PRODUCTION AND OPTIMIZATION OF AMYLASE, CELLULASE AND PECTINASE BY SELECTED BACTERIA AND FUNGI USING YAM PEELS AND CASSAVA PEELS AS A SUBSTRATE

BY

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ABSTRACT

Everyday large amount of waste material is lodged into the environment by industries, agriculture farm land and other ways leading to environmental pollution and these wastes materials from different sources can be dangerous to man. However, not much work has been done on utilization of these wastes (agricultural wastes) to generate useful products which necessitated this study. Agricultural wastes such as yam peels and cassava peels were used as solid substrate on solid state fermentation for the production of amylase, cellulase and pectinase. Isolation and identification of *Lactobacillus bulgaricus*, *Saccharomyces cereviciae*, *Aspergillus niger* and *Zynomonas mobilis* from milk, fresh palm wine, soil and spoilt orange sample respectively were done, Biochemical and molecular identification of the test organisms were carried out. The organisms were screened for enzymatic activity, the organisms were assayed at different incubation period, different optimum condition such as temperature, pH and substrate concentration were also performed. The result revealed a clear zone of inhibition in the agar plates. The highest amylase activity was obtained on the 4th day (8.5×10^{-2} mg/ml/sec.) by *Aspergillus niger* using cassava peels as a substrate. The highest cellulase activity (4×10^{-3} mg/ml/sec.) was obtained on the 4th by *Aspergillus niger* using cassava peels. The highest pectinase activity was obtained on the 4th day (3.7×10^{-2} mg/ml/sec.) by *Aspergillus niger* using yam peels as a substrate. The optimum pH for cellulase production was at pH 6 with the highest enzyme activity of (3.5×10^{-4} mg/ml/sec.) by *Aspergillus niger* using yam peels as a substrate, the optimum pH for cellulase activity was at pH 6 with the highest activity of (2.7×10^{-2} mg/ml/sec.) by *Aspergillus niger* using yam peels as a substrate. The optimum pH for pectinase activity was at pH 7 with the highest activity of (2.6×10^{-2} mg/ml/sec.) by *Aspergillus niger* the optimum temperature was at 50 °C with the highest activity (8.5×10^{-2} mg/ml/sec.) by *Aspergillus niger* using cassava peels as a substrate. The optimum pH for cellulase enzyme activity was 60°C. With the highest activity of ($1.7\text{x}10^{-4}$ mg/ml/sec.) using cassava peels as a substrate. For pectinase production the optimum temperature of 50 °C gave the highest pectinase activity of $1.5\text{x}10^{-2}$ mg/ml/sec. Substrate concentration for amylase cellulase and pectinase activity were also determined, points of saturation occurred between 2.5 % to 3 % in all the organisms tested *Aspergillus niger* have the highest concentration of substrate. This study revealed that all the test isolates using agro wastes as a substrate have the ability to produce amylase, cellulase and pectinase, hence it may be considered as a source for the production of industrial amylase, cellulase and pectinase. Serious work should be done on the abundant of agro wastes that are generated annually in Nigeria which could be utilized for the production of enzymes.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Agricultural wastes are amongst the main sources of environmental pollution which may be dangerous to man, animals and other living organisms in a particular environment. Their transformation into essential products may drastically reduce the problems they cause. Rice husks, cassava peels, yam peels, corncob are all sources of agricultural waste. In Nigeria huge amount of these waste materials are left on farm land to be decomposed by microorganisms such as Bacteria and fungi. Since these wastes are sources of environmental pollution, therefore, it is necessary for these wastes to be transformed into useful products (Enzymes) which is the main focus of this study.

Enzymes are proteins molecules found in the cells of living organisms which have the ability of speeding up chemical reactions but do not get used up in the process. Enzymes are the most essential products in industries obtained for human needs by microbial sources, many industries such as biotechnology, chemical industries and food industries make judicious use of enzymes at one stage or the other for the production of useful products (Oyeleke *et al.,* 2010). Enzymes are biological catalysts which are an indispensable component of biological reactions. The use of chemical catalysts has been followed for a very long time. Chemical catalysis though widely used was very common. The disadvantages that this method poses include need for high temperature and pressure for catalysis and the moderate specificity. These limitations were overcome by the use of enzymes. Enzymes work at milder conditions when compared to that required by chemical catalysts for operation. Also, enzymes are highly specific and catalyse reactions faster than chemical catalysts (Prasad, 2011).

Enzymes are now being used in various sectors of industry. They are used in detergents, paper industry, textile industry, food industry and many others industrial applications. Enzymes have been in use since ancient times (Gupta *et al*., 2003) and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk (Drauz *et al*., 2010). Among the many enzymes that are widely used Cellulase, pectinase, amylase has been in increasing demand due to their crucial roles and the applications in industries.

According to Burhan *et al.* (2003) there are three major sources of enzymes, those that are derived from a variety of plant (pappain), those that are derived from animal's gland (Trypsin and pepsin), and those that are derived from microorganisms (fungi and bacterial). The potential use of microorganisms for the production of enzymes are preferred than those from both plant and animal's sources, because they are cheaper, easy to produce and also their enzyme content is more predictable, controllable and reliable (Burhan *et al.*, 2003).

Cellulase is an enzyme that is capable of degrading cellulose. It has several industrial applications such as malting use in brewing of beer, wood processing, textile industries, maceration of protoplasts from plant tissues and deinking process in recycling of printed papers. The major obstacle to the exploitation of Cellulase is its high cost of production which includes other factors like complexity of cellulose, the type and source of cellulose employed for the production and low amounts of Cellulase production by cellulolytic organisms due to catabolic repression influence economics of Cellulase production. One effective approach to reduce the costs of enzymes production is to replace pure cellulose by relatively cheaper substrates such as lignocellulose materials (Oyeleke *et al*., 2012).

Pectinase is an enzyme that breaks down pectin, commonly referred to as peptic enzymes. pectinase is one of the known terms for the preparation of industrial enzyme that breaks

down pectin; a polysaccharide substrate found in the cell wall of plants. It is useful because pectin is the jelly-like matrix which helps in binding plant cells together and with other cell wall components, such as cellulose fibrils, are embedded (Oyewole *et al.*, 2011). This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages, through this process, it softens the cell wall and increase the yield of juice extract from the fruits. Two major sources of enzyme pectinase are plant and microorganism, but for both technical and economic point of view, microbial source of pectinase has becoming increasing important. A great variety of strains of bacteria, yeast and mold are capable of producing peptic enzymes (Oyewole *et al*., 2011).

Amylase is an enzyme that catalyse the hydrolysis of starch into sugar. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Amylase are example of hydrolases and functions in the hydrolysis of molecules. Amylase are of the most important enzymes use in industries (Burhan *et al.*, 2003). The applications of amylase in industrial setting includes, hydrolysis of starch to yield glucose syrup, amylase-rich floor and in the formation of dextrin during baking in food industries, they are also used for removal of starch sizing and additives in detergents (FAO, 2001).

Despite the wide applications of pectinase, amylase, Cellulase mentioned above, there are not widely produced in Nigeria but imported from other countries. The local production of Cellulase, pectinase, amylase from microorganism isolated from agricultural wastes (rice husks, Yam peels, cassava peels) in Nigeria will certainly help to minimize the huge amount of money spend on the importation of Cellulase, pectinase, amylase from other countries and in reducing the environmental pollution caused by these wastes products. This study is therefore aimed at, potential use of agricultural wastes for the production of Cellulase, pectinase, amylase.

1.2 Statement of the Research Problem

Everyday large amount of waste material is lodged into the environment by industries, agriculture farm land and other ways leading to environmental pollution and these wastes materials from different sources can be dangerous to both aquatic and terrestrial organisms. However, not much work has been done on utilization of these wastes (agricultural wastes) to generate useful products which necessitated this study.

1.3 Justification of The Study

The need for the production of industrial product (enzymes) is rapidly increasing, most of the industries depend on enzymes for the production of useful products. Because of the increasing demands of enzymes in food, chemical, and others industrial applications and also because of huge foreign exchange spent on the importation of enzymes, novel methods have to be adapted for its production which can be readily and cheaply available for industrial use.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to produce enzymes (Cellulase, pectinase, amylase) from agricultural wastes

1.4.2 Objectives

The Objectives of this Study were to:

I. Isolate and characterize

II. the selected microorganisms;

III. screen amylase, pectinase, and Cellulase producing microorganisms using agro wastes;

IV. Produce and assay amylase, pectinase and Cellulase, from the selected microorganisms using yam peels and cassava peels as substrate under solid state fermentation; and

V. optimize the growth conditions of the selected microorganisms for amylase, pectinase, and Cellulase production;

CHAPTER TWO

LITERATURE REVIEW

2.0 Agricultural Waste

In Nigeria, huge amount of waste and crops residues are made available everywhere. Which results to an environmental pollution. Agricultural waste such as cassava peels, Rice husks, Yam peels, sweet potatoes peel, remains the major environmental threat in Nigeria which are disposed of indiscriminately during their processing in domestic and industrial settings which can be harmful to our health (Oyeleke *et al*., 2012). According to Pandey (2000), some of these waste materials from agriculture are fibrous material which contains about 30–50 % starch on dry weight basis due to their rich organic nature and low ash content, they can serve as an ideal substrate for microbial processes for the production of value-added products. Several attempts have been made to produce several products such as enzymes, organic acids, flavour and aroma compounds, mushrooms from agricultural wastes. Solid-state fermentation has been mostly utilizing for biotransformation, agricultural waste generated as a by-product from farm, home and food industries, would be processed into useful products such as enzymes which are useful in chemical, food and others industries in Nigeria.

About three million cassava producers would benefit from the sale of cassava peels and undersized roots, as well as increased investment in the sector. Garri producers and their employees stand to benefit from the sale of the waste, both financially and in terms of a cleaner working environment. The nascent processing sector would attract investment, creating businesses and job opportunities throughout the processes (Chellapandi *et al*., 2008).

2.1 Classes of Agricultural Wastes

The main purpose of utilizing agricultural waste product is because of its stability, safety and economic feasibility of product/process development. Apart from the high carbohydrate content, the additional nutrients found in food industrial wastes make them ideal media components for microbial growth. Food processing waste can be categorized into six types, based on its source of origin: (i) vegetable trimmings and pulp; (ii) starch-based waste; (iii) fruit peels and pulp; (iv) spent grains/vegetable oil cakes; (v) meat and fish waste and (vi) dairy waste. All the other categories are raw materials for enzymes production and differ from one another with respect to factors such as structure, chemical composition, moisture content, etc. While the majority of the composition is dominated by non-starch carbohydrates and lignin, grain waste varieties also contain high amounts of proteins, lipids, starch and glucans. The exact percentage composition of grain varieties differs according to the season of harvest (Musatto *et al.*, 2016). Apart from polysaccharides, proteins and lipids food industrial wastes, such as apple pomace, are also a major source of dietary fibre, polyphenols and bio-active compounds (Pirmohammadi *et al*., 2006; Bhushan *et al*., 2008). The diversity of compounds found in industrial waste can act as growth enhancers for microbial processes. Banana and plantain pulp extract was found to promote the growth of gram-positive bacterial (Oyewole *et al.*, 2010).

2.2 Amylase

Amylases are hydrolases that function by the breakdown or hydrolysis of starch into reducing fermentable sugars, mainly maltose and reducing non-fermentable or slowly fermentable dextrin's. Numerous microorganisms like *Saccharomycopsis capsularia, Bacillus. coagulans, Bacillus sp*. HOP-40, and *Bacillus megatarium* 16 M, *Aspergillus* spp, *Bacillus subtilis* etc. have been used for amylase production by solid state fermentation (SSF) using agro-industrial residues. Amylases are important enzymes used

in the food industry for hydrolysis of starch to produce glucose syrups amylase –rich flour and proper formation of dextrin during baking. Also, they are employed in textile industry for removal of starch sizing. In the production of detergents, amylases are used to dissolve starches from fabrics (Oyeleke and Oduwole, 2009).

Amylases constitute class of industrial enzymes, which alone form approximately 25 % of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Oudjeriouat *et al*.,2003). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. evidences of amylase in yeast, moulds and bacteria have been reported and their properties had been documented. Studies on bacteria amylase especially in the developing countries like Nigeria have concentrated mainly on *Bacillus* probably because of the simple nature and nutritional requirements of this organisms (Omemu *et al*., 2005; Ajayi and Facade, 2006; Oyeleke and Oduwole 2009).

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. Members of the genus *Bacillus* are heterogeneous and they are very versatile in their adaptability to the environment. There are various factors that influence the nature of their metabolic processes and enzymes produced (Ajayi and Fagade, 2006; Oyeleke and Oduwole, 2009). A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes (Ajayi and Fagade,2006; Oyeleke and Oduwole, 2009). *Bacillus species* produce a large variety of extracellular enzymes, such as amylases, which have significant industrial importance (Cordeiro*al*.,2003). In the same

vein, bacterial enzymes are known to possess more thermostability than fungi amylase (Oyeleke *et al.* ,2012).

2.1.1 α-Amylase

α-Amylase is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4 glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity (iota *et al.*, 2014). There are 2 types of hydrolases: endo-hydrolase and exohydrolase. Endo-hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non-reducing ends (Gupta *et al*., 2003). Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25 % of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1,4-glycosidic linkage. Amylopectin constitutes 75-80 % of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α- 1, 4-glycosidic linkage while branching occurs every 15-45 glucose units where α-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for activity is found to be 7.0 (iota *et al* 2014). α-Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch.

α-Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by

enzyme hydrolysis of starch to yield high fructose syrup. The use of enzymes in detergents formulations has also increased the environmental protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of this enzyme which is also the main purpose in carried out this research work in order to produce this enzyme in a safe and convenient manner.

2.2.2 β – Amylase

β-Amylase (EC 3.2.1.2) is an exo-hydrolase enzyme that acts from the nonreducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of β-Amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-Amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. β-Amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. In the industry it is used for fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups (Sivaramakrishnan *et al*., 2006).

2.1.3 γ – Amylase

γ-Amylase (EC 3.2.1.3) cleaves α (1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, unlike the other forms of amylase, yielding glucose. γ- amylase is most efficient in acidic environments and has an optimum pH of 3 (Sivaramakrishnan *et al*., 2006).

2.2 Cellulase Enzyme

Cellulase is a complex enzyme composed of cellobiohydrolases, endoglucanases and β-glucosidases. These enzymes act effectively in the transformation of complex carbohydrate present in lignocellulose cell into glucose. This enzyme is also needed in different industrial application and been sold in huge volumes. Cellulase is used for various industries such as starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruits and vegetable juices, pulp and paper industries and textile industries (Abo-State *et al.,* 2010). Usually, the production of Cellulase is produced from submerged fermentation (SMF) method but the production cost of Cellulase and low yield of these enzyme are the major problems in the industrial applications (Kang *et al.,* 2004). Therefore, in order to optimize the production of Cellulase and also lowering the cost of production of Cellulase, solid state fermentation (SSF) is being used. This method has been reported that has an attractive process which is economical due to its lower capital investment and lower operating expenses (Yang *et al.*, 2010).

2.3 Screening of Cellulase Producing Bacteria

Screening for bacterial Cellulase activity in microbial isolates is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1-0.5 %(w/v). After incubation of a suitable period, a zone of clearing surrounding the colonies will be indicated that cellulose producer. The colonies of cellulolytic Cytophaga spp. did not shown any clearing zone (Schlegel, 2013). So, the diameter of the clearing zone may not accurately reflect the true Cellulase activity. For a rapid screening of Cellulase producing bacteria, after the incubation of the agar medium are containing 0.5% (W/V) carboxymethyl cellulose (CMC) as sole carbon source and flooded with 1% (W/V) Congo red (Sangrina & Tushar, 2013). After 20

minutes, the dye is decanted and the plates are again flooded with 5M NaCl which is decanted after 20-30 minutes. Positive colonies are detected to be surrounded by a pale orange to clear zone against red background. The cellulolytic bacteria can be screened directly on such plate, but replica plating from master plate is preferred for isolation of active colonies as flooded reagent impairing isolation. Plant et al. (Plant *et a* 2008*)* has reported a semi-quantitative assay for Cellulase activity in bacteria by using cellulose-azure into the upper two layers of agar tubes. The dye released from the substrate is determined densitometrically.

(Kasana *et al.,* 2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernible result. However, plate-screening methods using dyes are not quantitative method for the poor correlation between enzyme activity and halo size. This problem solved by the development of short cellooligosaccharides possessing modified reducing terminal with chromogenic/fluorogenic groups e.g. fluorescein, resorufin and 4-methylumbelliferone for higher sensitivity and quantification (Fia *et al.*, 2005). But a major limitation of the use of fluorescent substrates into agar plates is the tendency for hydrolysis products to diffuse widely and therefore are not as readily used such compounds. So, new substrates, 2-(2'- benzothiazolyl)-phenyl (BTP) cellooligosaccharides were synthesized for the screening of cellulolytic microorganisms in plate assays (Ivanen *et al.*, 2005).

