20^{a}	2.1±0.10^{b}	2.2±0.20^{b}	3.1±0.10^{a}	2.2±0.10^{b}	1.8±0.20^{b}	2.0±0.10^{b}
2	4.4±0.30^{a}	2.3±0.10^{b}	2.5±0.30^{b}	4.7±0.10^{a}	2.4±0.20^{b}	1.9±0.10^{b}	2.1±0.10^{b}
3	5.1±0,10^{a}	2.8±0.20^{b}	2.6±0.10^{b}	5.4±0.20^{a}	2.6±0.10^{b}	2.0±0.20^{b}	2.3±0.20^{b}
4	5.8±0.20^{a}	2.9±0.10^{b}	2.9±0.10^{b}	6.2±0.20^{a}	2.9±0.20^{b}	2.3±0.10^{b}	2.4±0.00^{b}
5	6.5±0.00^{a}	3.2±0.20^{b}	3.2±0.10^{b}	6.7±0.10^{a}	3.2±0.20^{b}	2.6±0.20^{b}	2.6±0.10^{b}
6	7.5±0.30^{a}	3.6±0.20^{b}	3.6±0.10^{b}	7.1±0.10^{a}	3.4±0.10^{b}	2.7±0.10^{b}	2.7±0.10^{b}
7	8.6±0.30^{a}	3.7±0.20^{cde}	4.5±0.30^{c}	7.5±0.10^{b}	3.9±0.20^{cd}	2.8±0.20^{e}	3.4±0.20^{de}
8	8.5±0.50^{a}	3.7±0.30^{b}	4.1±0.10^{b}	7.8±0.20^{a}	3.7±0.20^{b}	2.8±0.20^{b}	2.7±0.30^{b}
9	7.4±0.20^{a}	3.2±0.20^{cd}	3.6±0.10^{c}	6.2±0.20^{b}	3.8±0.20^{c}	2.7±0.20^{d}	2.6±0.10^{d}

Key: Means along each row that do not share at least a superscript are significantly different P≤0.05

ZMA = Acetate adapted *Zymomonas mobilis*, ZM3 = *Zymomonas mobilis* isolate 3, BSS= *Bacilluslicheniformis* standard strain ATCC 14580, SCFB = Soil xylose fermenting and cellulose hydrolysing *Bacilluslicheniformis*, ZMSS = *Zymomonas mobilis* standard strain ATCC 29191

The physicochemical parameters of the bioethanol produced were determined to ascertain the quality of bioethanol produced (Table 4.12). The pH of the bioethanol produced (for the different set ups) ranged from 6.0-6.8 and 6.4 for the commercial 98% ethanol, the density of bioethanol produced ranged from 0.77-0.84 g/cm^3(for the different set ups) and 0.76 g/cm^3 for the commercial ethanol.

The boiling point of the bioethanol produced varied for each sample analyzed which ranged from 78.6-78.9ºC while that of the commercial 98% ethanol was 78.6ºC.

The flash points of the bioethanol produced were slightly higher than that of the commercial ethanol. The flash point of the commercial ethanol was 13ºC while that of the bioethanol produced ranged from 14-16ºC. All the bioethanol produced alongside the commercial ethanol burned with a blue flame.

TABLE 4.12: Mean values of the physicochemical properties of the bioethanol produced and commercial Ethanol

ETHANOL PROPERTIES	Bioethanol Using ZM3 ALONE	Bioethanol Using B22 ALONE	Bioethanol Using ZMA+ SCFB22	Bioethanol Using ZM3+ SCFB22	Bioethanol Using ZMA	Bioethanol Using ZMSS	Bioethanol Using BSS	Commercial 98% Ethanol (control)
Appearance	Colourless	Colourless	Colourless	Colourless	Colourless	Colourless	Colourless	Colourless
pH	6.0	6.3	6.2	6.2	6.2	6.3	6.9	6.4
Relative Density(g/cm^3)	0.79	0.82	0.77	0.77	0.78	0.79	0.84	0.76
Boiling points (^oc)	78.8	78.9	78.6	78.7	78.7	78.7	78.8	78.6
Burning Characteristics	burns with a blue flame	burns with a blue flame	burns with a blue flame	burns with a blue flame	burns with a blue flame	burns with a blue flame	burns with a blue flame	burns with a blue flame
Flash point ^oc	15	16	14	14	15	15	16	13

Key: Means along each row that do not share at least a superscript are significantly different P≤0.05

ZMA = Acetate adapted *Zymomonas mobilis,* ZM3 = *Zymomonas mobilis* isolate 3, BSS= *Bacilluslicheniformis* standard strain ATCC 14580

SCFB = Soil xylose fermenting and cellulose hydrolysing *Bacilluslicheniformis*, ZMSS = *Zymomonas mobilis* standard strain ATCC 29191

CHAPTER FIVE

5.0 DISCUSSION

Proximate analysis of corn cob was carried and the results revealed that the substrate had the following composition: moisture (13.50%),ash (14.90%), crude protein(3.50%),lipid(1.00%) and carbohydrate(67.10%).The moisture content was a bit high in the corn cob samples. This may be attributed to the corn cob samples not been properly dry before been analysed. Moisture content of plant based samples gives an indication of the subatrates' freshness and it is important for microbial growth (Adepoju and Onasanya, 2008).

The ash content of the corn cob samples was relatively high. High ash content may suggest high mineral content as reported by Bello (1999). Ashing of plant samples reveals their mineral constituents that are required in small amount by microorganisms. This is an advantage for the fermentation process. The presence of minerals in the corn cob sample could also play important role in the replication of the bacteria that are been used during fermentation.

The protein content of the corn cob sample was quite low. Proteins play important roles in the replication of bacteria although they are a less useful source of energy for fermentation than carbohydrates (Devi *et al*., 2017).

The lipid content of the corn cob sample was quite low. However, this could be attributed to the fact that samples of animal origin have higher lipid content than those of plant

origin.The Lipid present in the corn cob could be metabolised by bacteria that are been used for fermentation as they could serve as energy source. The carbohydrate content of the corn cob samples analyzed in this research work was high. This could be attributed to the fact that samples of plant origin have high carbohydrate content compared to those of animal origin. The corn cob used in this research work was rich in carbohydrates and has great potential for bio-ethanol production.

A total of thirty bacterial isolates were obtained from palm sap. Of the thirty presumptive *Zymomonas* species isolated, only isoloates ZM2,ZM3,ZM4 and ZM5 had morphological and biochemical characteristics that were similar to that of *Zymomonas mobilis* that were in agreement with the characteristics of *Zymomonas mobilis* published by other authors (Laura *et al*., 2014, Zhang *et al*.,1995 and Dworkin *et al*.,1996) . The four isolates were therefore further characterized using molecular methods.

Genomic DNA was isolated from *Zymomonas mobilis* ATCC 29191, ZM2, ZM3 and ZM4. A fragment of 900bp that encodes the 16SrRNA gene that was specific for *Zymomonas mobilis*was amplified. Another fragment of 1200bp that encodes the alcohol dehydrogenase I gene that was specific for Z*ymomonas mobilis* was alsoamplified. Isolates ZM2, ZM3 and *Zymomonas mobilis* ATCC 29191 all had clear bands at 900bp and 1200bp regions for the two genes amplified whereas isolate ZM4,ZM5 and the negative control did not have any bands at all. The results of the amplification of the two genes specific for*Zymomonas mobilis* further confirmed the identity of isolates ZM2 and ZM3 as *Zymomonas mobilis.* The result coincided with the studies carried out by other authors (Laura *et al*., 2014; Coton *et al*., 2005 and Zhang *et al*., 2014)

A total of ten (10) *Bacillus* species were identified to the specie level by sequencing of the 16SrRNA gene. The sequences were blasted and aligned with other gene sequences in the Gene bank database to compare their similarities.

The percentage nblast identities of the isolates after sequencing revealed that the highest nblast identity of all the sequenced isolates ranged from 97.77-99.93 %. The percentage nblast identy is a quantitative measurement of similarity between two sequences and closely related species are expected to have a higher percent identity for a given sequence than would distantly related species. The percent identity therefore reflects relatedness. A good percent identity in a blast falls in the range of 98-100% sequence similarity (Faly *et al*.,2020).

The E-values of sequence alignment of the sequences of all the isolates were zero (0). The E-value gives a measure of the similarity of sequences. It is a measure of the statistical significance of a blast search result. An E-value of zero or close to zero means that there is an exact match for that sequence.