2.4 Cellulase Production Using the Submerged Fermentation (SMF) and Solid-State Fermentation (SSF)

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of cellulase for their wide uses in industry. Over the years, fermentation techniques have

gained immense importance due to their economic and environmental advantages. Two broad fermentation techniques have emerged as a result of this rapid development: Submerged Fermentation (SMF) and Solid-State Fermentation (SSF).

2.4.1 Solid-state fermentation (SSF)

SSF utilizes solid substrates, like bran, bagasse, paddy straw, other agricultural waste and paper pulp (Subramaniyam and Vimala 2012). The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as cheaper substrates. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria (Sangrila and Tushar 2013).

2.4.2 Submerged (SMF)/liquid fermentation (LF)

Submerged fermentation utilizes free flowing liquid substrates, such as molasses and broth (Subramaniyam and Vimala 2012). This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier.

2.4.3 Submerged (SMF) / solid (SSC) state fermentation.

Cellulases are produced using the submerged fermentation (SMF) method traditionally, in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. An alternative to this traditional SMF method is the solid-state fermentation (SSC) method, which involves the growth of microorganisms on solid materials in the absence of free liquids. Since SSC involves relatively little liquid when compared with SMF, downstream processing from SSC is theoretically simpler and less expensive. During the past ten years, a renewed interest in SSC has developed due, in part, to the

recognition that many microorganisms, including genetically modified organisms (GMO), may produce their products more effectively by SSC (Pandey *et a*l., 2012). SSC has three advantages viz. i) lower consumption of water and energy, ii) reduced waste stream and iii) more highly concentrated product (Zhuang *et al.*, 2007). Moreover, the biosynthesis of cellulases in SMF process is strongly affected by catabolic and end product repressions (Via *et al.*, 2005) and on the overcoming of these repressions to significant extent in solid state fermentation (SSF) system (Sangrila *et al.*, 2013), therefore, are of economic importance.

The amenability of solid state fermentation technique to use up to 20-30 % substrate, in contrast to the maximum of 5 % in SMF process, has been documented (Subramaniyam and Vimala, 2012) The SSF is generally preferred as it offers many advantages such as two-three times higher enzyme production as well as protein rate, higher concentration of the product in the medium, direct use of air-dried fermented solids as source of enzyme which lead to elimination of expenses on downstream processing, employment of natural cellulosic wastes as substrate in contrast to the necessity of using pure cellulose in submerged fermentation (SMF) and the possibility of carrying out fermentation in non-aseptic conditions (Ivanen *et al.*, 2009).

2.5 Cellulase Producing Bacteria and their Characterization

Both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases. Most emphasis has been placed on the use of fungi because of their capability to produce copious amounts of cellulolytic enzymes and often less complex than bacterial Cellulase and easy for extraction and purification. It can therefore be more readily cloned and produced via recombination in a rapidly growing bacterial host. However, the isolation and characterization of novel Cellulase from bacteria are now becoming widely exploited. There are several reasons for these shifts

viz. i) bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes, ii) bacterial cellulases are often more complex and are in multi-enzyme complexes providing increased function and synergy iii) bacteria inhabit a wide variety of environmental and industrial niches like thermophilic or psychrophilic, alkaliphilic or acidophilic and halophilic strains, which produce cellulolytic strains that are extremely resistant to environmental stresses. These strains can survive and produce cellulolytic enzymes in the harsh conditions which are found to stable under extreme conditions and which may be used in the bioconversion process (Mirander *et al*., 2009). This may increase rates of enzymatic hydrolysis, fermentation, and, product recovery.

Researchers are now focusing on utilizing, and improving these enzymes for use in the biofuel and by-products industries. Many bacteria can grow on cellulose and many produce enzymes that catalyse the degradation of soluble derivatives of cellulose or the amorphous regions of crystalline cellulose. However, few bacteria synthesize the complete enzyme system that can result in extensive hydrolysis of the crystalline material found in nature. These few bacteria should be called "true cellulolytic "bacteria and those bacteria that produce some endoglucanases and ß-glucosidases, but not the complete system, are called "*pseudocellulolytic*" (Coughlan and Mayer, 2013). Such pseudocellulolytic bacteria may have picked up the genes encoding these enzymes from true cellulolytic species by horizontal transfer. There are different types of bacteria isolated from different environment produced Cellulase. Some of the importance bacterial for cellulase production are *Bacillus licheniformis, Bacillus sp* (alkaliphilic), *Bacillus subtilis*, Clone *E. Coli*

2.6 Pectinase Enzyme

Pectinase is also a well-known term for commercial enzyme preparation that break down pectin; a polysaccharide substrate, found in the cell wall of plants (Oyewole *et al*., 2011). This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. Through this process, it softens the cell wall and increase the yield of juice extract from the fruits. The two major sources of the enzyme pectinase are plant and microorganism. But for both technical and economic point of view microbial source of pectinase has become increasingly important. A great variety of microorganisms such as bacteria, yeast and mold are capable of producing peptic enzymes. The composition of peptic enzymes differs among species of microorganisms (Oyewole *et al*., 2011). Currently, pectinase is one of the most important enzymes in food processing industries mainly for extraction and clarification of fruit juices and wines (Oyewole *et al*.,2011).

2.7 Lignocellulose as a Raw Material

According to Rugauskas *et al* (2014), Lignocellulose is essentially a complex polymer which is made up of polysaccharides and phenolic polymers (lignin). All plant matter is composed of lignocellulose, and lignin is the most recalcitrant substance found in them. It provides physical support and rigidity to the plant cell wall. It is a complex polymer made up of coniferyl alcohol, snappy alcohol and p-hydroxyphenyl alcohol (Rugauskas *et al.*, 2014). The presence of lignin in lignocellulose prevents its effective enzymatic degradation and subsequent utilization of fermentable sugars by microbes (Zeng *et al*., 2014). This calls for effective measures for lignin removal through various pre-treatment techniques (Ghaffar & Fan 2013). The crystalline nature of lignocellulose is another major hurdle in its efficient utilization. Crystallinity of the biomass material is imparted by the presence of crystalline cellulose, which, if not converted into amorphous form, is not susceptible to enzymatic hydrolysis (Bansal *et al*., 2010; Gurgel *et al*., 2012).

Cellulose and hemicellulose are two components in the plant cell wall, which, when hydrolysed, result in their lease of fermentable sugars (glucose, xylose, galactose, etc.) (Wange *et al.*, 2014). Enzyme producing microbes depend on glucose as their main carbon source. High cellulose content is therefore desired in a potential lignocellulosic substrate. In such cases, the presence of hemicellulose can interfere with cellulose breakdown, glucose formation and uptake (Demartini *et al.*, 2013). However, the production of different enzymes is susceptible to the presence of specific substrates in the media composition. For example, the presence of xylans and arabinoxylans in substrates has been reported to boost xylanase production (Motta *et al*., 2013). Also, uncommon sugars, such as fructooligosaccharides and inulin based fructans, facilitate better inulin production by several microbial species (Chesini *et al.*, 2013). The availability of the lignocellulosic substrate and its cost are also important aspects in the choice of substrate.

2.8 Pre-treatment of lignocellulose.

The utilization of cellulose in biomass as a carbon source involves the adsorption of enzymes on the substrate surface, the synergistic effects of other protein components on hydrolysis, and the release of hydrolyse product into the bulk liquid. Along with lignin and hemicellulose, pectin and acetyl groups are some of the compositional factors that influence lignocellulose degradation (Xiao and Anderson 2013). The removal of pectin by the enzymatic treatment of lignocellulose with the pectinase enzyme along with cellulolytic enzymes has a positive effect on biomass utilization (Olesen, 2013). Degree of polymerization, plant protein enzyme interaction, structural rigidity, accessible surface area for enzymatic degradation and porosity and the residual surface area of biomass also have a pronounced effect on substrate utilization and assimilation (Singh *et al*., 2015). Pre-treatments are necessary to address these issues by bringing in structural and compositional changes in lignocellulose. Pre-treatment strategies employ high pressure

or temperature, or a combination, for the reduction of recalcitrant components from lignocellulose. Sophisticated disruption techniques, such as ultra-sonication, microwave exposure, treatment with corrosive liquids, such as acids and alkali, and enzymatic hydrolysis can also be part of effective pre-treatment measures (Mai *et al.*, 2014). However, pre-treatment techniques involving heat and acid hydrolysis give rise to enzyme and microbial growth inhibitors that can affect the production process (Bellid *et al*., 2011; Ravidran *et al*., 2016).

2.9 Choice of Microorganism for Enzyme Production.

Considerable research has been conducted to reveal several microorganisms belonging to the fungi, yeast, bacteria and actinomycetes categories. Screening of microorganisms is one of the most efficient means of finding new enzymes viable to the industry. This is particularly true in the case of thermophilic microorganisms that are isolated from exotic locations and subsequent extraction of enzymes. One of the greatest advantages of employing thermophilic microorganisms for enzyme production is reducing the risk of contamination due to bioprocessing operations being conducted at higher temperatures. Furthermore, elevated temperatures also result in lesser viscosity and greater solubility of substrates, subsequently resulting in increased product yields due to favourable displacement of the equilibrium in endothermic reactions (Vandenberge *et al*., 2016).

Enzymes of microbial origin employed in the industry are commercially available as enzyme preparations. These preparations not only contain the desired enzyme, but also other metabolites of the production strain, along with additional preservatives and stabilizers that are food grade and comply with applicable regulatory standards. While evaluating the safety of an enzyme, the safety of the production strain remains the primary consideration. Toxigenic potential is defined as the ability of a microorganism to produce chemicals (toxins) that can cause food poisoning (Arslan *et al.*, 2014). Strains that are

meticulously characterized to be non-pathogenic and non-toxigenic, particularly those with a history of being safe, are reasonable choices for the production of industrial enzymes (Pariza *et al.*, 2001). A majority of the industrial important enzymes has been derived from a rather small group of bacterial and fungal strains, primarily Bacillus subtilis, Bacillus licheniformis, *Aspergillus niger* and *Aspergillus oryzea*. These microbes have historically been used for the commercial production of various metabolites, leading to a thorough understanding of their characteristics and metabolic reactions, and have been documented to be efficient for industrial scale production. They can also be genetically manipulated easily and are known for their ability to overexpress proteins of interest in fermentation media. These features make these microorganisms extremely desirables host for a variety of heterologous enzymes. Furthermore, genetic engineering has enabled several microorganisms with no history of use in the industrial production of native enzymes, such as *Escherichia coliK-12, Fusarium venenatum* and *Pseudomonas fluorescens* to be successfully utilized as hosts for expression of industrially important enzymes (Olenpska-Beer *et al.*, 2006). Wild-type strains produce a variety of extracellular enzymes, which may naturally produce enzymes that have industrial importance.

A common method exploited to find these microbes is bioprospecting. Microbes are collected from specific environmental niches and their ability to hydrolyse specific substrates is investigated. Subsequently, the best candidate is selected based on screening for the production of an enzyme of interest. Another method is analysing the genetic composition using metagenomics tools. Probes and group-specific primers are employed to find new enzymes. The major drawback with this method is qualitative: the metabolic potential cannot be measured since the isolation and culturing of the microorganism is not performed. Comparative genome analysis of microbial strains assists in the screening

of prospective microbes in a short time. This facilitates the evaluation of the proteome of the microorganism (Youssef *et al.*, 2013)

The concept of metabolic engineering was introduced by Bailey in 1991 and relates to 'the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology (Nevoigt. 2008). A metabolic pathway (or pathways) associated with the production a desired chemical compound by fermentation is/are over-expressed in a cell by genetic engineering employing classical mutagenesis and selection and/or recombinant techniques. Cells are "evolved" in the laboratory to make them tolerant to high product concentrations by removing the normal genetic and biochemical regulation of the genes and enzymes associated with the pathway by genetic manipulation. Finally, a robust fermentation process is developed that allows mass production of the desired compound (Keasling, 2012). While traditional metabolic pathway engineering approaches have been successful in producing engineered microbes, novel techniques need to be called upon to develop microorganism and processes that are cost-competitive with existing large-scale, low-cost chemical manufacturing processes, to produce the same compounds in high-volume and at low-cost. Techniques, like genome sequencing, and new fields of study, like bioinformatics, systems biology and metabolomics, have greatly helped researchers to embellish the competences of metabolic engineering over the past decades to deliver new, highly engineered organisms that are capable of high throughput performance using renewable resources as substrates, lowering the cost of production even further (Agrimi *et al.*, 2012; Buschke *et al.*, 2013).

2.10 Isolation, Purification and Recovery of Enzymes

Once the production stage ends, enzymes are formed in the media in the form of crude extract. Although the crude extract exhibits the desired enzyme activity, low rates of reaction and the presence of impurities, such as media components used for microbial cultivation, metabolites and toxins released by the microorganisms, prevent its use as a commercial entity. Therefore, isolation and purification are the last steps of any enzyme production process. Purifying the enzyme increases its specific activity and removes any unwanted factors from the finished product. Protein purification techniques have been an area of research for many decades owing to the finalized product requires strenuous measures, which start with cell disruption (depending on the source), extraction, fractionation and the final product (Oyeleke *et al*., 2010).

2.11 Source of Enzyme

All biological systems are a source of enzymes and there is bound to be considerable variation in the concentration of the concerned enzyme, its activity, stability, availability and presence of inhibitory factors. Traditional animal, plant and microbial sources have given way to genetically-engineered organisms with the introduction of recombinant DNA technology. Eukaryotic proteins cloned and expressed in bacterial hosts, such as Escherichia coli and Bacillus subtilis, may be located in different locations within the cell (cytoplasm, periplasmic space) or may be truly extracellular. Enzymes accumulating in the periplasm may be released into the fermentation media by changing the culture conditions (Fakruddin *et al.,* 2013). However, recombination techniques allow the gene of interest to be equipped with an “affinity tag”, such as His-tag, which will help in the purification of the enzyme. This tag can later be removed by using highly-specific proteolytic enzymes (Young *et al.*, 2012).

2.12 Isolation of Enzymes

Isolating the protein from a solid source is a compromise between quality and quantity. The best isolation measure should facilitate the release of the enzyme of interest while leaving behind tough contaminants (nucleic acids, bacteria and viruses). Care should also be taken whereby the protein extracted is not degraded/denatured during the process. Homogenization is the most popular method for protein extraction from the cellular environment. Another method used for cell disruption is ultra-sonication. Ultrasonication facilitates the disruption of cells and exposes internal proteins to the growth medium. Ultrasonication techniques use high-frequency waves to cause cavitation on the microbial cell wall, thereby destroying it. However, prolonged exposure to ultrasound can denature the protein released upon cell lysis. Therefore, sonication cycles should be optimized in a manner where only cell disruption is achieved while the protein of interest is left intact (Liu *et al*., 2014). The cell disruption technique is usually followed by centrifugation or filtration aimed at the clarification of the extract prior to column chromatography. Characteristics of the isolation medium are determined by the conditions that are necessary for the stability of the protein released. The main factors that govern the preparation of the isolation medium are pH, detergents, reducing agents, chelators or metal ions, proteolytic inhibitors and bacterial contamination. The pH is usually chosen to be the value in which the enzyme exhibits maximum activity. However, this may not always be the case. In the case of trypsin, maximum activity is attained at pH 8–9, while the enzyme is most stable at pH 3 (Ham *et al* 2013). Detergents are used to relieve the enzyme of bonds to membranes by hydrophobic interactions. Several of the detergents used for isolation (such as Triton X-100 and Sodium dodecyl sulphate, SDS) do not denature the globular proteins or affect their catalytic activity. The use of detergents is usually limited to the isolation medium. Detergents, being amphipathic molecules,

aggregate to form ‘micelles’ at the critical micelle concentration (CMC). This can interfere with the purification process during column chromatography. Therefore, the concentration of detergent used during isolation must be lesser than CMC.

Many enzymes have exposed thiol groups which can oxidize when the protein is released from the cytosol to the growth medium during isolation. This is prevented by the addition of reducing agents such as mercaptoethanol, dithiothreitol (DTT), or ascorbic acid. The concentration of these reducing agents can normally be kept as low as 10–25 mM, while keeping the internal disulphide bonds intact (Mathieso and Thomas, 2013). Metal ions, proteases and bacterial contamination are three problems faced during enzyme isolation. The presence of metal ions leads to the enhanced oxidation of thiol groups and may form complexes with specific groups, which can cause problems. Heavy metals can be removed by treatment with chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetra acetic acid (EGTA). EDTA is a buffer whose addition can change the pH of the buffer. Therefore, care should be taken to adjust the pH post addition of EDTA (Campden *et al*., 2015).