All the ten (10) *Bacillus* isolates were identified to the species level by sequencing the 16SrRNA gene. The isolates sequences and their various accession numbers were obtained and deposited at the Gene bank. Different Phylogenetic trees were constructed for the ten (10) *Bacillus* isolates to show evolutionary relationship of the different *Bacillus* species obtained from soils in Jos, Nigeria with others strains available in the GenBank database and all the trees were rooted using an *Escherichia coli* isolate from Nigeria. The tree for *Bacillus thuringiensis*showed that all three *Bacillus thuringiensis* isolates SXFB3, SXFB7 and SXFB8 clustered together with the strains gotten from the Genbank. This means that

they share a similar evolutionary relationship that is different from the outdoor *E. coli* group. The three *Bacillus thuringiensis* isolates were not closely clustered together which could be so if they have evolved from different ancestors.

The tree for *Bacillus amyloliquifaciens* shows that all two *Bacillus amyloliquifaciens* isolates SXFB12, SXB16 clustered together with the strains gotten from the Gene bank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group.

The two *Bacillus amyloliquifaciens* isolates were not closely clustered together which may be so if they have evolved from different ancestors. Also, SXFB16 seems to be more recently evolved *Bacillus* specie than SXB12 which was indicated by the branching to the right as shown on the tree. The tree for *Bacillus subtilis* showed that the *Bacillus subtilis* isolate SXB18 clustered together with the strains gotten from the Genbank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group.

Also, SXFB16 seems to be more recently evolved *Bacillus* specie than the strains from the Genbank which was indicated by the branching to the right as shown on the tree. The tree for *Bacillus licheniformis* showed that all two *Bacillus licheniformis* isolates SCFB21, SCFB22 clustered together with the strains gotten from the Genbank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree.

The two *Bacillus licheniformis* isolates were closely clustered together which may be due to the fact that they may have evolved from the same ancestor. The tree for *Bacillus velezensis*showed that the *Bacillus velezensis* isolate SCFB23 clustered together with the

strains gotten from the Gene bank. This suggests that they share a similar evolutionary relationship and belong to the same phylogeny group that is different from the outdoor *E. coli* group as shown on the tree.

The phylogenetic evolutionary tree for all the ten sequenced *Bacillus* species revealed that the isolates clustered together with one another which suggests there similarities at the genetic level and that they belong to the same phylogeny group that is different from the outdoor *E.coli* strain.

Sequencing of the gene encoding 16SrRNA has allowed the identification of ten (10) *Bacillus* isolates. Of the bacteria sequenced 2 (20%) were *Bacillus amyloliquifaciens,* 2 (20%) were *Bacillus licheniformis,* 3 (30%) were *Bacillus thuringiensis*, 1 (10%) was *Bacillus cereus* and 1 (10%) was *Bacillus subtilis.Bacillus thuringiensis* was the predominant bacteria (30%) of the isolates sequenced.

The result of the biochemical characterization of *Bacillus* isolates using Bacillus Microgen kit was not in total agreement with the result obtained after sequencing of the 16SrRNA genes. Only isolates SXFB5, SCFB21 and SCFB22 that were characterized biochemically as *Bacillus cereus, Bacillus licheniformis* and *Bacillus licheniformis* respectively were also characterized molecularly as same organisms, the remaining isolates had different identities. Biochemical tests alone cannot confirm the identity of strains satisfactorily to the specie level especially in the genera of organisms that are very similar in their genetic makeup like the *Bacillus* genus; Hence the need for molecular characterization.

The two confirmed *Zymomonas mobilis* isolates were tested for ethanol tolerance by measuring their growth at different ethanol concentrations and their ethanol yields from fermentation of glucose in rich media to select the best isolate for bioethanol production.

From the results, ZM3 grew better in the presence of different alcohol concentrations which were evident by the higher optical densities the isolate had as compared to ZM2; the isolate had a better alcohol tolerance than ZM2. High alcohol tolerance in microbes that would be used for fermentation is an important characteristics that is been considered when selecting strains for fermentation. ZM3 also had the highest ethanol yield from the fermentation of glucose when compared to the ethanol yields of ZM2. ZM3 was therefore chosen as the best isolate for bioethanol production.

All the ten (10) sequenced *Bacillus* isolates were screened for xylose fermentation and cellulase production to select the best isolate for bioethanol production.Xylose is a major component of hemicellulose in most biomass feedstock, it is therefore essential for the fermentation of biomass to contain microorganisms that can utilize this sugar for a more efficient ethanol production (Pronk and Scheffers, 2006).

The results revealed that isolate SCFB22 (*Bacillus licheniformis*22) had the highest ethanol yieldfrom the fermentation of xylose (which was significantly different); the isolate also had the highest cellulase activity. SCFB22 (*Bacillus licheniformis* 22) was therefore selected for bioethanol production.

ZM3 was treated with different acetate concentrations in Rich media using the principle of adaptive laboratory evolution (ALE) to boost the isolates ability to withstand high acetate concentrations and adapt the ethanologenic bacterium to improve its ethanol productivity in the presence of some inhibitory substances.

The results of this research work revealed that ZM3 was able to grow in varying acetate concentrations although there were some observable changes in the cell shapes as well as the colony sizes. The colony sizes increased with increase in acetate concentration while

the morphology of the cells also changed with increase in the acetate concentration (increase in cell length). Although the causes of the changes in cell and colony morphologies were not clearly understood, it could be a result of the stress response of the organism to the inhibitory substance (acetate). Similar morphological changes have been described previously in *Zymomonas mobilis* when exposed to compounds frequently present in hydrolysate (Mohogheghi *et al*.,2015). The isolate may have increased its fatty acid composition thereby decreasing the cell membrane permeability to higher acetate concentrations as reported by Berger *et al.* (1980). The Acetate adapted isolates were selected for bioethanol production.

Hydrolyzed corn cob biomass was fermented using two different bacterial isolates in co-culture fermentation. The results revealed that the co-culture fermentation using acetate adapted Zymomonas *mobilis* and *Bacillus* Isolate SCFB22 yielded the highest ethanol content, ZMA alone yielded ethanol content that was lower than the ethanol yield from the fermentation using co-culture, *Zymomonas mobilis* ATCC 29191 (standard strain) yielded ethanol content that was also lower than the ethanol yield obtained from the fermentation using co-culture.

The co-culture fermentation using non acetate adapted isolate ZM2 and SCFB22 also yielded ethanol content that was slightly lower than the ethanol yield from the fermentation using co-culture of acetate adapted ZM and SCFB22. The fermentation using only the non acetate adapted isolate ZM2 yielded ethanol content that was lower than the co-culture fermentation with acetate adapted isolate and SCFB22. The fermentations using only the *Bacillus* isolate SCFB22 and only the *Bacillus* standard strain (*Bacillus licheniformis* ATCC 14580) yielded very low ethanol contents.

The high ethanol content produced by co-culture fermentation using acetate adapted isolate ZMA and *Bacillus* isolate SCFB 22 could be as a result of the synergistic effect of the utilization of reducing sugars by the two isolates, it could also be as a result of the effect of acetate adaptation of the ZMA isolate which may have resulted in the isolate been resistant to higher concentrations of inhibitors like acetic acid and that enabled it perform better in fermentation of the available sugars. The results of the present work has revealed also that the acetate adapted *Zymomonas mobilis* isolate performed slightly better than the parent non acetate adapted isolate.

During acid pretreatmentof lignocelluloses biomass, several inhibitory compounds like acetate, hydroxymethylfurfural (HMF), and furfural are produced from the biomass pretreatment process leading to hydrolysate toxicity which is a major problem for microorganisms to achieve complete sugar utilization (Mohogheghi *et al*.,2015).Therefore, development of more robust microorganisms to utilize the sugars released from biomass under toxic environment is critical. Z*ymomonas mobilis* strain ZMA with a slightly enhanced tolerance to the toxic acetate present in corn cob hydrolysate was obtained. The adapted strain ZMA had a higher ethanol yield than the parent strain (ZM3).

The effect of fermentation time on the ethanol yield was monitored, there was no yield after 24h fermentation (Day 1).This could be due to the fact that the organisms were trying to adapt to the new media (corn cob hydrolysate) before they began to carry out metabolism. There was progressive increase in ethanol yields from day 2 to day 7. This could be attributed to the continuous utilization of the reducing sugars by the bacteria and is in agreement with the findings by Ado *et al*. (2009) and Chen *et al*. (2010). We recorded the highest ethanol yields after 7 days fermentation. On the 8^{th} day, there was no increase in the

ethanol yields, instead there was decrease in the ethanol yields for some of the set ups from day 8-9.The decrease in ethanol yield at day 8 could be attributed to depletion of available nutrient and accumulation of toxic metabolites in the fermentation media which may have inhibited the organisms ability to grow and carry out metabolism.

A high ethanol yield of 38.6g/l was obtained from the co-culture fermentation of corn cob hydrolysate using acetate adapted *Zymomonas mobilis* (ZMA) and xylose fermenting*Bacillus licheniformis* (SCFB22). Co-culture fermentation using ZMA and SCFB22 yielded a considerably higher ethanol content as compared to other set ups. The value obtained was higher than what was recorded by Ado *et al*. (2009) who produced 17.6g/l of bioethanol from corn cob using a co-culture of *Saccharomyces cerevisiae* and *Bacillus cereus*.

The result of the statistical analysis carried out using one way ANOVA showed that there was significant difference in the mean ethanol yields of the co-culture fermentation of corn cob hydrolysate using acetate adapted ZMA and SCFB22 as compared to co-culture fermentation using non acetate adapted ZM and SCFB22, statistical significant difference also exists when compared to the other set ups containing single strains. The ethanol yields obtained in this research work were comparable to the ethanol yields obtained by other workers. Mojtaba *et al*. 2017 reported ethanol yields of 12 and 14g/l in the fermentation of a glucose and xylose rich media using native *Zymomonas mobilis* isolates. Laura *et al*., 2011 also reported an ethanol yield of 79.79g/l in the fermentation of sugarcane molasses using a native *Zymomonas mobilis.*

The reducing sugar concentrations of the fermentation media were measured using the DNS method of Zambere to ascertain the sugar consumption abilities of the fermenting Bacteria. The results showed that after fermentation, the reducing sugar quantities of the fermentation media decreased significantly. This confirmed that the fermenting bacteria had utilized the sugar present in the media for bio-ethanol production.

The total bacterial count (TBC) of the fermentation media revealed that the combination (co-culture) of acetate adapted *Z. mobilis* (ZMA) and *B. licheniformis* (SCFB22) had the highest initial TBC of 3.8×10^8 CFU/ml and the highest TBC of 8.6×10^8 CFU/ml at 7 days fermentation. The fermentation set up that contained SCFB22 alone had the lowest initial TBC of 1.8×10^8 CFU/ml and the lowest TBC of 2.4×10^8 CFU/ml at 7 days fermentation. The TBC of all the set ups increased with increase in fermentation time but decreased after seven days fermentation. The result showed that each type of bacterium in the co-culture fermentation set up was growing actively and metabolizing its substrates during fermentation irrespective of the presence of the other which was an advantage for the co-culture fermentation.

The pH of the fermentation media were monitored during co-culture fermentation of hydrolysed corn cob. The result revealed that the fermentation media pH for the various set ups decreased during the fermentation. This could be attributed to the fact that during fermentation, alcohols as well as some organic acids are produced due to utilization of the substrate which led to a decrease in the fermentation media pH. This further confirmed that fermentation had occurred.

The physicochemical parameters of the bioethanol produced were determined to ascertain the quality of bioethanol produced. The pH of the bioethanol produced varied but were all similar to the pH of the commercial ethanol with few discrepancies. The variation could be attributed to the different concentrations of bioethanol produced. The densities of bioethanol produced were slightly higher than the density of commercial ethanol. This could be attributed to the fact that the bioethanol was more diluted compared to the commercial ethanol.

The boiling points of the bioethanol produced varied for each sample analyzed but were all similar to that of the commercial ethanol but with slight differences. The difference in the boiling points of the samples could be as a result of the difference in the densities and alcohol yields. The bioethanol produced all had a colourless appearance and burned with blue flames which were also similar to the properties of the commercial ethanol.

The flash points of the bioethanol produced were slightly higher than that of the commercial ethanol, this may be attributed to the fact that the bioethanol produced was more diluted, hence the higher flash point. The lower the flash point, the better and faster the ignition of fuel. The bioethanol produced in this research work have shown that plant derived ethanol have similar properties with the commercial ethanol.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Two*Zymomonas mobilis* were characterized using molecular techniques (amplification of the 16SrRNA gene and alcohol dehydrogenase gene specific for *Zymomonas mobilis* using PCR). A total number of ten xylose fementing and cellulose hydrolysing *Bacillus* specieswere characterised by sequencing their 16SrRNA genes. The ten isolates sequences have been submitted to the gene bank with their accession numbers of OM648225, OM648226, OM6482267, OM6482268, OM648229, OM648230, OM648231, OM648232, OM648233 and OM648234.

Corn cob, a cheap and readily available substrate that is rich in nutrients has shown immense potential as a substrate for bioethanol production.

Zymomonas mobilis with enhanced tolerance to acetic acid was obtained using Adaptive laboratory evolution. The adapted strain ZMA had a slightly higher ability to utilize reducing sugars and produce a higher ethanol yield than the parent strain ZM3.The co-culture fermentation of corn cob hydrolysate using *Zymomonas mobilis* isolates ZMA and *Bacillus licheniformis* isolate SCFB22 yielded bioethanol content that was relatively high (38.6g/L). Co-culture fermentation has been shown to produce higher ethanol yields(35.3 g/L and 36.8g/l) as compared to fermentation using single strains (32.4,31.8 and 12.5 g/L).

Zymomonas mobilis isolate ZMA, ZM3 and *Bacillus licheniformis* isolate SCFB22 have great potential for industrial bioethanol production usingcheap Agricultural wastes.

The yield and bioethanol properties of the bioethanol produced varied .This may be attributed to the different set ups containing different strains of bacteria used for the fermentation process. A good quality bioethanol was produced with pH, density, boiling points and flash point with ranges from 6.0-6.9, 0.77-0.84g/cm^3, 78.6-78.9^{o}C, 15-16^{o}C respectively and appearances that were similar to that of a commercial 98% ethanol. The bioethanol produced can therefore serve as alternatives to the commercial ethanol.

6.2 Recommendations

1. Although using molecular approaches like PCR to detect specific genes for the characterization of Bacteria like *Zymomonas mobilis* to species level is good, partial or whole genome sequencing of isolates would be a better approach with available funds.
2. *Zymomonas mobilis* was isolated from Palm sap, there were no isolates from apple juices and spoiled orange juices but results from other studies suggest that the bacterium could be isolated from other sources. It is therefore recommended that other samples like local alcoholic beverages and fermenting fruits should be explored for isolation of the bacterium
3. Although the concentration of the ethanol produced were relatively high, optimization of the growth parameters for the fermentation of corn cob hydrolysate for both *Zymomonas mobilis* and *Bacillus licheniformis* could probably give a better yield of the product. Attention can be focused on this.

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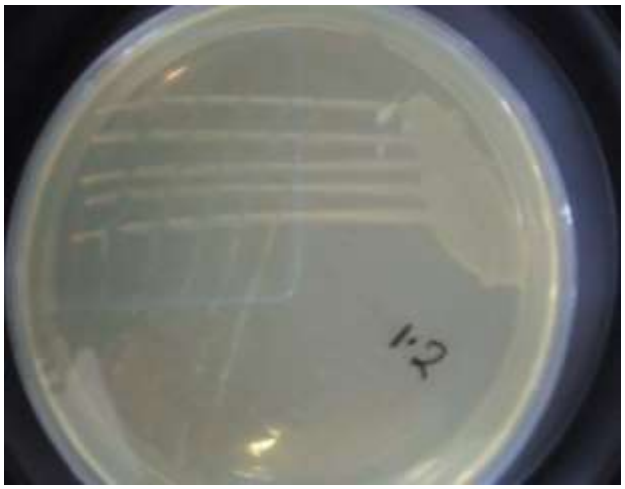
APPENDICES



Appendix I: Colony morphology of *Bacillus licheniformis* on nutrient agar plate



Appendix II: Colony morphology of *Zymomonas mobilis* on ZSM standard media plate