Proteases that are naturally present in the cell lysate pose a serious threat to the protein of interest. The simplest way to prevent the proteolytic action is by adding protease inhibitors. This can be a bit of a problem in large-scale processes, since proteolytic inhibitors are expensive. Nonetheless, other pragmatic measures to tackle this problem include the adsorption of proteases onto hydrophobic adsorbents and adjusting the pH to a value where proteases are rendered ineffective. Researchers have recently established that the addition of doxycycline indirectly inhibits proteolytic activity of tryptic peptidases (Kanada *et al*., 2012). The key to avoiding bacterial growth in enzyme preparation is following measures to ensure sterility. Some buffers, such as phosphate and acetate, among others, are more prone to supporting the growth of bacteria at neutral

pH. The addition of antimicrobial agents to buffers whenever feasible is also a tactic to prevent contamination (Yang *et al*., 2013).

2.14 Precipitation, Centrifugation and Ultrafiltration

Clarification of the cell lysate to remove all cell debris and other particles is achieved by centrifugation at high speeds. This is achievable at laboratory scale, where crude cell preparations can be attained at high speeds of 40,000 g to 500,000 g under refrigerated conditions. In order to complement centrifugation, microfiltration and ultrafiltration have emerged as advancements in filtration techniques to remove contaminating insoluble. Ultrafiltration is a protein separation technique that can separate protein fractions according to their molecular sizes (ranging from >1m to 300 kDa). This method is fast, reliable and inexpensive, while separating salts and other small molecules from protein fractions of larger molecular weight (He *et al*., 2013). Precipitation of a desired protein is achieved by the addition of salts, organic solvents, or polymers, or by varying the pH or temperature of the solution. Antichlorotic salts are the most widely used salting out agents. They bind to water molecules, thereby increasing the hydrophobic effect, leading to the aggregation of protein molecules. The most common antichlorotic salt used for protein precipitation is ammonium sulphate (Mirica *et al*., 2013). Organic solvents, such as ethanol and acetone, are also used for protein precipitation. Organic polymers function the same way as organic solvents. Polyethylene glycol (PEG) is the most widely used organic polymer for this purpose. The solution, being viscous at high concentrations, can be diluted with buffer. PEG, being uncharged, can be used directly for ion exchange chromatography, to separate proteins (Sim *et al*., 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples Collection Site

Agricultural waste (Yam peels and cassava peels) were collected from a waste bin at Kpakugun, Mobil and Bosso market Minna, Niger state, and transported to Microbiology Laboratory of Federal University of Technology Minna, for further analysis.

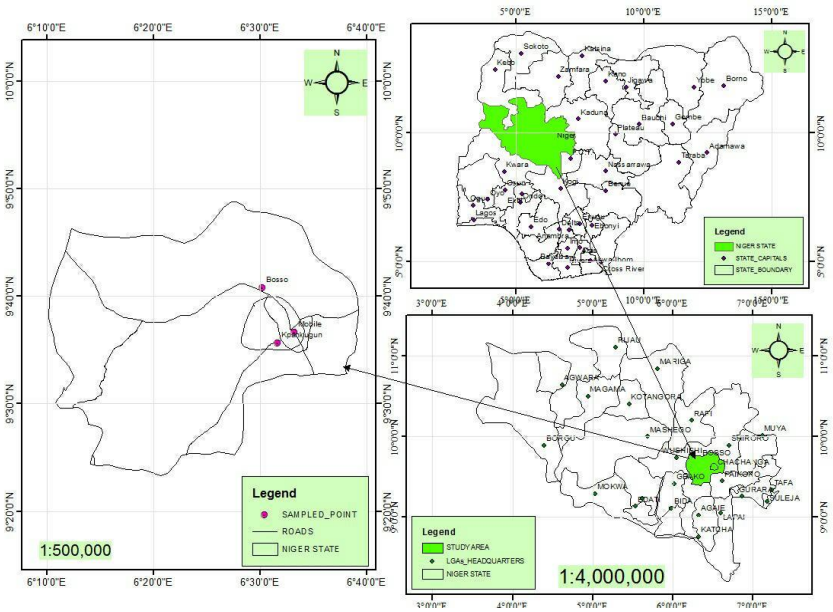


Figure 3.1 The map show the samples collection site in Minna, Niger State.

3.2 Pre-treatment of Substrates.

200 kg of yam peels and cassava peels were dried individually at room temperature fourteen days at 25 °C to reduce the moisture content and to make them more susceptible for crushing. The crushed substrates were sieves individually to get powder form. The sieved substrates were store in plastics bags at room temperature until analysis was done (Barnett *et al*., 2018)

3.3 Media Use

Nutrient agar and deman Rogosa Sharp agar (MRSA) medium were used for short term preservation of bacterial cultures and for subculture purposes. Potato destrose agar (PDA) was used for short term preservation of fungi cultures and for subcultures purpose. Luria Bertani (LB) broth was used for the growth of bacterial culture before DNA extraction. Starch agar medium, carboxylmethyl cellulose (CMC) and pectin agar were used to screen and produce amylase, cellulase and pectinase producing bacteria respectively. A selective media was used during the fermentation of bacteria and fungi for amylase, cellulase and pectinase production in a shake flask (Barnett *et al*., 2018).

3.4 Isolation of *Zymomonas mobilis* from spoilt orange

The isolation of Zymomonas *mobilis* was carried out according to the method described by Obire (2005). The spoit orange samples were washed and then squeezed to obtain the juice. The juice was serially diluted from tube 1 (10^{-1}) to tube 5 (10^{-5}). Then 0.1 ml aliquot from the 10^{-5} tubes was plated on nutrient agar medium using spread plate techniques. Each medium was treated with actidione (cycloheximide) to inhibit yeast growth. The plates were incubated in an anaerobic jar in which Gas pak sachet was placed to exhaust the oxygen in the jar and incubated at 37 °C for 2 days. Colonies suspected to be those of *Zymomonas mobilis* were characterized on the basis of their cultural and morphological

characteristics. The isolates were purified by streaking on freshly prepared media and incubated for 2 days at 37 ℃ in an anaerobic jar. The ability of *Zymomonas mobilis* to ferment various carbohydrates using glucose, fructose, sucrose, maltose, lactose and arabinose was determined by growing the isolates in liquid standard medium (Yeast glucose broth pH 6.8) containing 1 % (w/v) of the particular carbohydrate. Durham tubes were inverted into culture tubes for gas collection. The tubes were incubated at 37 ℃ for 24 hours. Uninoculated broths were used as Control (Yaser *et al.*, 2013).

3.5 Isolation of *Lactobacillus bulgaricus* from Fermented Milk Sample

1ml of a fresh milk sample was mixed with 9 ml sterile physiological saline (0.85 % w/v, NaCl) to make an initial dilution. Serial dilutions were made for each sample and then 1mL of the appropriate dilution was mixed with the melted MRS agar to enumerate the total *LAB* with the pour plate method. Cycloheximide at a concentration of 0.01 % (v/v) was added to the MRS plates in order to prevent the growth of fungi. Meanwhile, the appropriate dilution was evenly spread onto the MRS (Oxoid Ltd., UK) plates before being incubated under anaerobic condition at 30 ℃ for 48-72 h. Colonies with distinct morphological differences (based on colour, shape, size, rough or smooth surface) were selected and then purified using another agar plate of the same culture medium. The catalase activity and Gram reaction of all the isolates were assessed. Gram-positive, catalase-negative and non-motile microorganisms were preserved in 10 % (w/v) skim milk containing 0.1 % (w/v) sodium glutamate and stored at 80℃

3.6 Isolation of *Saccharomyces cereviciae* from Fresh Palm Wine

palm wine samples were collected early in the morning and was shaken vigorously by hand within 1-2 hrs of collection and plated on triplicate potato dextrose agar (PDA) plates containing 0.05 mg/ml of chloramphenicol and gentamicin to inhibit bacterial

growth. Plates were incubated at room temperature (25 ± 2 °C) for a maximum of 5 days. Representative colonies (confirmed to be yeasts by microscopy) were purified by restreaking on PDA plates and isolates were stored on slopes of the same medium. Isolates were sub cultured as needed for the various experiments conducted in this work. Yeast isolates were identified by standard morphological and physiological methods and identification (Barnett *et al*., 2018).

3.7 Isolation and Identification of *Aspergillus niger*

Isolates of *Aspergillus niger* was obtained from soil. The soil samples were seeded in sterile distilled water, serially diluted and inoculated on Potato Dextrose Agar (PDA). Fungal growth suspected to be *Aspergillus niger* based on macroscopic observation (carbon black or dark brown conidia) were further subcultured on fresh PDA plates. Those that exhibited characteristic *A. niger* growth (initially white, quickly turning black) were subjected to microscopic observation with reference to the manuals of Barnett and Hunter (1972) and Mycology online of Ellis, (2006). For the microscopic identification a drop of lactophenol blue was placed on a clean slide, a bit of the fungal growth was removed and placed inside the drop of lactophenol blue, covered with a cover slip and observed under the microscope using x10 and x40 objective lens.

3.8 Identification of Bacterial Strain

The selected bacterial isolate was identified via the following methods.

3.8.1 Microscopic observation of the isolated bacteria and fungi

The bacterial isolate underwent microscopic observation after different staining methods.

3.8.2 Gram staining

A bacterial smear was prepared in on a clean glass slide. It was allowed to air dry and heat fixed by passing the slide through the flame of a Bunsen burner very swiftly. At first it was stained with crystal violet for 1 minute and washed. Gram's iodine was added and washed off. After that it was decolorized with 95% ethyl alcohol. The smear was stained again with safranin for 45 seconds and washed. The smear was then observed under the microscope with 1000X magnification (Yaser *et al.*, 2013).

3.8.3 Spore staining

A bacterial smear was made in sterile conditions. The smear was air dried and heat fixed. The slide was flooded with malachite green and placed on top of a beaker containing boiling water and left for 3 minutes. After washing, it was stained with safranin for 30 seconds and washed. The smear was air dried and observed under the microscope (Yaser *et al.*, 2013).

3.8.4 Cultural characterization

The colony morphology of the bacteria was determined by observing the size, form, colour, pigmentation, elevation, margin, texture and opacity of the colony. In this experiment, the bacterial cultures where freshly grown in nutrient agar media by three-way streaking method and their colony morphology was observed. Photographic images were taken for visual preservation. (Yin *et al*., 2012)

3.8.5 Biochemical characterization

In this experiment, different biochemical tests were carried out for the bacterial isolate. The following biochemical tests were performed using 24 hours fresh bacterial cultures

grown on nutrient agar media. The media were autoclaved at 121°C for 15 minutes at 15 psi, wherever required (Barnet *et al*., 2018).

3.8.6 Catalase test

A clean glass slide was marked with the name of the bacterial isolate. To the opposite side, a drop of hydrogen peroxide was added. To the drop, a loopful of bacterial isolate was mixed. Any immediate formation of bubbles was observed (Oyeleke *et al*., 2012).

3.8.7 Oxidase test

A clean filter paper was placed on a Petri dish. A drop of oxidase reagent was added on the filter paper. Using an inoculating loop, a small amount of bacterial culture was streaked on the drop of oxidase reagent. Any formation of dark purple colour of the reagent was observe. (Yaser *et al*., 2013)

3.8.8 Protein hydrolysis:

The bacteria were inoculated in skimmed milk and agar containing medium. If a clear zone is observed after incubation at 370C, then it is confirmed that the bacteria are able to hydrolysed protein (Zeng *et al*., 2014).

3.8.9 Citrate utilization test:

Citrate utilization test was carried out by inoculating the bacteria in Simmon's Citrate agar slant. Formation of blue colour of the media indicates the positive result (Oyeleke *et al* 2012).

3.8.10 Urease production test:

Slant containing urea agar was inoculated with the bacteria. Urea agar is prepared by autoclaving a media containing 1 gm of peptone, 5gm of NaCl, 2gm of Di potassium

hydrogen phosphate, and 20 gm of agar in 1000 ml of water, at pH-6.8. Then 1gm of glucose and 6.0ml of 0.2 % phenol red solution were added to the media after bringing it at 50 °C. Then steamed the media for 1hr and cooled it to 50 °C. 20 % urea solution was added to it after sterilization by filtration. Final media was used for the slant. If the media converted to red from yellow, the result was taken as positive (Oyeleke *et al*., 2012).

3.8.11 Methyl red and voges proskauer (V.P) test

Glucose phosphate broth at pH 7 was sterilized for 20min. the tube was inoculated with the test organism. After incubation at 37 °C. for a week the culture broth was treated with a drop of 0.02 % methyl red in 50 % alcohol. For VP test, 0.5 ml of α-napthol solution in 95 % alcohol and 0.5ml of 16% KOH were added to each tube containing 2 ml of the liquid culture. Development of red colour in broth of both cases indicated positivity of reaction (Yaser *et al* 2014).

3.8.12 Indole production

The test cultures were grown in sterile 1 % tryptone broth for 5days and then few drops of Kovac"s reagent (5 gm of β-dimethylamino benzaldehyde in a mixture of 75 mil amyl alcohol and 25 ml H_2SO_4) was added and shaken. A rose –pink colour indicates the formation of indole. (Oyeleke *et al*., 2012)

3.9 Molecular Identification of Isolates.

3.9.1 Deoxyribonucleic acid (DNA) extraction

Single bacterial colonies and fungi mycelia were moved from nutrient agar and potato dextroxe agar respectively into 1.5 ml medium that is liquid and cultured on an orbital shaker for 48 h 72 h respectively under a temperature of 28 ºC. following this was, the centrifugation of the cultures for 5 minutes at a revolution of 4600rpm. After that the

granules were then placed into 520 litters of the buffer TE (10 m MTris-HCl, 1 ml EDTA, pH 8.0). After that, 15 microliters containing 20% sodium dodecyl sulfate (SDS) with 3 litres Proteinase enzyme K (20 mg/ml) was incorporated. The combination had to be incubated at 37°C for an 1 h before being vortexed with 100L of NaCl 5 M and 80L of a 10% CTAB solution in NaCl 0.7 M. The suspension was held on ice for 15 minutes after being incubated at 65 °C for 10 minutes. A 24:1 ratio of chloroform to iso amyl alcohol was added, then incubation for 5 minutes on ice and 7200g centrifugation for 20 minutes. After that, the phase that is aqueous was moved into a fresh tube incorporating isopropanol in the ratio 1:0.6, and DNA was precipitated for 16 hours at –20 °C. the harvest of DNA was through centrifugation at 13000 g for 10 minutes, then purified using 500L of 70 % ethanol, dried under air for 3 hours under room temperature, and lastly dissolved into 50 L of buffer (TE) (Trindade *et al.*, 2007).

3.9.2 PCR analysis

ITS universal primer set which flank the ITS1, 5.8S and ITS2 region was used for pcr. PCR reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each ITS 1: 5’ TCC GTA GGT GAA CCT GCG G 3’and - ITS 4: 5’ TCC TCC GCT TAT TGA TAT GC 3’’. primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl was added to sterile distilled water 8µl DNA template. PCR carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) (Trindade *et al*., 2007).

3.9.3 Integrity

The integrity of the amplified 1.5 Mb gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliters (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel. (Trindade *et al*., 2007)

3.9.4 Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95 % ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70 % ethanol and mix then centrifuge for 15 min at 7500 g and 4 °C. Again, remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15

min. then resuspend with 20 µl of sterile distilled water and kept in -20 ℃ prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific (Trindade *et al*., 2007)

3.9.5 Sequencing of the amplified products

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software was used for all genetic analysis (Trindade *et al*., 2007).

3.10 Screening of Amylase Producing Strain

All the microorganisms used in this research work were tested for amylase production by starch hydrolysis. Twenty-four (24) hours old isolated bacteria culture and 5 days old fungi culture were transferred into different starch agar medium (pepton- 0.5g, beef extract-0.15g, yeast extract-0.15g, NaCl-0.5g, starch-1g, and distilled water-100ml) and the plates were incubated at 37 °C for 48 hrs for bacteria and 5 days for fungi. After 48 hours and 5 days of incubation the culture plates were flooded with iodine which revealed a clear zone of inhibition on the agar plate (Palaniyappan *et al*., 2009).

3.11 Screening of Cellulase Producing Strain

A loopful of grown culture of isolated colonies were inoculated on deman Rogossa sharp agar (MRS) and sabouraud dextrose agar (SDA) amended with 0.1 % Carboxymethyl cellulose (CMC). The SDA plates were incubated for 5 days at 25 ºC for fungi isolates then observe for growth while MRS Agar plate were incubated for 24 hours for bacteria isolate and observed for growth. Following incubation, the plates showed zone of clearance around the line of growth (Singh *et al*., 1988).