A



B

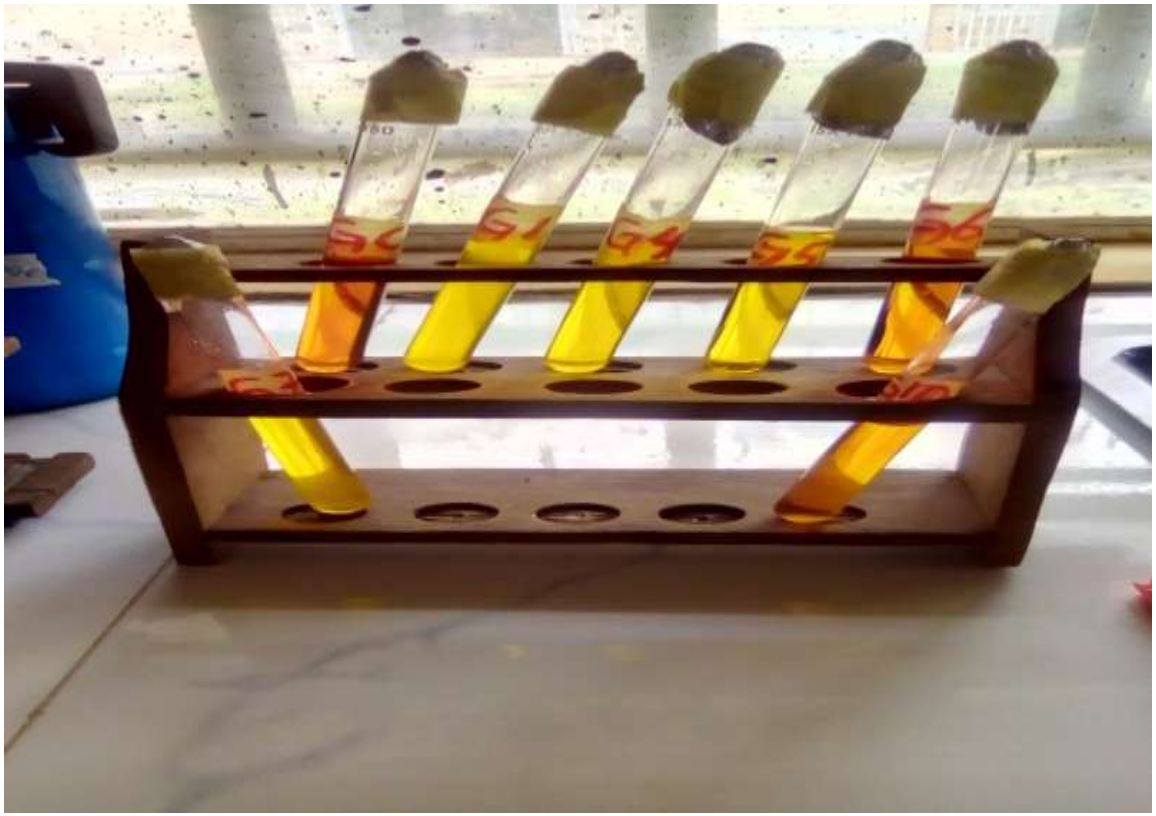
Appendix III: *Zymomonas mobilis* (ZM3) colony sizes after treatment with different acetic acid concentrations. A: Pin head colonies of ZM3 after treatment with 1.2% acetate, B: Larger colonies of ZM3 after treatment with 1.4% acetate



Appendix IV: Cell morphology of *Zymomonas mobilis* viewed under 100X objective of the light microscope after Gram staining



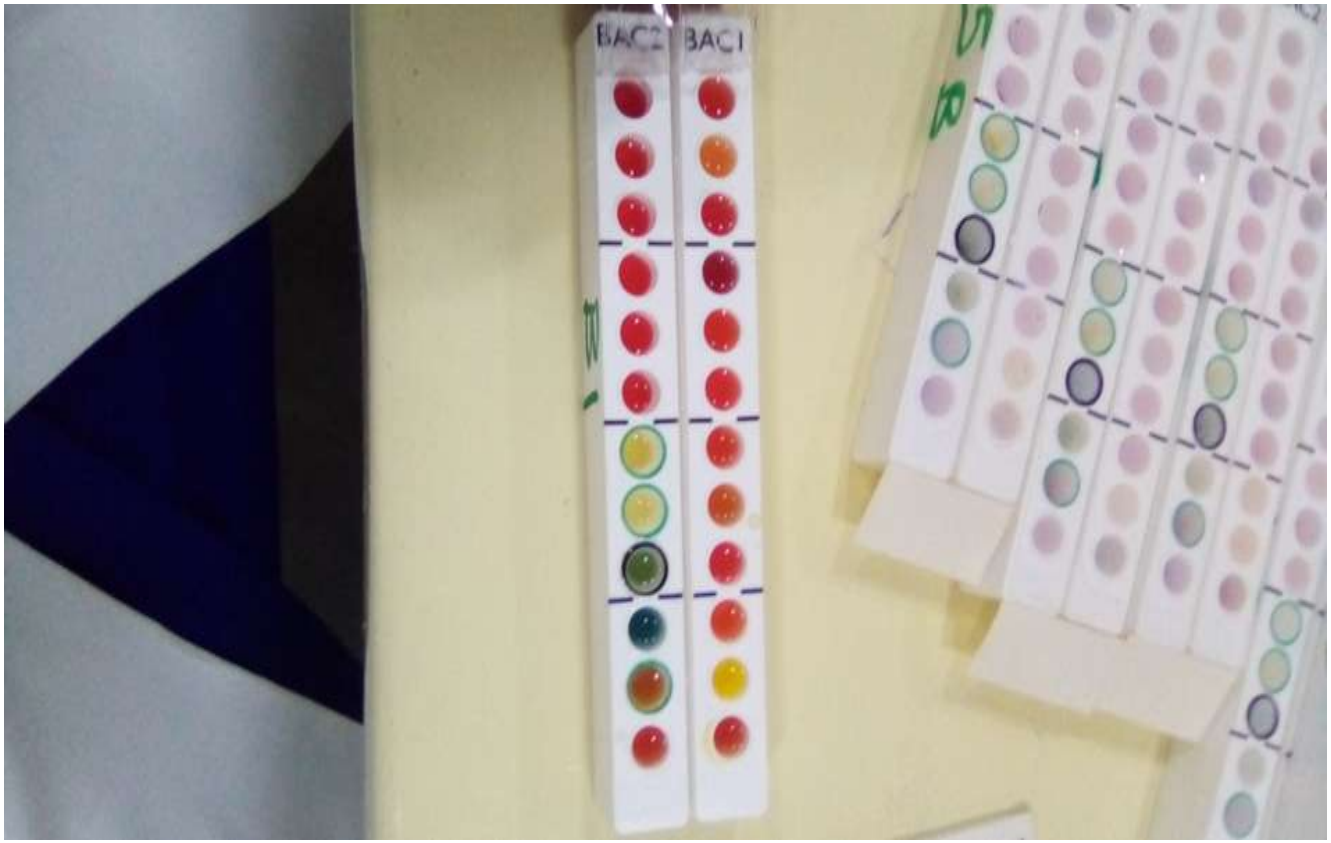
Appendix V: Xylose fermentation by *Bacillus* isolates



Appendix VI: Glucose fermentation by *Zymomonas mobilis* isolates



Appendix VII: Caboxymethyl cellulose (CMC) agar plates showing zones of cellulose hydrolysis by *Bacillus* isolates



Appendix VIII: Some Microgen Bacillus-ID system strip test showing colour change reactions.



Appendix IX: Pulverization of corn cob using a laboratory mortar and pestle



Appendix X: Co-culture fermentation of corn cob hydrolysate by *Zymomonas mobilis* and *Bacillus licheniformis* isolates



Appendix XI: Laboratory analysis



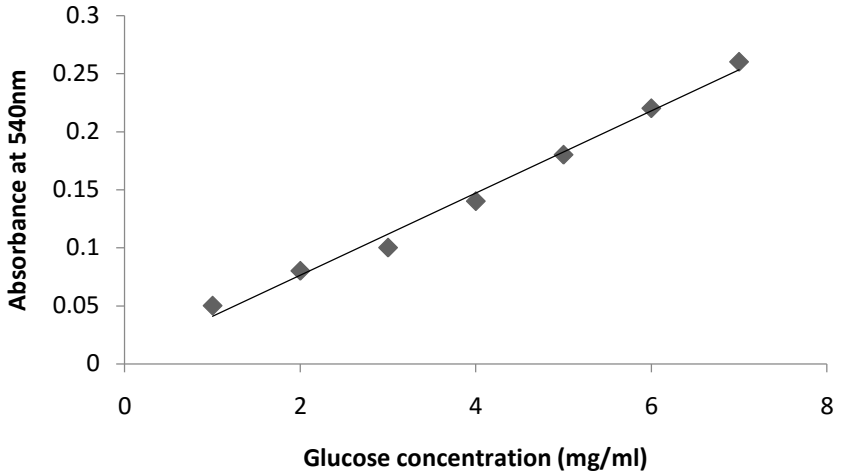
Appendix XII: Palm sap sample collection



Appendix XIII: Cultures of *Zymomonas mobilis* in anaerobic jar containing anaerobic gas pack

Appendix XIV: Cultural and Biochemical propertiesthe presumptive *Zymomonas* spp from palm sap samples.

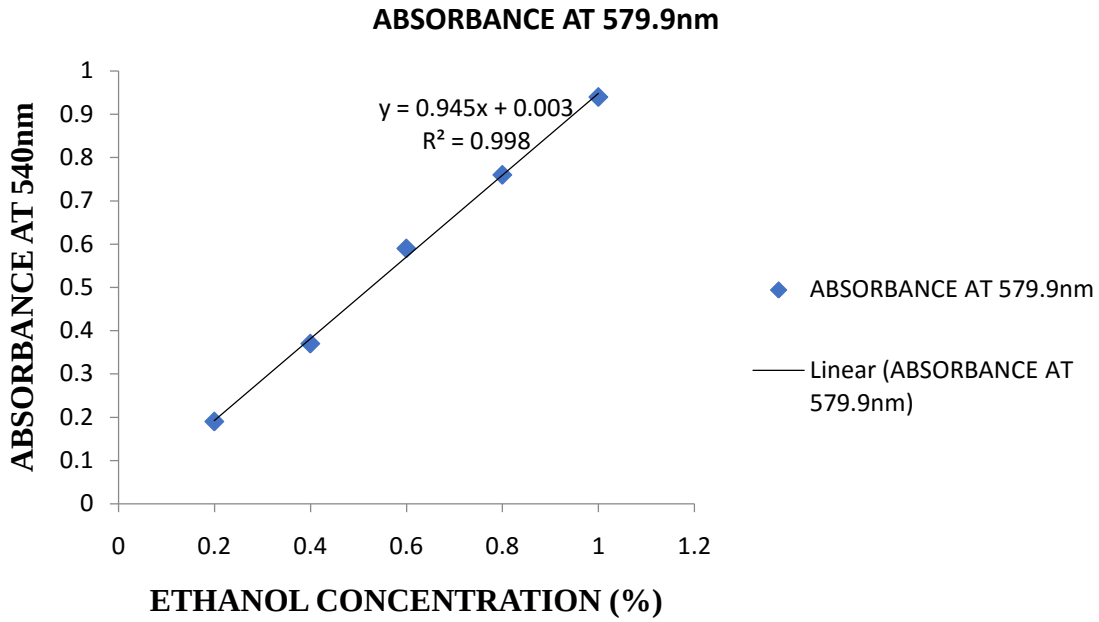
Isolate	Cell shape	Colony Morphology	Gram Reaction	Lactose	Maltose	Xylose	Glucose	Fructose	Sucrose	Motility	Oxidase	Indole	Urease
ZM1	Rod shape	Moist and whitish	_	_	+	_	+	+	+	+	_	_	_
ZM2	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
ZM3	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
ZM4	Rod shape	Moist and whitsh	_	_	_	_	+	+	+	+	_	_	_
ZM5	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
ZM6	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
ZM7	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
ZM8	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
ZM9	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
ZM10	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_



Appendix XV: Standard glucose curve for reducing sugar determination.

Appendix XVI: Reducing sugar quantity before and after fermentation

SAMPLE	REDUCING SUGAR BEFORE FERMENTATION (g/l)	REDUCING SUGAR AFTER FERMENTATION (g/l)
ZM2 ALONE	79.0	41.0
B21 ALONE	81.0	53.0
ZMA + B21	79.3	22.4
ZM2 + B21	80.4	27.5
ZMA	80.4	40.0
ZMSS	78.7	30.4
BSS	78.1	34.7



Appendix XVII: Ethanol standard curve for ethanol concentration determination

Appendix XVIII: Effect of varying fermentation time on the bioethanol yield

Bioethanol yields (g/l) for different Organism (s)

Fermentation Time (Days)	ZMA + SCFB22	ZMA	ZM ATCC 29191	ZM3 + SCFB22	ZM2	SCFB22	*B. licheniformis* ATCC 14580
1	0	0	0	0	0	0	0
2	5.8	3.3	3.4	3.6	3.8	2.2	2.6
3	10.4	6.8	8.6	8	7.2	4.8	5.7
4	20.6	13	14.2	15.8	12.5	7.2	10.3
5	25.5	19.6	16.8	22.9	18.8	10.2	12.6
6	32.2	26.8	25.5	28.8	22.5	11.6	11.9
7	38.6	32.1	31.8	35.3	31.9	12.8	14.5
8	38.7	32.4	31.3	30.8	31.8	12.8	14.7
9	36.6	30.1	31.2	30.5	30.2	12.5	13.6

Appendix XIX: pH of fermentation media during fermentation

Organism (s)	pH								
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
ZMA + B21	6.0	5.8	5.6	5.3	5.2	4.9	4.6	4.2	4.0
ZMA	6.0	5.8	5.7	5.6	5.4	5.1	4.8	4.4	4.3
Zymomonas mobilis ATCC 29191	6.0	5.9	5.6	5.5	5.4	5.1	4.9	4.4	4.2
ZM3+ B21	6.0	5.8	5.4	5.3	5.2	4.9	4.7	4.5	4.2
ZM3	6.0	5.8	5.7	5.4	5.4	4.9	4.7	4.6	4.3
B21	6.0	5.9	5.8	5.8	5.6	5.6	5.3	5.2	5.0
Bacillus licheniformis ATCC 14580	6.0	5.9	5.8	5.7	5.7	5.6	5.2	5.1	5.0

Appendix XX: Statistical analysis Results

Results for: Worksheet 1

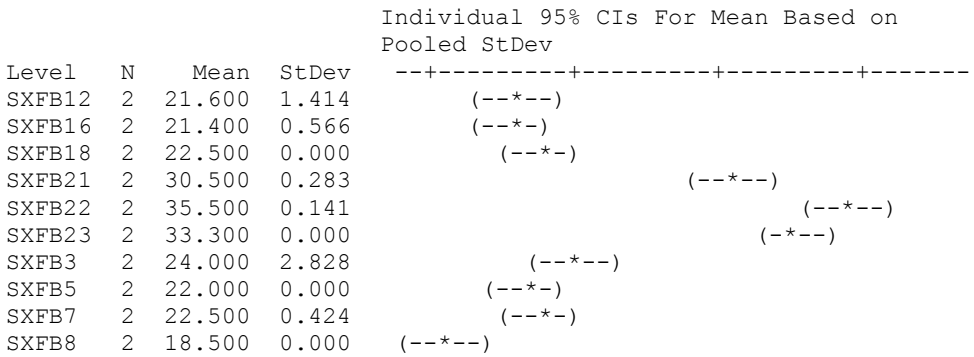
Descriptive Statistics: ETHANOL YIELD AFTER XYLOSE FERMENTATION WITH Bacillus strains

Variable	ISOLATE	Mean	SE Mean	StDev	Minimum	Maximum
ETHANOL YIELD	SXFB12	21.60	1.00	1.41	20.60	22.60
	SXFB16	21.400	0.400	0.566	21.000	21.800
	SXFB18	22.500	0.000000	0.000000	22.500	22.500
	SXFB21	30.500	0.200	0.283	30.300	30.700
	SXFB22	35.500	0.100	0.141	35.400	35.600
	SXFB23	33.300	0.000000	0.000000	33.300	33.300
	SXFB3	24.00	2.00	2.83	22.00	26.00
	SXFB5	22.000	0.000000	0.000000	22.000	22.000
	SXFB7	22.500	0.300	0.424	22.200	22.800
	SXFB8	18.500	0.000000	0.000000	18.500	18.500

One-way ANOVA: ETHANOL YIELD versus ISOLATE

Source	DF	SS	MS	F	P
ISOLATE	9	596.67	66.30	62.54	0.000
Error	10	10.60	1.06		
Total	19	607.27			

S = 1.030 R-Sq = 98.25% R-Sq(adj) = 96.68%



```
                          --+---------+---------+---------+-------
                           18.0      24.0      30.0      36.0

Pooled StDev = 1.030


Grouping Information Using Tukey Method

ISOLATE  N    Mean  Grouping
SXFB22   2  35.500  A
SXFB23   2  33.300  A B
SXFB21   2  30.500    B
SXFB3    2  24.000      C
SXFB7    2  22.500      C D
SXFB18   2  22.500      C D
SXFB5    2  22.000      C D
SXFB12   2  21.600      C D
SXFB16   2  21.400      C D
SXFB8    2  18.500        D

Means that do not share a letter are significantly different.
```