3.12 Screening of Pectinase Producing Strain

Pectin, (1gm) was added into sabouraud dextrose agar and deman Rogossa sharp agar respectively. This media was sterilized and distributed aseptically into Petri dishes, each of the isolated microorganism were inoculated unto the plates. The plates were observed for a zone of clearance after 2 days for bacteria and 5 days for fungi as described by Palaniyappan *et al*. (2009)

3.13 Inoculum Preparation for Amylase Production

The bacterial and the fungi isolates were streaked on separate fresh nutrient and potatoe destroxe agar media respectively. The plates were incubated at 37 °C for 24 hours. A loop full of the freshly grown colony was inoculated in separate screw cap test tubes containing a liquid media (Yaser *et al.,* 2013) selective for amylase producing bacteria and fungi. The media were prepared using the following g/l 5g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H2O$, 3 g/L KH_2PO_4 and 0.25 g/L $CaCl_2.2H2O$ (Yaser *et al.,* 2013). The test tubes were incubated at 37°C for 24 hours to allow bacterial growth.

3.14 Inoculum Preparation for Cellulase Production

The inoculum was prepared by growing the organisms in 250 ml Erlenmeyer flask with 100 ml of Czapek-Dox broth containing g/l sucrose, 30; sodium nitrate, 3; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, trace; agar, 15; and yeast 7. The medium was inoculated from the Czapek-Dox agar slants and incubated at 30 ºC for five days in a shaker (200 rpm) before it was used for the fermentation process (Oyeleke *et al.,* 2011).

3.15 Inoculum Preparation for Pectinase Production

The bacterial and the fungi isolates were streaked on separate fresh nutrient and potatoe destroxe agar media respectively. The plates were incubated at 37 °C for 24 hours for bacteria and 25°C for 5 days for fungi isolates. A loop full of the freshly grown colony was inoculated in separate screw cap test tubes containing a liquid media (Yaser *et al.*, 2013) selective for amylase producing bacteria and fungi. The media were prepared using the following g/l: 5g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)2SO_4$, 0.$_2$ g/L $MgSO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4 and 0.25 g/L $CaCl_2.2H_2O$, 1g/l of pectin (Yaser *et al.*, 2013). The test tubes were incubated at 37 °C for 24 hours to allow bacterial growth.

3.16 Fermentation Medium for Amylase Production

Fermentation was carried out using modified method of ((Khan & Yadav, 2011). (5g) each of grounded yam peels and cassava peels were dispensed into different 250ml conical flasks and 85ml of minimal salt medium (MS) which contained the following in g/l (8 KCl 0.8 NaCl, 2.0 Na_2HPO_4, 0.1 $CaCl_2$, 0.1 $FeSO_4$, 8.0 fructose, 2.0 NH_4Cl) was added. The conical flasks were autoclave, cooled at room temperature, inoculated with 1ml of overnight bacteria culture and 5 days of fungi culture at a separate medium and incubate at 37 °C. and 25 °C for 7 days respectively

3.17 Fermentation Medium for Cellulase

Each of the test isolate were placed in a separate basal medium used for cellulase production just like the modified medium of Deacon (1985) containing (in g/l), 5g each of yam peels and cassava peels, yeast extract, 2.0; $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; and $FeCl_3$, 0.001. Carboxymethyl cellulose (CMC) was added at 1% concentration. The culture was grown for 7 days at 25 ºC. And 37 °C. For 7 days respectively for fungi and bacteria culture

3.18 Fermentation Medium for Pectinase Production

A basic liquid medium showing the following composition (g/l): Cassava waste 5, Sucrose 10, pectin 2, KNO_3 0.6, KH_2PO_4 0.1, $MgSO_4$ 0.25, $CaCl_2$ 0.1, $NaNO_3$ 2, K_2HPO_3 0.5, KCl 0.5 and yeast extract1 used for the production of pectinase (Reda *et al.*, 2008). Erlenmeyer flasks containing 100ml of basal medium amended with 3% cassava waste were inoculated with 1ml of overnight grown bacterial culture and 5days fungi culture and incubated for 7 days at 37 °C. Under agitation (125rpm) (Khan and Yadav, 2011).

3.19 Separation of Crude Enzyme from Culture Media

After incubation of amylase, cellulase and pectinase, enough culture fluid was collected. The culture fluid was then centrifuged at 6000 rpm for 10 minutes. The supernatant obtained was the crude enzyme. It was stored at 4°C for further use (Oyeleke *et al*., 2009).

3.20 Pectinase Enzyme Assay

Pectinase assay was done according to the method of Mandels (1985). Half milliliter of 1 % pectin in 0.1M citrate buffer (pH 5.8) was placed in a test tube and 0.5 ml of culture filtrate was added. The reaction mixture was incubated at 50 °C for 30 min and the reaction terminated by adding 1.5 ml 3,5-dinitrosalisylic acid (DNSA) reagent. The tubes were heated at 100 °C in a boiling water bath for 15 min and then cooled at room temperature. The absorbance was read at 575 nm. Enzyme activity is expressed as mmol glucose released per min-1ml-1 of culture filtrate as enzyme solution. Culture filtrate was obtained by filtration through Whatman No. 1 filter paper and the culture filtrate served as the enzyme solution (Singh *et al.,* 1988; El-Naghy *et al.,* 1991) and One unit (U) of pectinolytic activity was defined as the amount of enzyme that catalyzes the formation of 1µmol galacturonic acid under the assay conditions (Minjares-Carranco *et al.*, 1997).

3.21 Cellulase Enzyme Assay

Cellulase assay was done according to the method of Mandels (1985). One milliliter of 1% CMC in 0.1M citrate buffer (pH 5.5) was placed in a test tube and 1ml of culture filtrate was added. The reaction mixture was incubated at 50 °C for 30 min and the reaction terminated by adding 1.5 ml 3,5-dinitrosalisylic acid (DNSA) reagent. The tubes were heated at 100 °C in a boiling water bath for 15 min and then cooled at room temperature. The absorbance was read at 540 nm. Enzyme activity is expressed as mmol glucose released per sec-1 ml-1 of culture filtrate as enzyme solution (Wang *et al*., 2014).

3.22 Optimization of production and Partial Characterization of Enzymes

3.22.1 Effect of incubation time on enzymes production

The different range of time of incubation was done by incubating the fermentation at different period of time. The fermentation was incubated and harvested at a day interval for a period of seven days for enzyme assay (Barnet *et al* 2016).

3.22.2 Effect of pH on the production of cellulase.

The optimum pH was determined by incubating 1ml appropriate crude enzyme, 1 % CMC in 1ml citrate buffer, pH 4.8 (Ani *et al.,* 2008) different pH ranging from 4-9 for 30 minutes at 5 ^{o}C. Reducing sugars thus released were estimated by the dinitrosalicylic acid reagent method (Bertrand *et al*., 2004)

3.22.3 Effect of temperature on the production of cellulase.

The optimum temperature of Cellulase activity was determined by incubating 1ml of appropriately diluted enzyme with 1ml CMC at different temperature range from 30 °C.-80 ^{o}C for 30 minutes. Reducing sugar were estimated by the dinitrosalicylic acids reagent method (Bertrand *et al.,* 2004).

3.22.4 Effect of pH on the production of amylase.

The optimum pH for enzyme activity was determined over a pH range of 4-9 on 1 % starch medium using 0.05M Na_2HPO_4 as buffer solution. The pH was determined by incubating the enzyme extract in a water bath at 70ºC and the residual enzyme activity was then measured. The pH was adjusted by the addition of 0.1N NaOH to achieve acidity and alkalinity respectively (Bertrand *et al.,* 2004).

3.22.5 Effect of temperature on the production of amylase.

The activity of amylase enzyme was examined by using soluble starch (dissolved) as substrates. A few drops of dinitro salicylic acid (NNSA) reagent were added and the absorbance of the reaction was measured on a spectrometer at a wavelength of 540nm. The temperature ranges between 30—80 ºC of the enzyme extract was determined by maintaining the enzyme extract in water bath for five minutes at 50 ºC (Yaser *et al.*, 2013).

3.22.6 Effect of pH for the production of pectinase

The optimum pH was determined by incubating the 1 ml centrifuged enzyme mixed with pectin in 1 ml citrate buffer, pH 5.8 (Bertrand *et al.*, 2004) buffer of different pH (4 – 9) for 30 minutes at room temperature (50 ºC). Reducing sugars thus released were estimated by the dinitrosalicyclic acid reagent method (Miller, 2014) and then boiled at 100 ºC for 15 minutes.

3.22.7 Effect of temperature on the production of pectinase

The optimum temperature of pectine was determined by incubating 1ml appropriately diluted enzyme with 2ml 3 % pectin in citrate buffer pH 5.8 (Bertrand *et al.*, 2004) at different temperature (30–80 ºC) for 2 hours. Reducing sugars were estimated by the dinitrosalicyclic acid reagent method (Miller, 2014).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Isolation and identification of selected microorganisms

Milk samples, Spoilt orange, soil, and palm wine samples were all used for isolation of Amylase, Cellulase, and Pectinase producing species. *Lactobacillus bulgaricus*, *Zynomonas mobilis*, *Aspergillus niger*, and *Sacharomyces cereviciea* were identified based on morphological and biochemical characteristics.

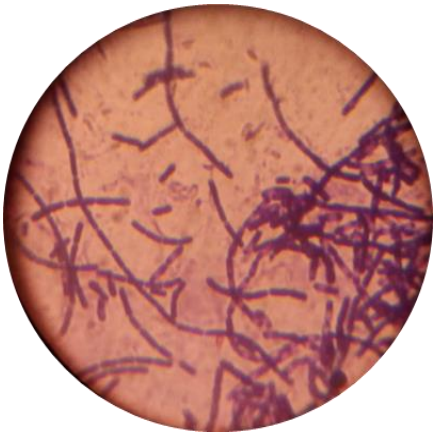


Plate I: ***Lactobacillus bulgaricus***

Plate II: ***Zymomonas mobilis***

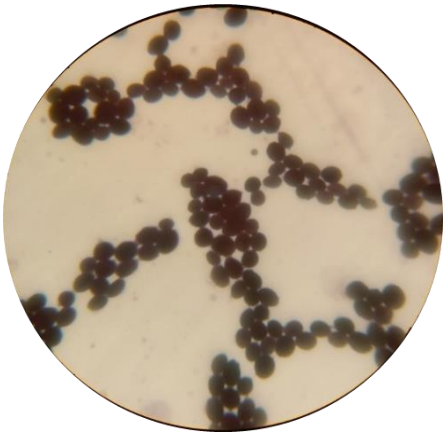


Plate III: ***Saccharomyces cerevisia*** **Plate IV:** ***Aspergillus niger***

Plate 1-4 microscopic view of bacteria and fungi used for the producción of amylase, cellulase and pectinase

Table 4.2 Morphological and biochemical characterization of isolated organisms from fresh palm wine and fresh cow milk

Gram reaction	Catalase	Oxidase	Protein hydrolysis	urease	citrate	Suspected/Organism
–	+	+	+	–	–	*Zymomonas mobilis*
+	–	–	+	–	–	*Lactobacillus bulgaricus*

Key: +: fermentation/positive; –: no fermentation/negative

4.1.2 Effect of incubation period on amylase activities using yam peels as a substrate

Figure 4.1.2 shows the incubation period of amylase activities of all the test isolates using yam peels as a substrate for an incubation period of 7 days. The highest amylase activity was obtained on the 4th day and the least on the 7 days. However, the highest enzyme activities were observed by Aspergillus *niger* (7.3×10^{-3} mg/ml/sec.) While the least was obtained by *Lactobacillus bulgaricus* (7.3×10^{-4} mg/ml/sec.). This corresponds to the day with the highest and lowest biomass activities

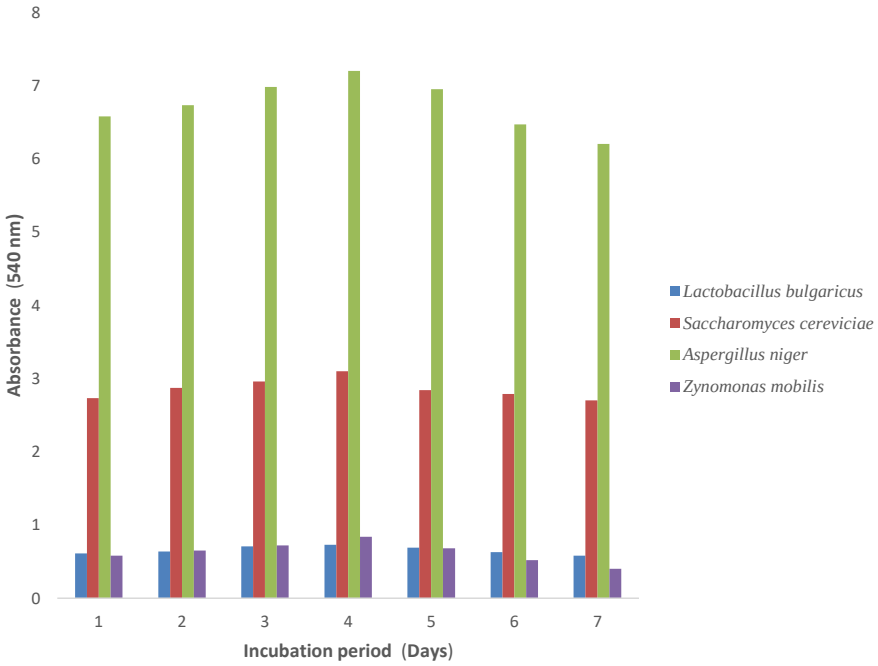
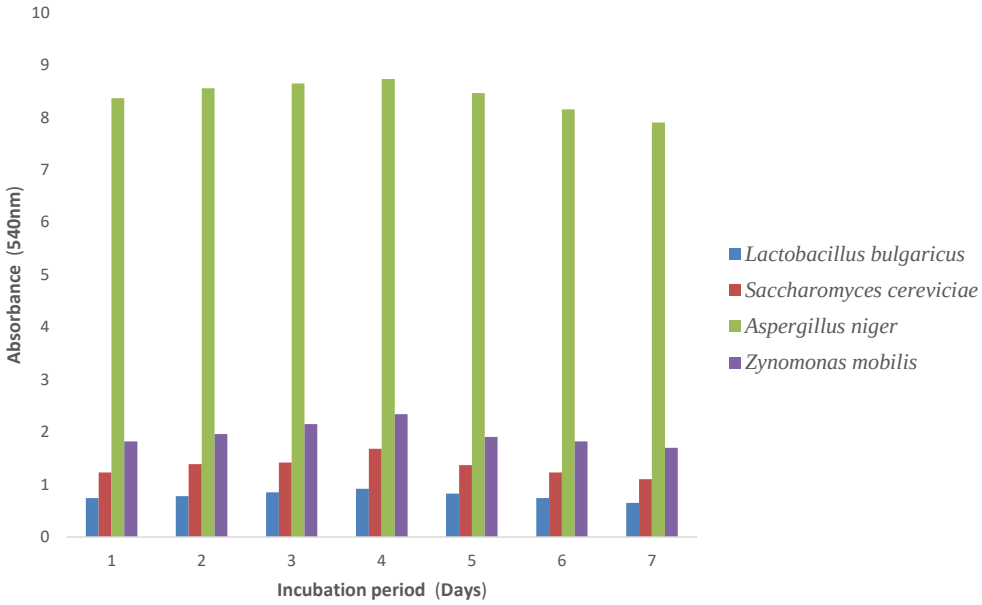


Figure 4.1 Enzyme activity of amylase at different incubation period using yam peels as a subtract

4.1.3 Effect of incubation time on amylase activity using cassava peels

Figure 4.1.3 shows the incubation period of amylase activities of all the test isolates using cassava peels as substrate for an incubation period of 7 days. The highest amylase activity was obtained on the 4th day for all the test organism and the least on the 7th day. However, the highest amylase activity was observed by *Aspergillus niger* (8.7×10^{-}



4.2 Enzyme Activity of Amylase are different incubation period

4.1.4 Effect of incubation time on cellulase activity using yam peels

Figure 4.1.4 shows the incubation period of amylase activities of all the test isolates using cassava peels as substrate for an incubation period of 7 days. The highest cellulase activity was obtained on the 4th day for all the test organism and the least on the 7th day. However, the highest cellulase activity was observed by Aspergillus niger (1.64×10-2 mg/ml/sec.) While the least cellulase activity was observed by *Lactobacillus bulgaricus* (8×10-4 mg/ml/sec.) This corresponds to the day with the highest and lowest biomass activities

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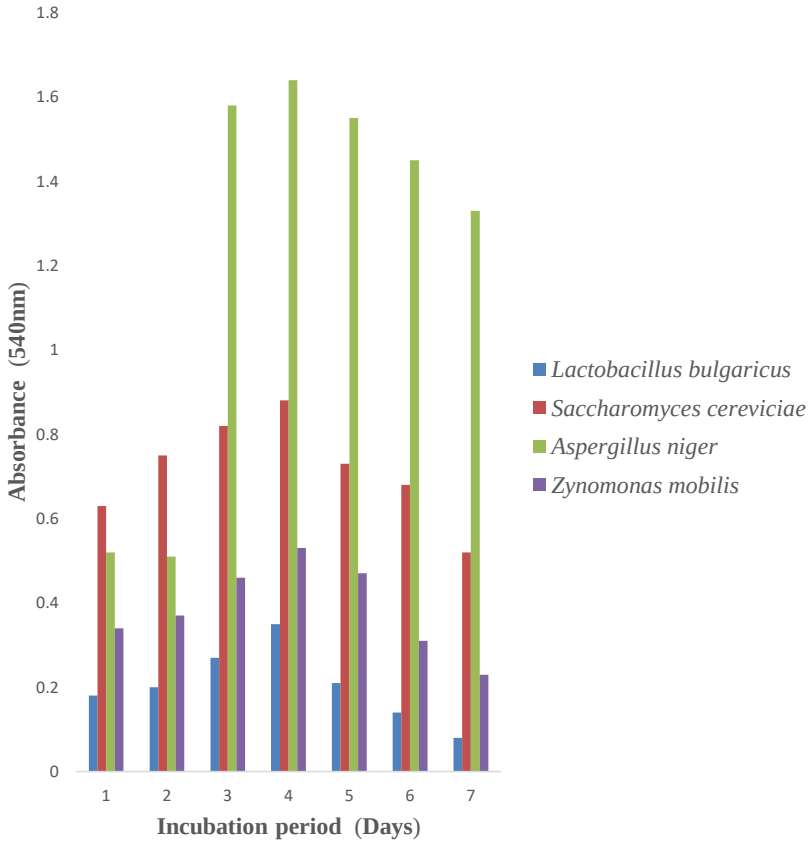

Figure 4.3 Different incubation period on a cellulase activity

4.1.5 effect of incubation time on cellulase activity using cassava peels

Figure 4.1.5 shows the incubation period of amylase activities of all the test isolates using cassava peels as substrate for an incubation period of 7 days. The highest amylase activity was obtained on the 4th day for all the test organism and the least on the 7th day. However, the highest amylase activity was observed by *Aspergillus niger* (6×10^{-2} mg/ml/sec.) While the least amylase activity was observed by *Zynomonas mobilis* (2×10^{-3} mg/ml/sec.) This corresponds to the day with the highest and lowest biomass activities

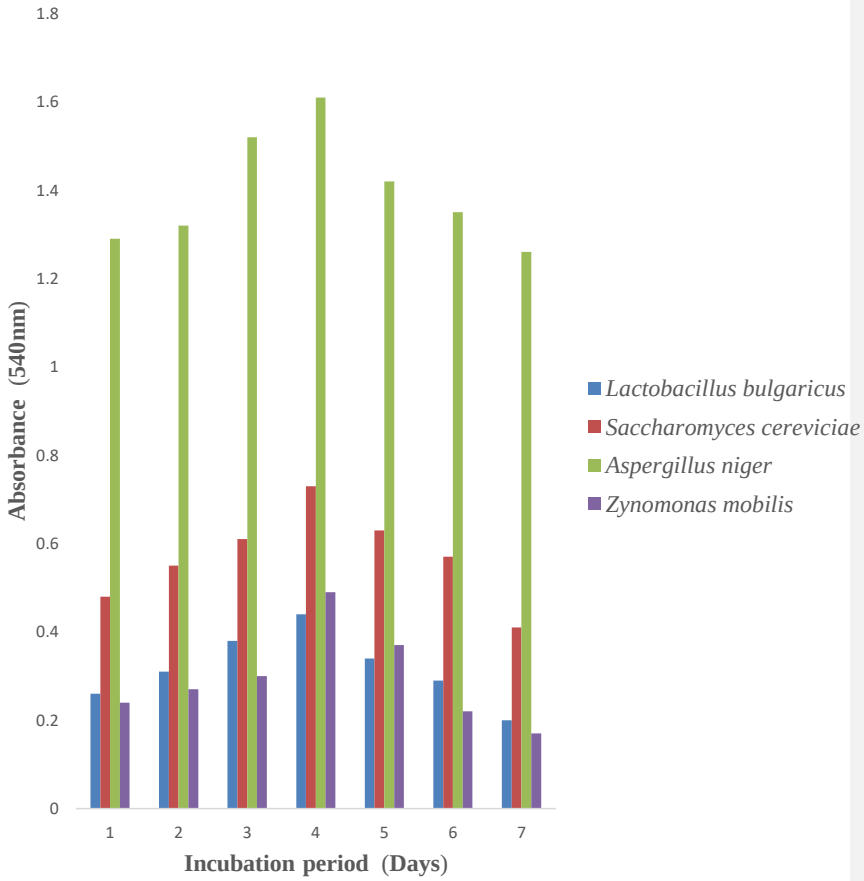

Figure 4.4 Different incubation period on a cellulase activity

4.1.6 Effect of incubation time on pectinase activity using yam peels

Figure 4.1.6 shows the incubation period of amylase activities of all the test isolates using cassava peels as substrate for an incubation period of 7 days. The highest amylase activity was obtained on the 4th day for all the test organism and the least on the 7th day. However, the highest amylase activity was observed by *Aspergillus niger* (3.74×10^{-2} mg/ml/sec.) While the least amylase activity was observed by *Lactobacillus bulgaricus* (1.6×10^{-3} mg/ml/sec.) This corresponds to the day with the highest and lowest biomass activities

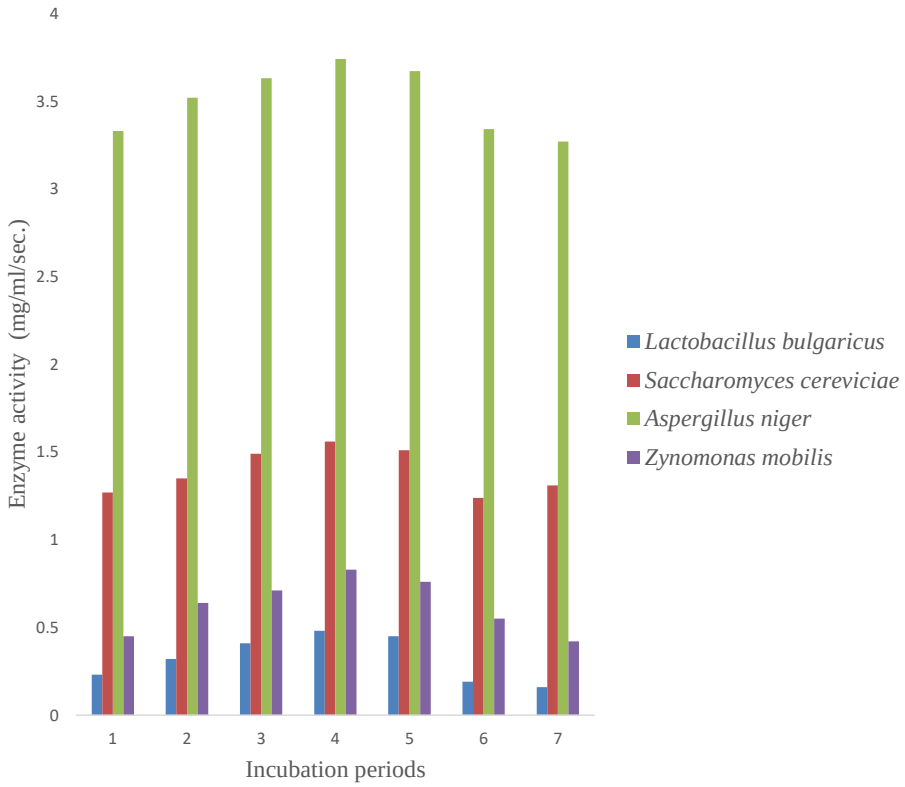

Figure 4.5 Effect of incubation period on pectinase activity using yam peels

4.1.7 Effect of incubation time on pectinase production using cassava peels

Figure 4.1.7 shows the incubation period of amylase activities of all the test isolates using cassava peels as substrate for an incubation period of 7 days. The highest amylase activity was obtained on the 4th day for all the test organism and the least on the 7thday. However, the highest amylase activity was observed by *Aspergillus niger* (3.64×10^{-2} mg/ml/sec.) While the least amylase activity was observed by *Lactobacillus bulgaricus* (3.1×10^{-3}mg/ml/sec.) This corresponds to the day with the highest and lowest biomass activity

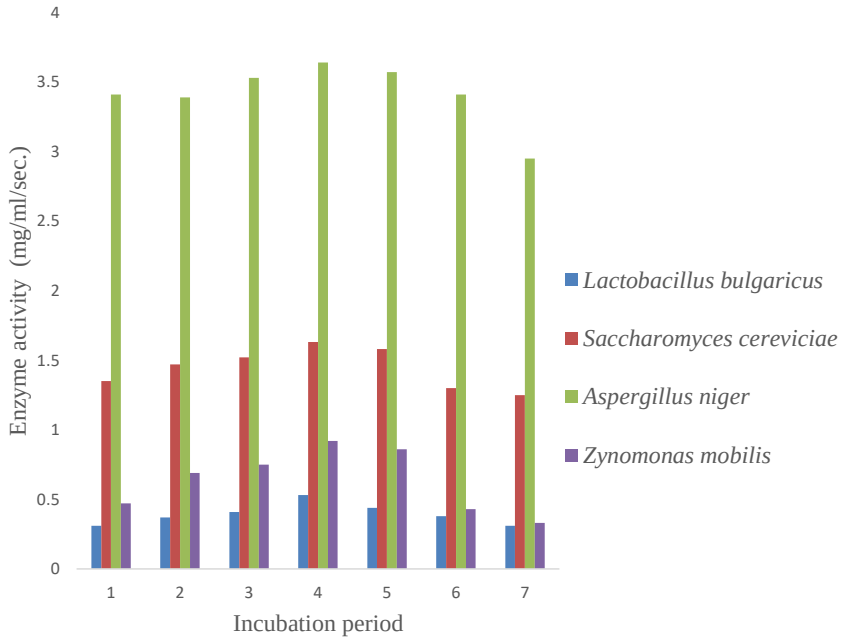

Figure 4.6 Effect of incubation period on pectinase activity using cassava peels

4.1.8 Effect of temperature on amylase activity using yam peels

Figure 4.1.8 shows the effect of temperature on amylase activities produced by all the test organisms. The optimum temperature of all the isolates was at 50° C. The highest amylase activity was observed by *Aspergillus niger* with an enzyme activity of 5.1×10^{-2} mg/ml/sec at a temperature of 50° C. and the lowest enzymatic activity of 2.3×10^{-3} mg/ml/sec was obtained at 80°C by *Lactobacillus bulgaricus*

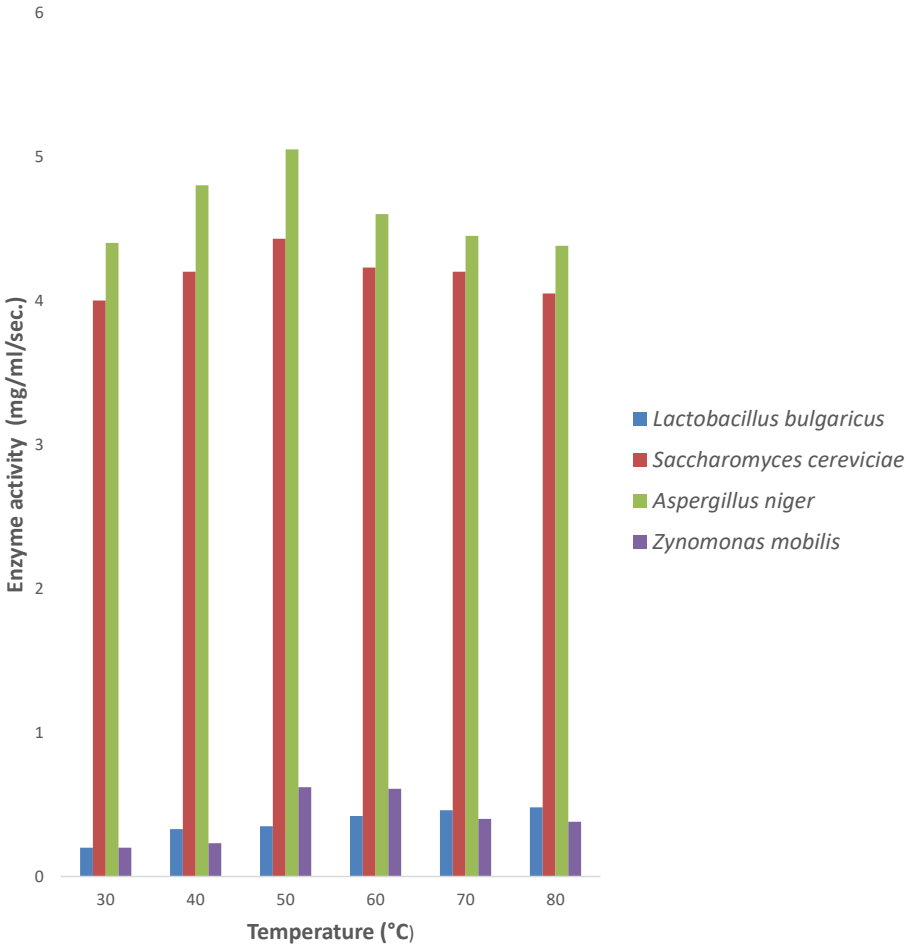

Figure 4.7 Effect of temperature on amylase activity using yam peels

4.1.9 Effect of temperature on amylase activity using cassava peels

Figure 4.1.9 shows that the effect of temperature on amylase activities produced by the test organisms. The optimum temperature of all the isolates was at 50 °C. The highest amylase activity was observed by *Aspergillus niger* with an enzyme activity of 8.5×10^{-2} mg/ml/sec at a temperature of 50°C. and the lowest enzymatic activity of 3.6×10^{-3} mg/ml/sec was obtained at 80°C by *Lactobacillus bulgaricus*

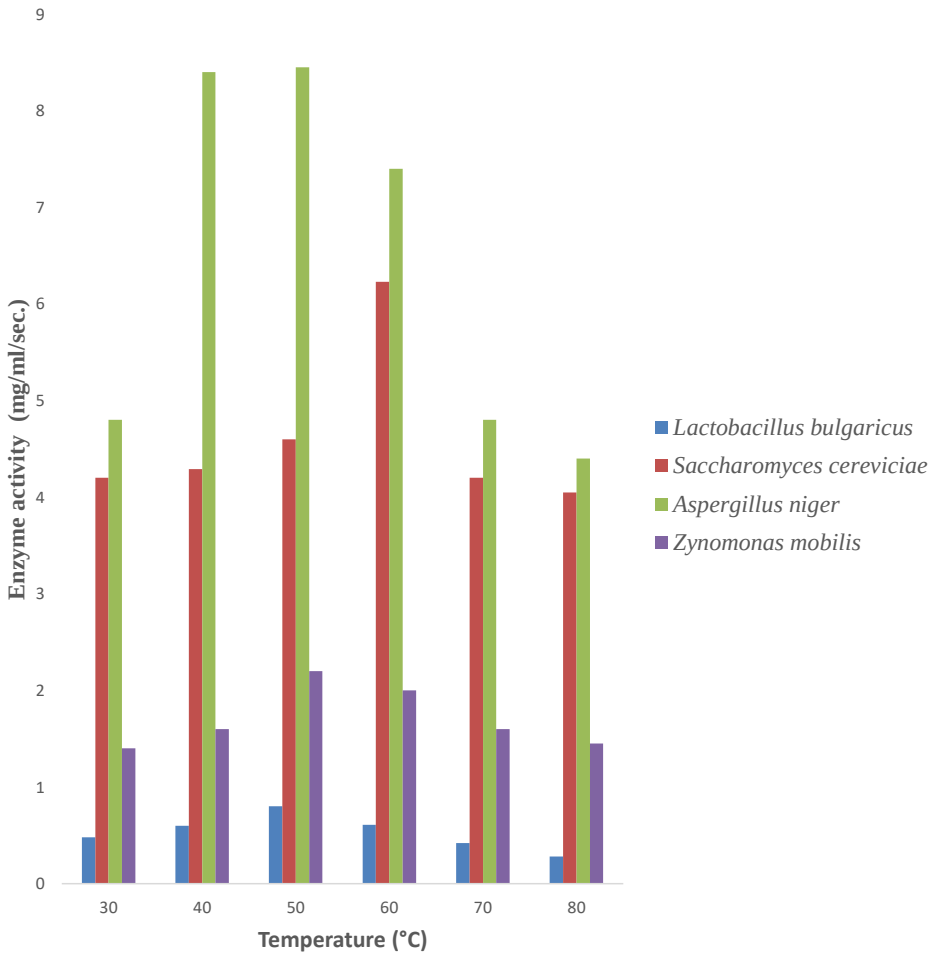

Figure 4.8 Effect of temperature on amylase activity using cassava peels

4.1.10 Effect of temperature on cellulase activity using yam peels

Figure 4.1.10 shows that the effect of temperature on cellulase activities produced by all the test organisms. The optimum temperature of all the isolates was at 60 °C. The highest cellulase activity was observed by *Aspergillus niger* with an enzyme activity of 1.04×10^{2} mg/ml/sec at a temperature of 60 °C. and the lowest enzymatic activity of 1.2×10^{-4} mg/ml/sec was obtained at a temperature of 30 °C. By *Lactobacillus bulgaricus*