Results for: Worksheet 2

Descriptive Statistics: Ethanol yield after glucose fermentation for 24HRS, 48HRS and 72HRS

Variable	Mean	SE Mean	StDev	Minimum	Maximum
24HRS	27.00	2.08	4.16	22.00	32.00
48HRS	58.50	6.06	12.12	48.00	69.10
72HRS	70.00	6.60	13.19	54.00	85.00

Descriptive Statistics: 24HRS, 48HRS, 72HRS

Variable	ORGANISM	Mean	SE Mean	StDev	Minimum	Maximum
24HRS	ZM2	24.00	2.00	2.83	22.00	26.00
	ZM3	30.00	2.00	2.83	28.00	32.00
48HRS	ZM2	48.000	0.000000	0.000000	48.000	48.000
	ZM3	69.000	0.100	0.141	68.900	69.100
72HRS	ZM2	60.00	6.00	8.49	54.00	66.00
	ZM3	80.00	5.00	7.07	75.00	85.00

Two-Sample T-Test and CI: 24HRS, ORGANISM

```
Two-sample T for 24HRS

ORGANISM  N   Mean  StDev  SE Mean
ZM2       2  24.00   2.83      2.0
ZM3       2  30.00   2.83      2.0


Difference = mu (ZM2) - mu (ZM3)
Estimate for difference:  -6.00
95% CI for difference:  (-18.17, 6.17)
T-Test of difference = 0 (vs not =): T-Value = -2.12  P-Value = 0.168  DF = 2
```

Two-Sample T-Test and CI: 48HRS, ORGANISM

```
* ERROR * All values in column are identical.
```

Two-Sample T-Test and CI: 48HRS, ORGANISM

```
Two-sample T for 48HRS

ORGANISM  N    Mean  StDev  SE Mean
ZM2       2  48.000  0.283     0.20
ZM3       2  69.000  0.141     0.10


Difference = mu (ZM2) - mu (ZM3)



Estimate for difference:  -21.000
95% CI for difference:  (-23.841, -18.159)
T-Test of difference = 0 (vs not =): T-Value = -93.91  P-Value = 0.007  DF = 1
```

Two-Sample T-Test and CI: 72HRS, ORGANISM

```
Two-sample T for 72HRS

ORGANISM  N   Mean  StDev  SE Mean
ZM2       2  60.00   8.49      6.0
ZM3       2  80.00   7.07      5.0


Difference = mu (ZM2) - mu (ZM3)
Estimate for difference:  -20.00
95% CI for difference:  (-119.24, 79.24)
T-Test of difference = 0 (vs not =): T-Value = -2.56  P-Value = 0.237  DF = 1
```

Results for: Worksheet 3

Descriptive Statistics: ETHANOL YIELD AFTER CO-CULTURE FERMENTATION OF CORN COB HYDROLYSATE

```
Variable        Mean  SE Mean  StDev  Minimum  Maximum
ETHANOL YIELD  28.14     2.62   9.81    12.75    38.70
```

Descriptive Statistics: ETHANOL YIELD

```
Variable       STRAINS                    Mean  SE Mean   StDev  Minimum
ETHANOL YIELD  BACILLUS LICHENIFORMIS  14.500    0.300   0.424   14.200
               SCFB22                  12.800   0.0500  0.0707   12.750
               ZM2                     31.900    0.100   0.141   31.800
               ZM2+SCFB22              35.300    0.300   0.424   35.000
               ZMA                     32.100    0.100   0.141   32.000
               ZMA+SCFB22              38.600    0.100   0.141   38.500
               ZYMOMONAS MOBILIS       31.800    0.100   0.141   31.700

Variable       STRAINS                  Maximum
ETHANOL YIELD  BACILLUS LICHENIFORMIS    14.800
               SCFB22                    12.850
               ZM2                       32.000
               ZM2+SCFB22                35.600
               ZMA                       32.200
               ZMA+SCFB22                38.700
               ZYMOMONAS MOBILIS         31.900
```

One-way ANOVA: ETHANOL YIELD versus STRAINS

```
Source   DF        SS       MS        F      P
STRAINS   6  1250.514  208.419  3278.50  0.000
Error     7     0.445    0.064
Total    13  1250.959

S = 0.2521   R-Sq = 99.96%   R-Sq(adj) = 99.93%



Level                    N    Mean  StDev
BACILLUS LICHENIFORMIS   2  14.500  0.424
SCFB22                   2  12.800  0.071
ZM2                      2  31.900  0.141
ZM2+SCFB22               2  35.300  0.424
ZMA                      2  32.100  0.141
ZMA+SCFB22               2  38.600  0.141
ZYMOMONAS MOBILIS        2  31.800  0.141

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level                     --+---------+---------+---------+-------
BACILLUS LICHENIFORMIS      (*
SCFB22                     *)
ZM2                                          (*
ZM2+SCFB22                                        *)
ZMA                                          (*
YYYUYYUUZMA+SCFB22                                                 *)
ZYMOMONAS MOBILIS                            *)
                          --+---------+---------+---------+-------
                         14.0      21.0      28.0      35.0

Pooled StDev = 0.252


Grouping Information Using Tukey Method

STRAINS                  N    Mean  Grouping
ZMA+SCFB22               2  38.600  A
ZM2+SCFB22               2  35.300    B
```

ZMA	2	32.100		C	
ZM2	2	31.900		C	
ZYMOMONAS MOBILIS	2	31.800		C	
BACILLUS LICHENIFORMIS	2	14.500			D
SCFB22	2	12.800			E

Means that do not share a letter are significantly different.

Appendix XXI: Journal Publication

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Migap HH
Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

Abdullahi IO
Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

Whong CMZ
Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

Benshak JA
National Veterinary Research Institute, Vom, Plateau, Nigeria

Jurbe FD
Department of Medical Microbiology, University of Jos, Plateau, Nigeria

Adamu RR
Federal College of Animal Health and Production Technology, NVRI, Vom, Plateau State, Nigeria

Dalhatu HA
Federal College of Animal Health and Production Technology, NVRI, Vom, Plateau, Nigeria

Uzoma S
Federal College of Animal Health and Production Technology, NVRI, Vom, Plateau, Nigeria

Corresponding Author:
Migap HH
Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria

Isolation and molecular characterization of *zymomonas mobilise* for bioethanol production from palm saps in some parts of Jos, Plateau state, Nigeria

Migap HH, Abdullahi IO, Whong CMZ, Benshak JA, Jurbe FD, Adamu RR, Dalhatu HA and Uzoma S

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Abstract
The search for a suitable organism for bioethanol production is still ongoing. *Zymomonas mobilis* is a bacterium belonging to the genus *Zymomonas* and is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. In the present research work *Zymomonas mobilis* isolates were obtained from fresh palm sap and characterized using conventional biochemical tests as well as molecular techniques through the PCR detection of the alcohol dehydrogenase gene (the primer sequences used targeted 16SrRNA and 23SrRNA genes). The results of the biochemical characterization of presumptive isolates of *Zymomonas mobilis* showed that out of ten (10) isolates only four (4) isolates (40%) which include ZM2, ZM3, ZM4, and ZM5 matched with the characteristics of *Zymomonas mobilis* according to available standard techniques. For the molecular characterization of the presumptive *Zymomonas mobilis* isolates, genomic DNA was isolated from *Zymomonas mobilis* (ATCC 29191), ZM2, ZM3, ZM4 and ZM5. Two different primers were used; a fragment of 900 bp was amplified using 16SrRNA(Z16p3) and 23SrRNA (Z23p5) gene primers that were specific for *Zymomonas mobilis* and another fragment of 1200bp was amplified using ADH 1 gene (ZADH IF and ZADH IR primers) that were specific for *Zymomonas mobilis* for each of the isolates. The results of the amplification with the specific primer pairs coincided with the studies carried out by other authors. The two confirmed *Zymomonas mobilis* isolates were also screened for ethanol production using rich media and they showed relatively high ethanol yields (24-60g/l for ZM2 and 30-80g/l for ZM3). The results of this research work revealed that the two *Zymomonas mobilis* isolates obtained in this research have good potential as candidates for industrial bioethanol production.