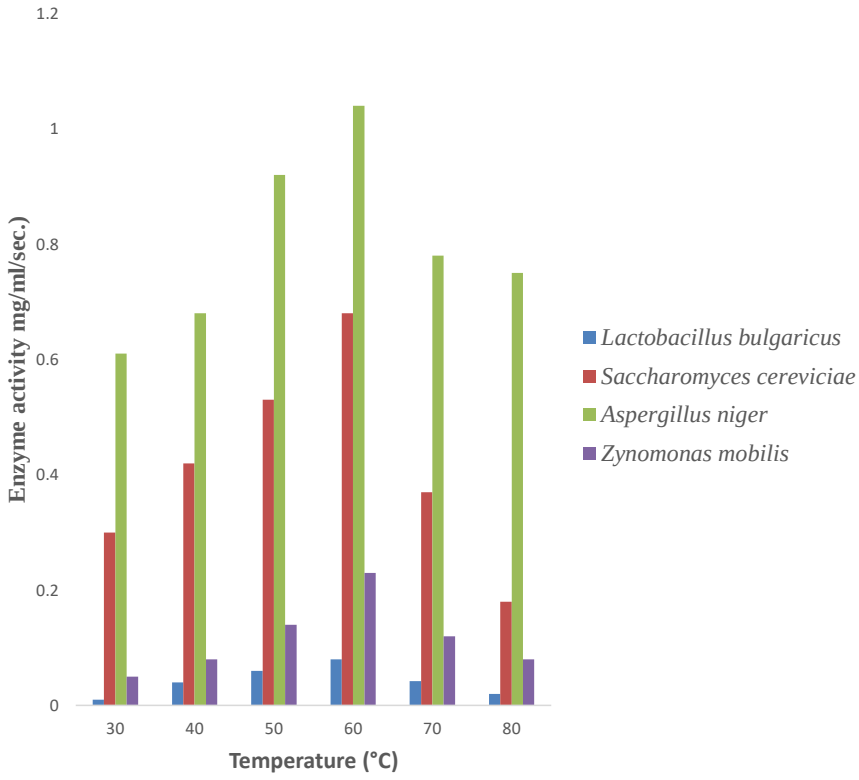

Figure 4.9 Effect of temperature on cellulase activity using yam peels as a substrate

4.1.11 Effect of temperature on cellulase activity using cassava peels as a substrate

Figure 4.1.11 shows that the effect of temperature on cellulase activities produced by the test organisms. The optimum temperature of all the isolates was at 60 °C. The highest cellulase activity was observed by *Aspergillus niger* with an enzyme activity of 1.65×10^{3} mg/ml/sec at a temperature of 60 °C. and the lowest enzymatic activity of 1.0×10^{-4} mg/ml/sec was obtained at a temperature of 30 °C. By *Lactobacillus bulgaricus*

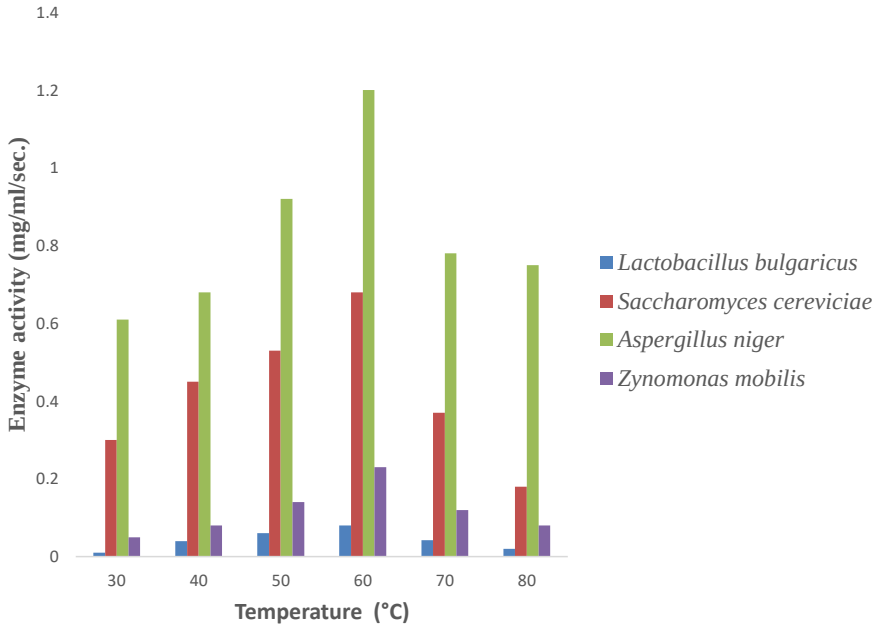

Figure 4.10 Effect of temperature on cellulase activity using cassava peels

4.1.12 Effect of Temperature on Pectinase Activity Using Yam Peels as a Substrate

Figure 4.1.12 shows that the effect of temperature on pectinase activities produced by all the test organisms. The optimum temperature of all the isolates was at 50 °C. The highest pectinase activity was observed by *Aspergillus niger* with an enzyme activity of 1.4×10^{-2}mg/ml/sec at a temperature of 50 °C. and the lowest enzymatic activity of 4.0×10^{-4}mg/ml/sec was obtained at a temperature of 30 °C. By *Lactobacillus bulgaricus*

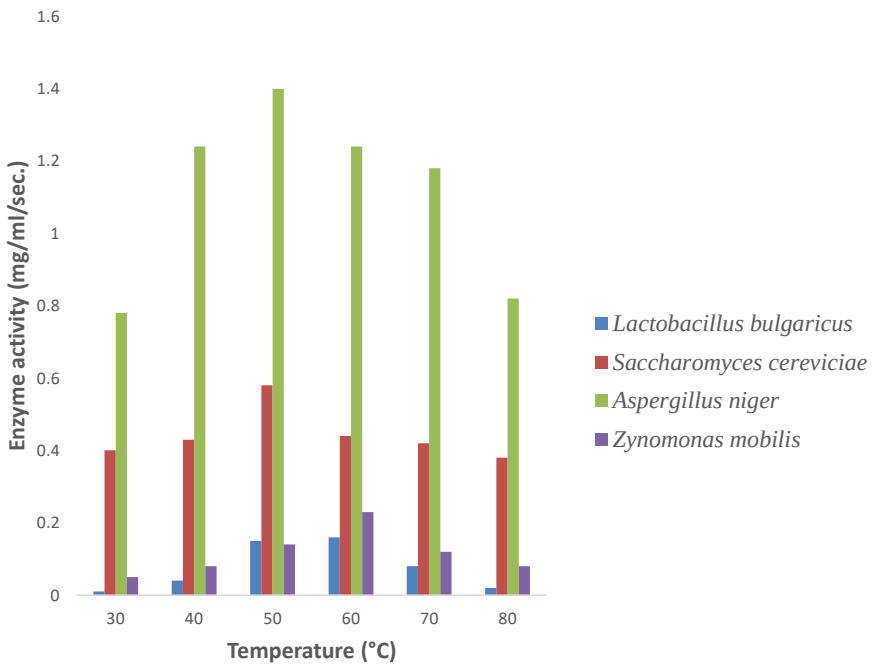

Figure 4.11 Effect of temperature on pectinase activity using yam peels

4.1.13 Effect of temperature on pectinase activity using cassava peels as a substrate

Fig. 4.1.13 shows that the effect of temperature on pectinase activities produced by all the test organisms. The optimum temperature of all the isolates was at 50 °C. The highest pectinase activity was observed by *Aspergillus niger* with an enzyme activity of 1.5×10^{-2} mg/ml/sec at a temperature of 50 °C. and the lowest enzymatic activity of 2.1×10^{-4} mg/ml/sec was obtained at a temperature of 30 °C by *Lactobacillus bulgaricus*.

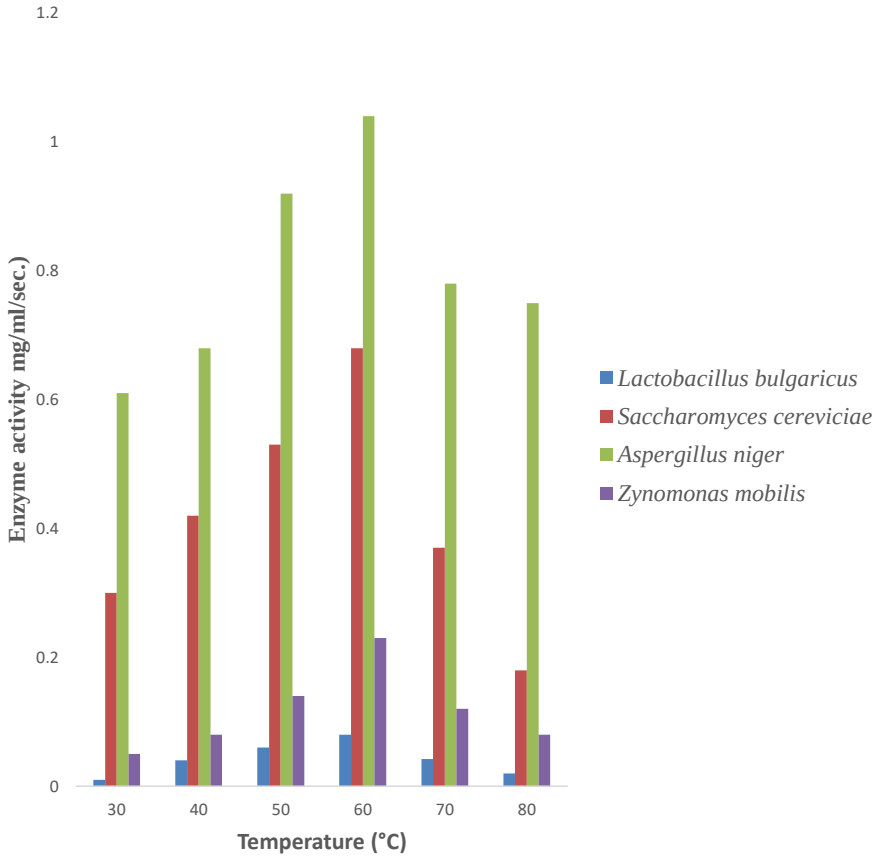

Figure 4.12 Effect of temperature on pectinase activity using cassava peels

4.1.14 Effect oF pH on amylase activity using yam peels

Figure 4.1.14 shows the effect of pH on amylase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 6.0 the highest amylase activity was observed by *Aspergillus niger* with an enzyme activity of 2.8×10^{-2} mg/ml/sec at pH 6. and the lowest enzymatic activity of 2.9×10^{-4} mg/ml/sec was obtained at pH 9.0 by *Lactobacillus bulgaricus*

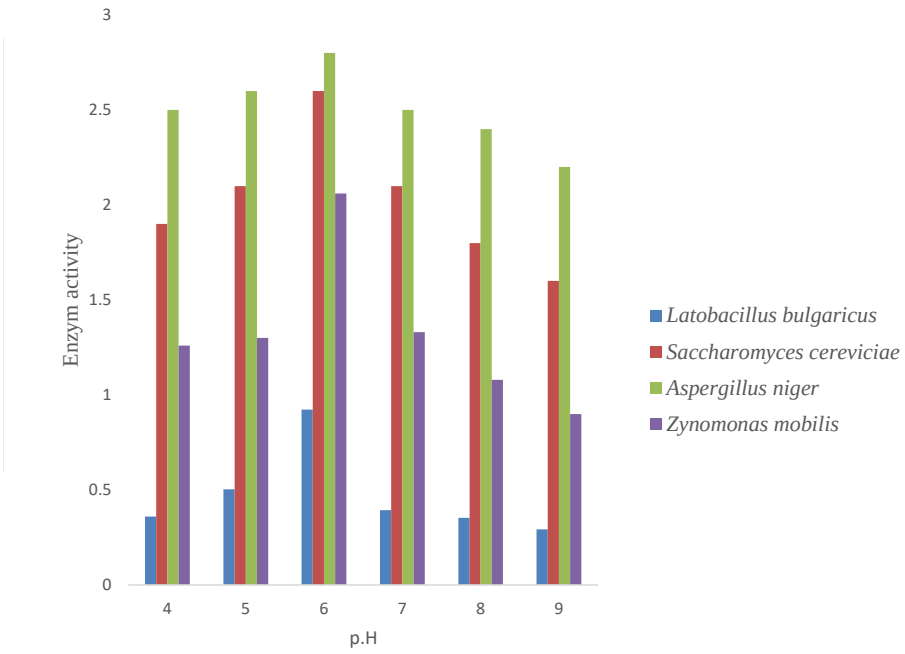

Figure 4.13 Effect of pH on amylase activity using cassava peels

4.1.15 effect of pH on amylase activity using cassava peels as a substrate

Figure 4.1.15 shows the effect of pH on amylase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 6.0 The highest amylase activity was observed by *Aspergillus niger* with an enzyme activity of 3.0×10^{-2} mg/ml/sec at pH 6. and the lowest enzymatic activity of 2.9×10^{-4} mg/ml/sec was obtained at pH 9 by *Lactobacillus bulgaricus*

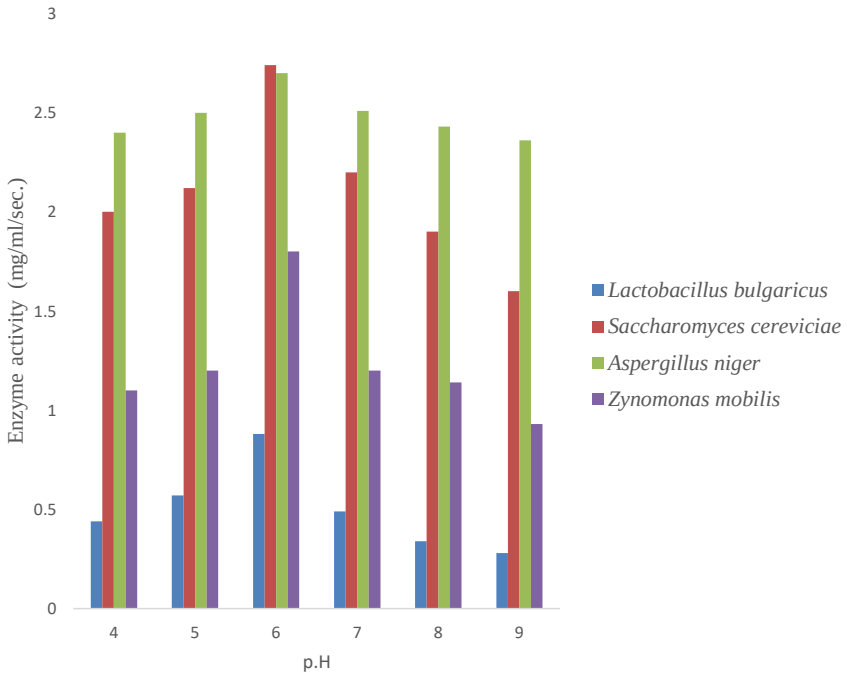

Figure 4.14 Effect of pH on amylase activity using cassava peels

4.1.16 Effect of pH on cellulase activity using yam peels

Fig. 4.1.16 shows the effect of pH on cellulase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 6.0 The highest cellulase activity was observed by *Aspergillus niger* with an enzyme activity of 2.7×10^{-2} mg/ml/sec at pH 6. and the lowest enzymatic activity of 1.9×10^{-4} mg/ml/sec was obtained at pH 9.0 by *Zynomonas mobilis*