Keywords: Bioethanol, palm sap, zymomonas, molecular

1. Introduction
Bioethanol is a distilled colorless liquid fuel obtained from numerous potential feedstock varieties such as sugar beet, wheat, corn, cassava, fruits, bagasse, barley, molasses, skim milk (whey), potatoes, sorghum, switchgrass and cellulose biomass such as wood, paper, straw and other cellulose wastes such as grasses, others include municipal solid wastes (Shell Global, 2001) [15].

Bioethanol is mostly obtained by fermentation of sugars which are converted into carbon dioxide and ethanol by microorganisms; *Saccharomyces cerevisiae* (yeast) is the most frequently used in this bioprocess (Dien *et al.*, 2003, Lin and Tanaka 2006) [3, 8]. Another promising microorganism for the production of bioethanol is *Zymomonas mobilis*. This is a bacterium belonging to the genus *Zymomonas*. It is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. It was originally isolated from alcoholic beverages like the African palm wine, and the Mexican pulque, and also as a contaminant of cider and beer in European countries (Shiva *et al.*, 2012) [16].

As an organism for bio-ethanol production, *Zymomonas mobilis* has shown some advantages over *Saccharomyces cerevisiae*, for example, higher specific rate of sugar uptake, high ethanol yield (up to 2.5 times higher), lower biomass production, higher ethanol tolerance up to 16% (v/v), non-requirement of controlled addition of oxygen during fermentation and amenability to genetic manipulations (Paneser *et al.*, 2006, Sahn *et al.*, 2006 and Rogers *et al.*, 2007) [10, 13, 12].

Extensive fundamental studies on *Zymomonas mobilis* over the last 30 years had also made this strain a promising ethanologenic organism for large-scale bio-ethanol production. *Zymomonas mobilis* degrades sugars to pyruvate using the Entner-Doudoroff pathway. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (analogous to yeast), (Paneser *et al*., 2006) [10].

Even though many microorganisms, including *Saccharomyces cerevisiae*, and *Clostridium* sp., have been considered as ethanologenic microbes, *Zymomonas mobilis* is considered the better candidate for industrial alcohol production (Rogers *et al*., 2007) [12].

There has been no report on the isolation and characterization of *Zymomonas mobilis* in Nigeria. This study seeks to isolate and characterize *Zymomonas mobilis* from palm sap, apple juice, and orange juice and to test the organisms ability to produce ethanol.

Materials and Methods

Isolation of *Zymomonas mobilis*

Media with the following compositions as described by Swings and De Ley (1977) [17] were used for isolation of *Zymomonas mobilis* from spoiled orange, fermented apple juice and fresh palm saps (1) Standard solid media that contained yeast extract 5.0 g, glucose 20 g, agar 20 g, distilled water 1 litre, pH 6.8, (2) Malt yeast peptone glucose (MYPG) medium contained malt extract 3.0g, yeast extract 5.0 g, peptone 5.0 g, glucose 20 g, distilled water 1litre, PH 4.8 was used. Each medium was treated with 10mg cycloheximide (actidion) per litre to inhibit yeast growth and autoclaved at 121 °C for 15min.

For isolation of *Zymomonas mobilis* from rotten *Citrus aurantium* (sweet orange), fermented apple juice and fresh palm saps, the method described by Obire (2005) [9] was adopted. The orange samples were washed thoroughly with water to remove dirt and some surface contaminants. The juice were squeezed out into a beaker, using different sterile syringes 1ml of each sample (apple, orange, and palm saps) were taken and diluted with 9ml sterile distilled water in a test tube and serially diluted in sterile distilled water from 10^1-10^6 dilutions. The spread plate technique was used to inoculate the samples on the sterilized media. Plates were incubated for 2 days at 30 °C in an anaerobic jar containing anaerobic gas packs (to exhaust the oxygen and produce CO_2 saturated atmosphere) after which presumptive *Z. mobilis* isolates were obtained. Presumptive colonies of *Zymomonas mobilis* were sub-cultured on fresh MYPG medium and incubated for 2 days at 30 °C. Isolates were preserved at 4 °C until required.

Identification and Characterization of Presumptive *Zymomonas mobilis*

Presumptive *Z. Mobilis* isolates were subjected to some conventional biochemical tests for identification. Gram stain, catalase test, motility, oxidase, urease, nitrate reduction, indole, carbohydrate fermentation tests as well as alcohol tolerance tests were carried out according to the method described by Cheesebrough, 2010 [1].

Confirmation of *Zymomonas mobilis* isolates using PCR

Zymomonas mobilis DNA extraction

Genomic DNA extraction was done by using DNA purification kit from Thermo Scientific Products. A single colony of *Z. mobilis* was inoculated into 10ml standard media broth and incubated overnight at 37 °C. The bacterial cells of 2×10^9 were harvested by centrifugation for 10 minutes at 500 × g and the supernatant was discarded. The pellet was resuspended in 180 µL of digestion solution with the addition of 20µL of proteinase K solution and mixed thoroughly by vortexing to obtain a uniform solution. The sample was incubated at 56 °C by shaking in a water bath until the cells were completely lysed for 30min. 20uL of RNase A solution was added and mixed by vortexing and incubated for 10 min at room temperature. 200ml of lysis solution was added and mixed by vortexing for about 15 s until a homogeneous mixture was obtained. To the mixture, 400 µL of 50% ethanol was added and mixed by vortexing. The DNA was purified using the GeneJET Genomic DNA Purification column with the aid of wash and elution buffers. The purified DNA was used immediately or stored at -20 °C.

Zymomonas mobilis Polymerase chain reaction

The target primers were for alcohol dehydrogenase I gene which include *Zad HIF* and *Zad HIR* with a primer sequence; ((5'CCCTCGAGGTAATCGGCTGGCAAT CGTTTTCC 3') and 23SrRNA (5' GTTCTAGAGATAGCGGCTTATAGCAACGAGTG 3') of about 1.2-kilobase size as well as 16SrRNA gene primers which include Z16P3-F and Z23P5-R. with sequences; (5'-CAAGCCTGCAAAGGTTAG-3') and (5'-ATGCTCTTACCTCACGCT-3') of about 900bp. The PCR reaction mixture of 50µl contained 2.5 mM dNTPs, 5 µg genomic DNA template and 20 µmol of each primer. The PCR mixture was subjected to the following thermal conditions using DNA thermal cycler: The Products were isolated after 40 cycles of amplification with initial denaturation at 94 °C for 30 s, 30 seconds at 58 °C and 2 minutes at 72 °C, final extension for 3 minutes at 72 °C. Purification to obtain PCR products was done with the QIA quick PCR purification kit (Qiagen). All amplification products were separated into 1% agarose gels which were stained with ethidium bromide. The PCR products were observed under an ultraviolet (UV) light Tran's illuminator (Sambrook *et al*., 1989) [14].

Results

The cultural and biochemical properties of the presumptive *Zymomonas mobilis* isolates (Table 1) that were gotten from palm sap, apple juice and orange juice revealed that all the isolates were rod-shaped, Gram-negative, had the ability to ferment glucose, fructose and sucrose. Only isolates ZM2, ZM3, ZM4 and ZM 5 could not ferment maltose, lactose and xylose. All the isolates were urease negative, indole negative and oxidase negative. Isolates ZM2, ZM3, ZM4 and ZM 5 were further characterized using molecular techniques (PCR Amplification of the 16SrRNA gene and Alcohol dehydrogenase (ADH I) gene of *Zymomonas mobilis*).

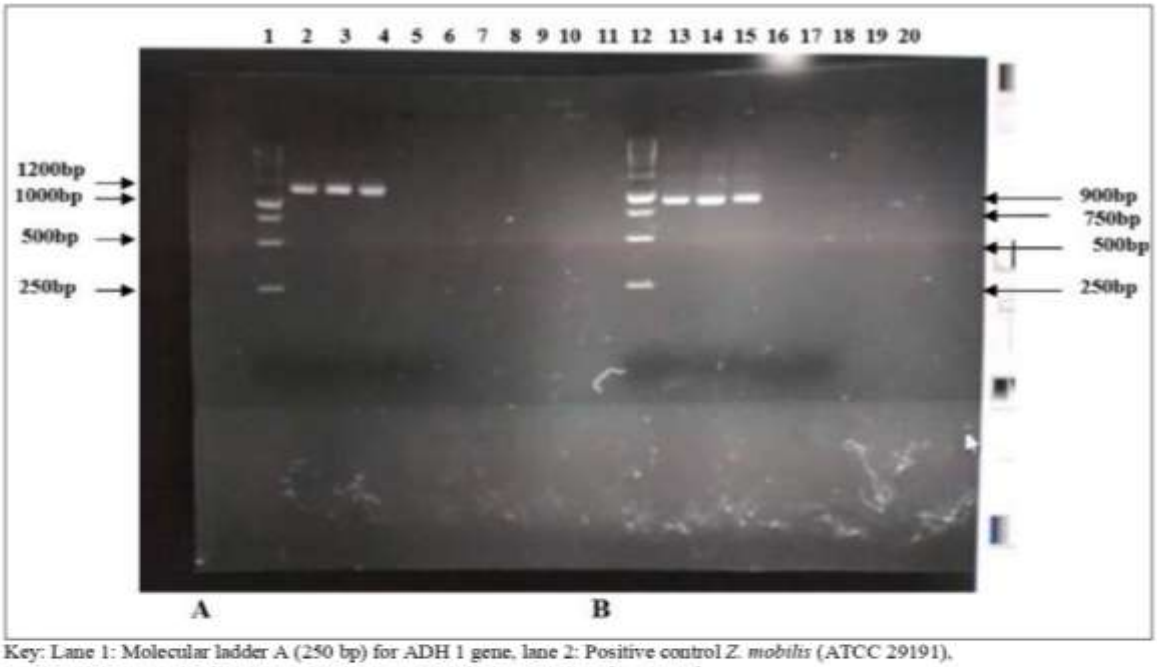
The results of the gel electrophoreses (Plate 1A and 1B) of the PCR Amplification of the Alcohol dehydrogenase gene (1200bp DNA fragment) and 16SrRNA gene (900bp DNA fragment) of *Zymomonas mobilis* ATCC 29191, bacterial isolates ZM2, ZM3, ZM4 and ZM5 showed that *Zymomonas mobilis* ATCC 29191 (positive control), isolates ZM2 and ZM3 had bands at 1200 bp position of the ladder, there were no bands for ZM4, ZM5 and the negative control. Similarly,

Zymomonas mobilis ATCC 29191, isolates ZM2 and ZM3 had bands at 900bp position of the ladder but there were no bands for ZM4, ZM5 and the negative control.

Some parameters were checked to choose the best *Zymomonas mobilis* isolate to use for bioethanol production. They include the growth of the isolates in the presence of varying ethanol concentrations and the isolate with the highest ethanol yield. The result (Table 2) showed the growth of *Z. mobilis* isolates at varying ethanol concentrations while Table 3 showed the ethanol yield in Rich medium. ZM2 had optical densities that ranged from 1.36-0.34, as the ethanol concentrations increased from 2.5-15.0% while ZM3 had optical densities that ranged from 1.40-0.66 as the ethanol concentrations increased from 2.5-15.0%. ZM3 had the highest ethanol yield of 80 g/l, while ZM2 had the lowest ethanol yield of 60 g/l. The result of the statistical analysis (two samples T-Test) carried out showed that statistically significant difference exists for the ethanol yields of ZM3 and ZM2 after 48 h and 72 h fermentation with $p \leq 0.05$.

Table 1: Cultural and Biochemical properties of presumptive *Zymomonas* spp from palm sap, apple juice and orange juice samples

Isolate	Cell shape	Colony Morphology	Gram reaction	Lactose	Maltose	Xylose	Glucose	Fructose	Sucrose	Motility	Oxidase	Indole	Urease
1	Rod shape	Moist and whitish	–	–	+	–	+	+	+	+	–	–	–
2	Rod shape	Moist and whitish	–	–	–	–	+	+	+	+	–	–	–
3	Rod shape	Moist and whitish	–	–	–	–	+	+	+	+	–	–	–
4	Rod shape	Moist and whitsh	–	–	–	–	+	+	+	+	–	–	–
5	Rod shape	Moist and whitish	–	–	–	–	+	+	+	+	–	–	–
6	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–
7	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–
8	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–
9	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–
10	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–
11	Rod shape	Moist and creamy	–	–	+	–	+	+	+	+	–	–	–



Key: Lane 1: Molecular ladder A (250 bp) for ADH 1 gene, lane 2: Positive control *Z. mobilis* (ATCC 29191), Lane 3: ZM2, Lane 4: ZM3, Lane 5:ZM4, Lane 6:ZM5, Lane 7: Negative control.

Plate 1B: Amplification of 16SrRNA gene (900bp) of *Zymomonas mobilis*

Key: Lane 12: Molecular ladder 2(250bp) for 16SrRNA gene, Lane 13: *Z. mobilis* (ATCC 29191), Lane 14: ZM2, Lane 15: ZM3, Lane 16: ZM, Lane 17:ZM5 and Lane 18: Negative control.

Plate 1A: Amplification of Alcohol dehydrogenase gene I (ADH I, 1200bp) of *Zymomonas mobilis*

Table 2: Alcohol tolerance of ZM2 and ZM3

Isolate	Growth (Optical Density at 540nm) at Varying Ethanol Concentrations					
	2.5%	5.0%	7.5%	10.0%	12.5%	15.0%
ZM2	1.36 ± 0.02^{b}	1.23 ± 0.01^{b}	1.22 ± 0.01^{b}	1.09 ± 0.01^{b}	1.00 ± 0.02^{b}	0.34 ± 0.01^{b}
ZM3	1.40 ± 0.02^{ab}	1.24 ± 0.01^{b}	1.21 ± 0.01^{b}	1.11 ± 0.01^{b}	1.06 ± 0.01^{b}	0.66 ± 0.01^{b}

Mean±SD that do not share at least a superscript are statistically significant ($p \leq 0.05$).

Table 3: Ethanol yield of ZM2 and ZM3 after fermentation of glucose in Rich medium

Ethanol yield in rich medium (g/l) Mean±*SD*			
Isolate	24 hr.	48 hr.	72 hr.
ZM2	24.0±2.83	48.0±0.00^{b}	60.0±8.49^{b}
ZM3	30.0±2.83	69.0±0.14^{a}	80.0±7.07^{a}

Mean±SD that do not share at least a superscript are statistically significant ($p \leq 0.05$)

Discussion

A total of thirty bacterial isolates were obtained from palm sap, apple juice and orange juice. Of the thirty presumptive *Zymomonas* species isolated, only isoloates ZM2, ZM3, ZM4 and ZM5 had morphological and biochemical characteristics that were similar to that of *Zymomonas mobilis* that were in agreement with the characteristics of *Zymomonas mobilis* published by other authors (Laura *et al.*, 2014, Zhang *et al.*, 1995 and Dworkin *et al.*,1996) [7, 18, 4]. The four isolates were therefore further characterized using molecular approach.

Genomic DNA was isolated from *Zymomonas mobilis* ATCC 29191, ZM2, ZM3 and ZM4. A fragment of 900bp that encodes the 16SrRNA gene that was specific for *Zymomonas mobilis* was amplified. Another fragment of 1200bp that encodes the alcohol dehydrogenase I gene that was specific for *Zymomonas mobilis* was also amplified. Isolates ZM2, ZM3 and *Zymomonas mobilis* ATCC 29191 all had clear bands at 900 bp and 1200 bp regions for the two genes amplified whereas isolate ZM4, ZM5 and the negative control did not have any bands at all. The results of the amplification of the two genes specific for *Zymomonas mobilis* further confirmed the identity of isolates ZM2 and ZM3 as *Zymomonas mobilis*. The result coincided with the studies carried out by other authors (Laura *et al.*, 2014; Coton *et al.*, 2005 and Zhang *et al.*, 2014) [7, 2, 19].

The two confirmed *Zymomonas mobilis* isolates were tested for ethanol tolerance by measuring their growth at different ethanol concentrations and their ethanol yields from fermentation of glucose in rich media to select the best isolate for bioethanol production. From the results, ZM3 grew better in the presence of different alcohol concentrations which were evident by the higher optical densities the isolate had as compared to ZM2. The isolate had a better alcohol tolerance than ZM2. High alcohol tolerance in microbes that would be used for fermentation is an important characteristics that is been considered when selecting strains for fermentation. ZM3 also had the highest ethanol yields from the fermentation of glucose when compared to the ethanol yields of ZM2. The result of this research work revealed that ZM2 and ZM3 both have good potential as candidates for bioethanol production.

Conclusion

Zymomonas mobilis were isolated and characterised morphologically, biochemically and by using molecular techniques through amplification of the 16SrRNA gene and alcohol dehydrogenase gene specific for *Zymomonas mobilis*.

Zymomonas mobilis isolate ZM2 and ZM3 demonstrated relatively high abilities to withstand high concentrations of ethanol and abilities to produce ethanol using glucose. The two isolates therefore have great potential for industrial bioethanol production.

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