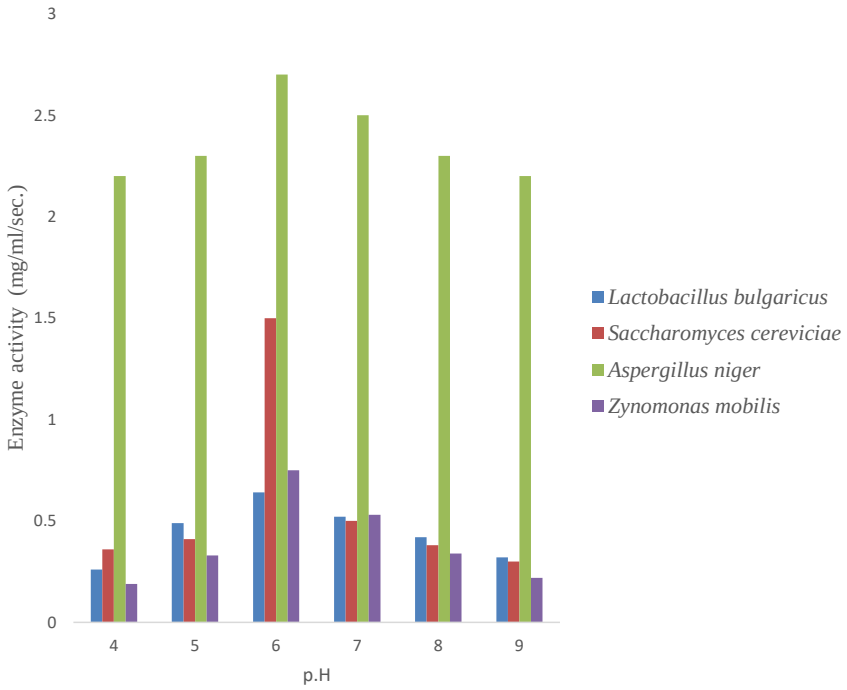

Figure 4.15 Effect of pH on cellulase activity using yam peels

4.1.17 Effect of pH on cellulase activity using cassava peels as a substrate

Figure 4.16 shows the effect of pH on cellulase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 6.0 The highest cellulase activity was observed by *Saccharomyces cereviciae* with an enzyme activity of 2.74×10^{-2} mg/ml/sec at pH 6. and the lowest enzymatic activity of 2.8×10^{-4} mg/ml/sec was obtained at pH 9 by *Lactobacillus bulgaricus*

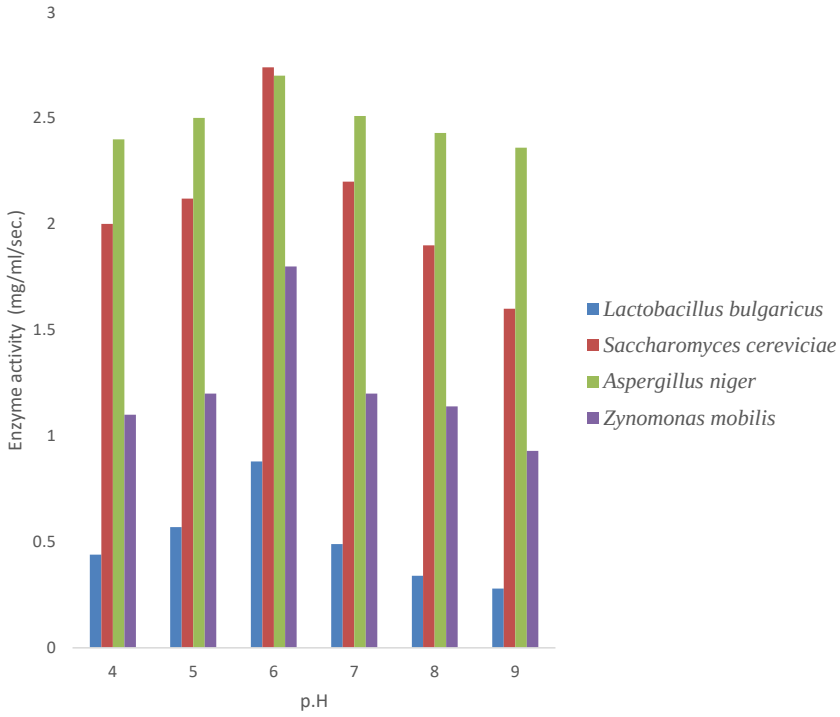

Figure 4.16 Effect of pH on cellulase activity using cassava peels

4.1.18 Effect of pH on pectinase activity using yam peels as a substrate

Fig. 4.1.18 shows the effect of pH on pectinase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 7.0 The highest pectinase activity was observed by *Aspergillus niger* with an enzyme activity of 1.3×10^{-2} mg/ml/sec at pH 7. and the lowest enzymatic activity of 9×10^{-4} mg/ml/sec was obtained at pH 9 by *Lactobacillus bulgaricus*

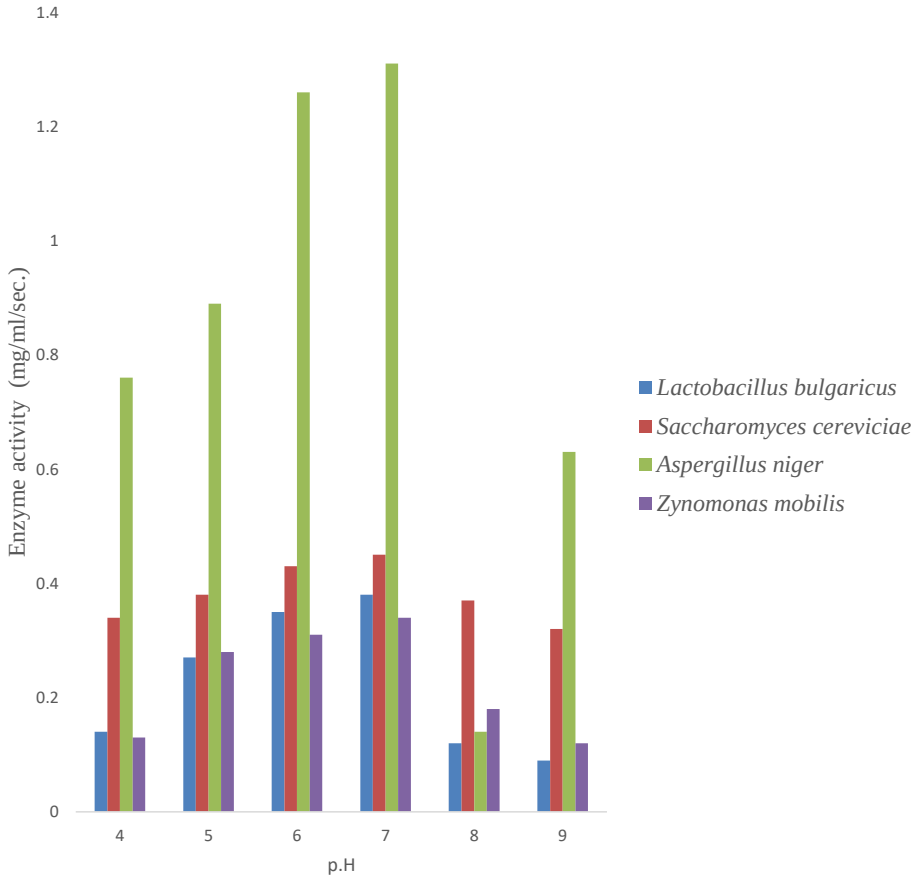

Figure 4.17 Effect of pH on pectinase activity using yam peels

4.1.19 Effect of pH on pectinase activity using cassava peels as a substrate

Figure 4.1.19 shows the effect of pH on pectinase activities produced by all the test organisms. The optimum pH of all the isolates was at pH 7.0 The highest pectinase activity was observed by *Aspergillus niger* with an enzyme activity of 2.6×10^{-2} mg/ml/sec at pH 7. and the lowest enzymatic activity of 1.5×10^{-4} mg/ml/sec was obtained at pH 9 By *Lactobacillus bulgaricus*

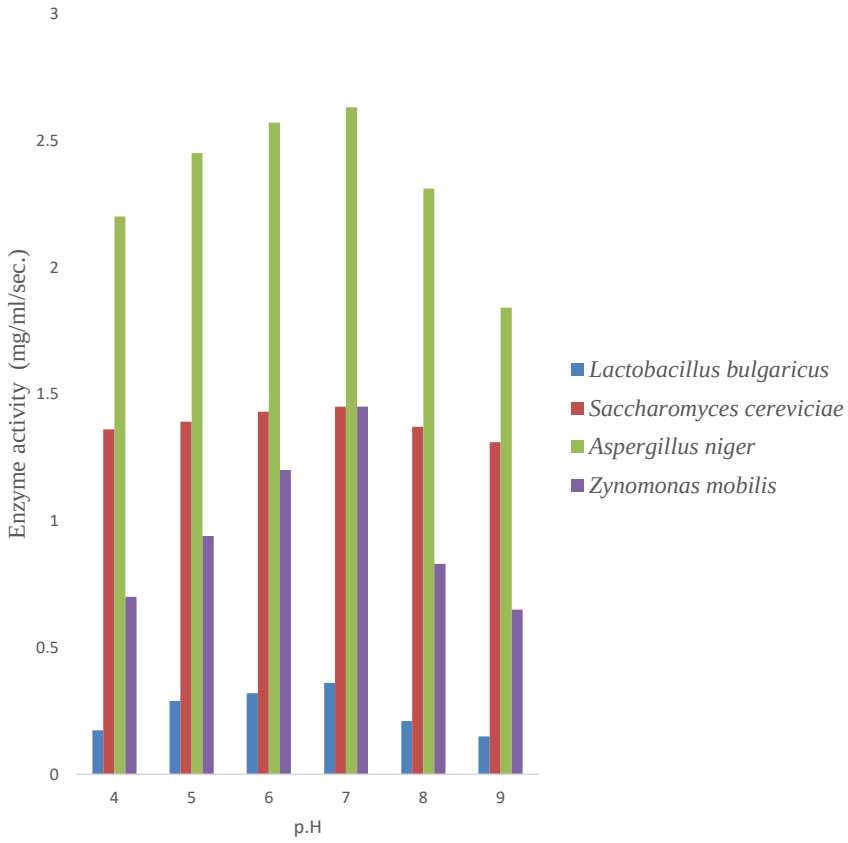

Figure 4.18 Effect of pH on pectinase activity using cassava peels

4.1.20 Effect of substrate concentration on amylase activity using yam peels as a substrate

Figure 4.1.20 shows the effect of substrate concentration on amylase activities produced by all the test organisms. At 2.5-3.0 % almost all test organisms were saturated. The highest substrated concentration from amylase activity was observed by *Aspergillus niger* with an enzyme activity of 3.7×10^{-2} mg/ml/sec at 3.0 % and the lowest substrate concentration of (1.6×10^{-4} mg/ml/sec.) was obtained at 0.5 % by *Zynomonas mobilis*

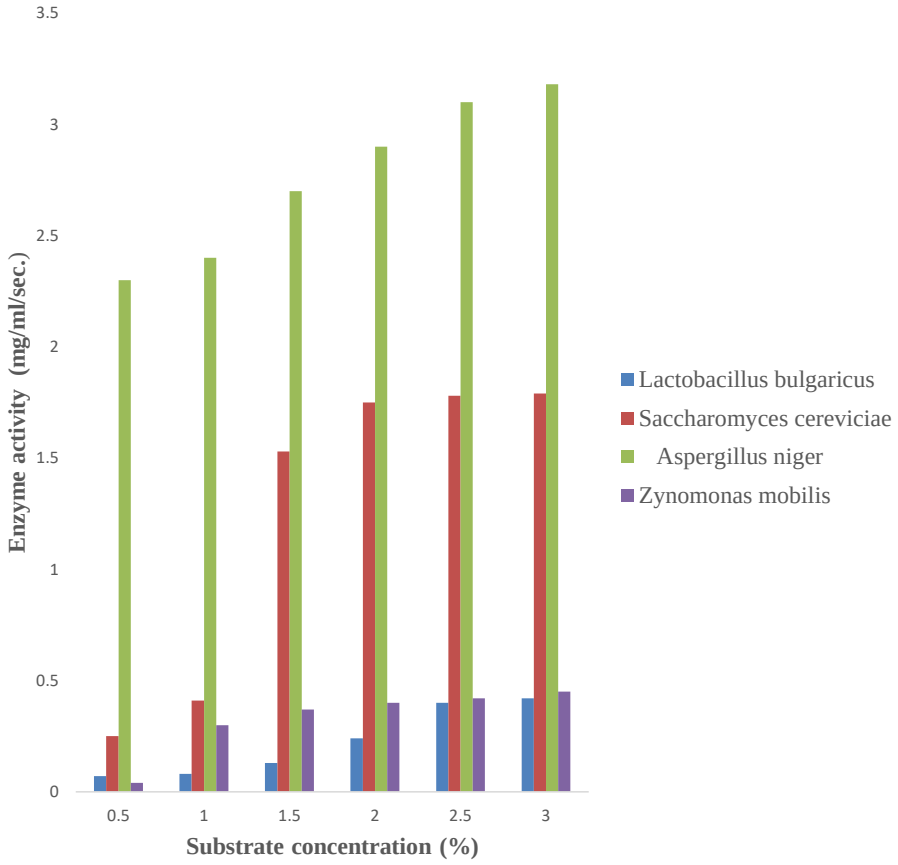

Figure 4.19 Effect of substrate concentration on amylase activity using yam peels

4.1.20 Effect of substrate concentrations on amylase activity using cassava peels as a substrate

Figure 4.1.20 shows the effect of substrate concentration on amylase activities produced by all the test organisms. At 2.5-3.0 % almost all test organisms were saturated. The highest substrate concentration from amylase activity was observed by *Aspergillus niger* with an enzyme activity of 1.6×10^{-2} mg/ml/sec at 3.0 % and the lowest substrate concentration of (1.0×10^{-4} mg/ml/sec.) was obtained at 0.5 % by *Lactobacillus bulgaricus*

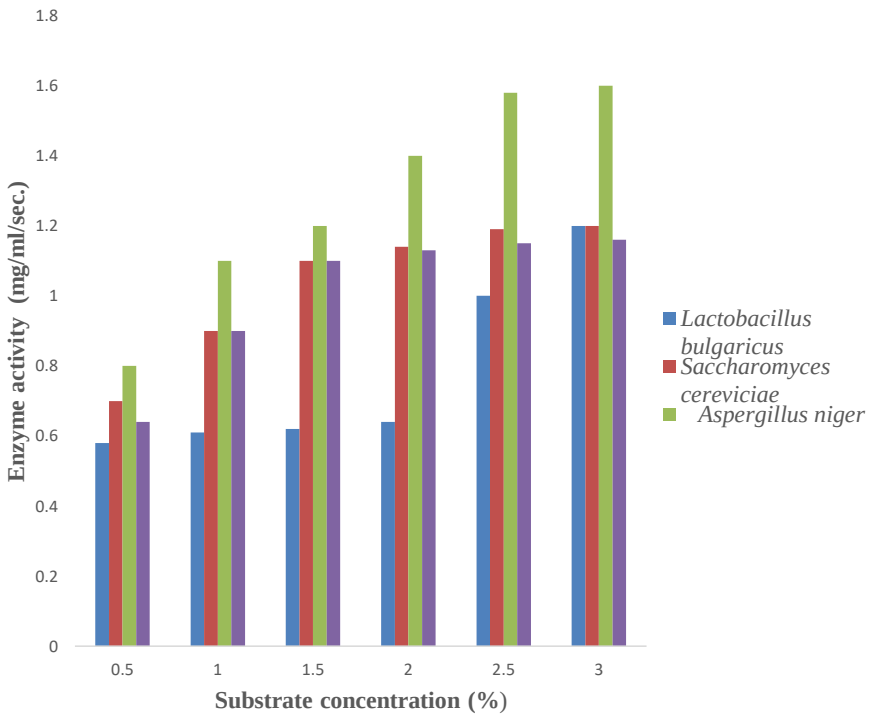

Figure 4.20 Effect of substrate concentration on amylase activity using cassava peels

4.1.22 Effect of substrate concentration on cellulase activity using yam peels as a substrate

Figure 4.1.22 shows the effect of substrate concentration on cellulase activities produced by all the test organisms. At 2.5-3.0 % almost all test organisms were saturated. The highest substrate concentration from cellulase activity was observed by *Aspergillus niger* with an enzyme activity of 3.3×10^{-2} mg/ml/sec at 3.0 % and the lowest substrate concentration of (2.4×10^{-4} mg/ml/sec.) was obtained at 0.5 % by *Lactobacillus bulgaricus*

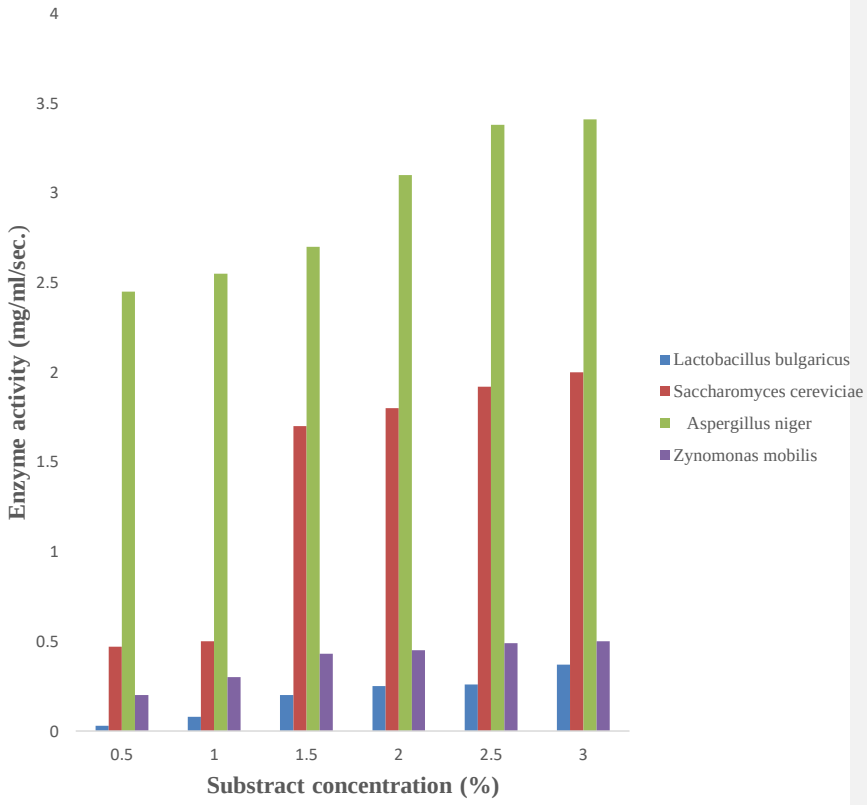

Figure 4.21 Effect of substrate concentration on cellulase activity using yam peels

4.1.24 Effect of substrate concentration on cellulase activity using cassava peels as a substrate

Fig. 4.22 shows the effect of substrate concentration on cellulase activities produced by all the test organisms. At 2.5–3.0% almost all test organisms were saturated. The highest substrate concentration from cellulase activity was observed by *Aspergillus niger* with an enzyme activity of 1.4×10^{-2}mg/ml/sec at 3.0% and the lowest substrate concentration of (1.3×10^{-4}mg/ml/sec.) was obtained at 0.5% By *Lactobacillus bulgaricus*

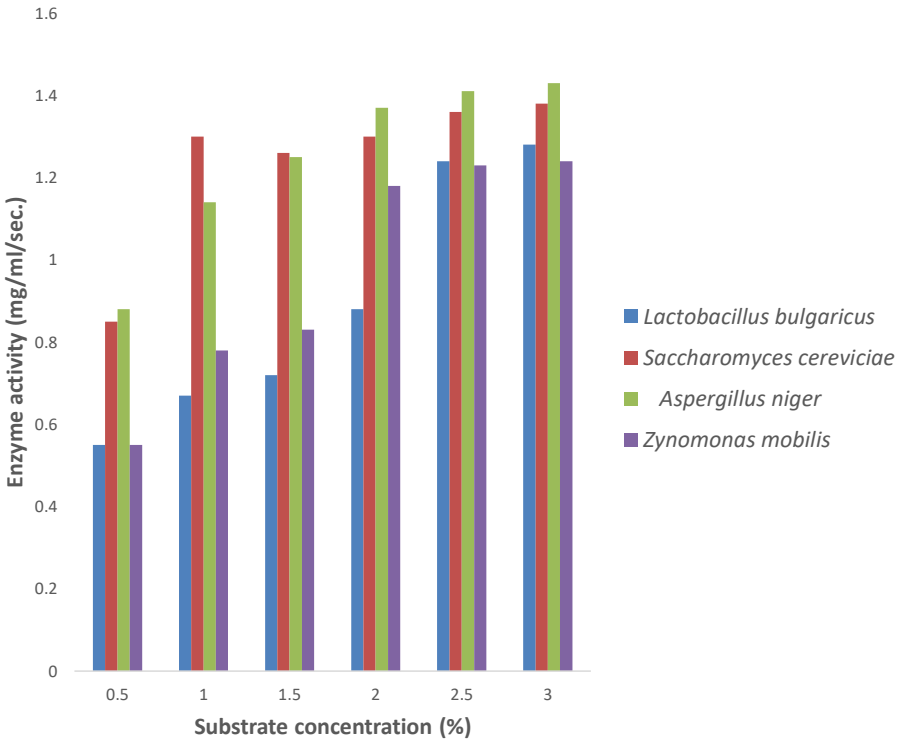

Figure 4.22 Effect Of substrate concentration on cellulase activity

4.1.25 Effect of substrate concentration on pectinase activity using yam peels as a substrate

Fig. 4.23 shows the effect of substrate concentration on pectinase activities produced by all the test organisms. At 2.5–3.0 % almost all test organisms were saturated. The highest substrate concentration from pectinase activity was observed by *Aspergillus niger* with an enzyme activity of 2.4×10^{-2}mg/ml/sec at 3.0 % and the lowest substrate concentration of (3.7×10^{-3} mg/ml/sec.) was obtained at 0.5% by *Zynomonas mobilis*

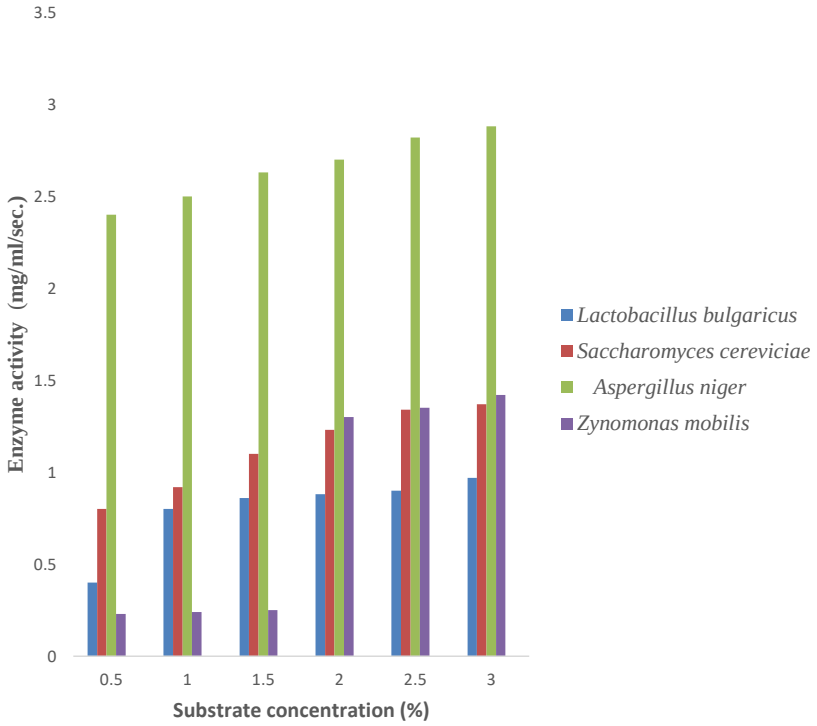

Figure 4.23 Effect of substrate concentration on pectinas activity using yam peels

4.1.26 Effect of substrate concentration on pectinase activity using cassava peels as a substrate

Fig. 4.24 shows the effect of substrate concentration on pectinase activities produced by all the test organisms. At 2.5–3.0 % almost all test organisms were saturated. The highest substrate concentration from amylase activity was observed by *Aspergillus niger* with an enzyme activity of 2.9×10^{-2}mg/ml/sec at 3.0 % and the lowest substrate concentration of (9.7×10^{-4} mg/ml/sec.) was obtained at 0.5 % by *Lactobacillus bulgaricus*

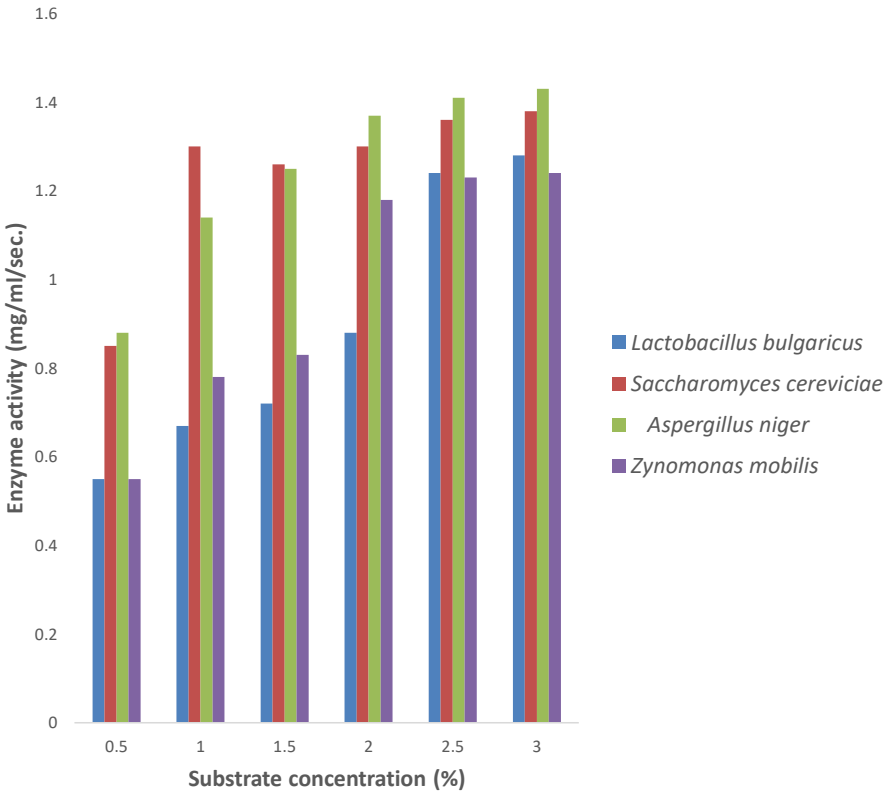

Figure 4.24 Effect of substrate concentration on pectinase activity using cassava peels

4.2 Discussion

Enzymes are not only essential for carrying out biochemical reactions within an organism, but their high specificity and catalytic characteristics have enabled them to be used in various industrial sectors for the production of a wide range of products. Amylase, cellulase and pectinase are among those enzymes. Globally, amylase, cellulase and pectinase are frequently used in food, textile, detergents and paper industries. In addition, pharmaceutical and chemical industries use amylase, cellulase and pectinase on a regular basis to yield their products (El-Fallal *et al.,* 2012). Enzymes are widely used in textile industries along with industries that manufacture food and pharmaceuticals products (Islam *et al.,* 2014). However, the local production of commercial amylase, cellulase and pectinase are limited in Nigeria and thus a lot of money have been spent on the process of enzyme importation. Harmful chemicals are also used as an alternative to amylase, cellulase and pectinase production (Dash *et al*., 2015). For this reason, it is necessary that a process is established to generate commercial amylase, cellulase and pectinase production in the country.

This study was aimed at the bioconversion of agricultural wastes to produce amylase, cellulase and pectinase from yam and cassava peels. The study also includes the partial characterization of the produced amylase, cellulase and pectinase enzyme by determining the optimum temperature, pH and substrate concentration at which the enzyme works best.

Cellulase producing microorganisms have due advantage over other sources because of their high rate of multiplication to increase yield of production (Bhardwaj and Garg, 2014). In this study, potential cellulase producing microorganisms were isolated using serial dilution, pour plating and streak plating techniques from the soil. Nayak*et al.*, (2012) and Sumitha *et al.,* (2013) were also followed similar procedures to isolate

microorganisms for cellulase production. the organic nature of yam peels and cassava peels makes it an ideal substrate for microbial processes for the production of value-added products (Pandey *et al.*, 2000).

Emerging new applications underline the importance of screening amylase, cellulase and pectinase producing microorganisms with novel properties, greater enzyme activity and large-scale production of these enzymes (Singh and Mandal, 2012). In this study, the isolates were subjected into plate agar screening methods to identify potent isolate with highest enzyme activity. Hitha and Girija (2014) used plate agar screening method to screen native isolates for amylase, Cellulase and Pectinase activity. Similarly, Mehta *et al.,* (2013) screened bacterial strains, isolated from soil and samples of vegetable using plate agar and submerged fermentation screening methods.

About four different microorganisms were used for this study which includes *Lactobacillus bulgaricus*, *Saccharomyces cereviciae*, *Aspergillus niger* and *Zynomonas mobilis*. In the screening processes of the four isolates, it was observed that all the test organisms were amylase, cellulase and pectinase producing strains. For amylase producing strain it was determined by growing the isolates on starch agar medium. For cellulase producing strains, deman Rogossa sharp agar medium (MRS) and sabouraud destrose agar (SDA) amended with 0.1 % of carboxymethyl cellulose (CMC) were used for the screening process for the bacteria and fungi isolates respectively and for pectinase producing strains, 1g of pectin added into deman Rogossa sharp agar medium and sabouraud dextrose agar medium were used for the screening processes for the Bacteria and fungi isolates respectively. detecting any clear zone produced around the bacterial colonies by adding Gram's iodine. The clear zones produced were due to the absence of starch, cellulose and pectin which were hydrolysed by the amylase, cellulase and pectinase enzymes excreted by the organisms (Oyeleke *et al.*,2012).

In order to determine the amount of amylase produced by the selected isolates for an incubation period of seven days enzyme assay was carried out. This was done using the method that involved the use of 3, 5 – dinitrosalisyllic acid (DNS). The amylase, cellulase and pectinase production by different isolates of microorganisms using cassava peels and rice husk were found maximum at the 4th day of incubation period. Further increase in incubation time marks the decrease of amylolytic activity (*Oyewole et al.,* 2012). The 7th day of incubation has the lowest amylase activity by all the four isolates. Using cassava peels and rice husk as a substrate *Aspergillus niger* have the best yields of amylase activity with an enzyme activity of 7.2×10^{-3} mg/ml/sec. and 8.7×10^{-4} mg/ml/sec. respectively at the 4th day of incubation period while the lowest amylase activity was recorded by *Lactobacillus bulgaricus* with an amylase activity of 5.8×10^{-4} mg/ml/sec. and 6.5×10^{-4}mg/ml/sec. respectively at the 7th day of incubation period. This study was in agreements with (Nur *et al*., 2012), who produce amylase and cellulase from agricultural waste by *Aspergillus sp* under solid state fermentation.

For cellulase enzyme production using yam peels and cassava peels by the four isolates, Aspergillus *niger* still had the highest yield of enzyme activity which was recorded as 1.6×10-2 mg/ml/sec. and 1.7×10-2 mg/ml/sec. respectively at the 4th day of incubation period while the lowest yield of enzyme activity was obtained from *Lactobacillus bulgaricus* which was recorded as 8.0×10-4 mg/ml/sec. and 1.0×10-3 mg/ml/sec. respectively at the 7th day of incubation period. This result agreed with (Oyewole *et al.,* 2012), who reported the highest cellulase activities at 4th day of incubation period for the production of cellulase and pectinase by *Aspergillus niger* isolated from corncorb.

For pectinase production the highest enzyme activity was obtained by *Aspergillus niger* using yam peels and cassava peels as a substrate which was recorded as 3.7×10^{-2}mg/ml/sec. and 3.6×10^{-2} mg/ml/sec. respectively at the 4th day of incubation period while

the lowest pectinase activity was obtained from *Lactobacillus bulgaricus* which was determined as followed 1.6×10^{-3} mg/ml/sec. and 3×10^{-3} mg/ml/sec. respectively at the 7th day of incubation period.

increase in incubation period did not show any significant increase in pectinase production rather it was decreased (Kumar *et al.*, 2012). Enzyme production increase with increase in time duration up to 4th day then it decreases (Mehta *et al.*, 2013). These investigations are in line with the present study. But in some studies incubation period of 48 hr for optimal production for some fungal strains was reported (Phutela *et al.*, 2005). While Fuijo and Eledago, (1993) reported a 72-hr incubation time for polygalacturonase production by *Rhizopus oryza*. These studies were also in line with Oyeleke *et al.*, 2012 who reported the highest incubation period on the fourth day for cellulase and pectinase potentials of *Aspergillus niger* isolated from corn cob

The second part of the study was based on the partial characterization of the amylase, cellulase and pectinase enzyme generated by the four test microorganisms. The rate at which starch, cellulose and pectin were broken down by amylase, cellulase and pectinase enzyme respectively depends on various parameters (Sivaramakrishnan *et al.*, 2006). Characterizing an enzyme leads to the determination of optimum fermentation conditions for that enzyme. The properties of amylase, cellulase and pectinase should meet its application and hence it is mandatory to check its optimum conditions which can be done via characterization (Sivaramakrishnan *et al.*, 2006). Some of the most important ones include optimum temperature and pH. Hence, the enzyme was characterized by carrying out enzyme assay at different temperatures and pH in order to detect the optimum conditions. There are various ways to characterize an enzyme. In this study, the DNS method was used which determines the amount of enzyme produced at different temperatures and pH.

It was detected that the activity of amylase was low at temperatures from 30 °C to 40 °C. However, a drastic elevation of activity was observed at 50 °C. At a higher temperature of 60 °C, the activity started to decrease. So, this particular amylase showed highest activity at 50 °C. Thus, it can be concluded that the amylase is most active at moderate temperature. In a study conducted by Cordeiro *et al* (2002) who worked with a thermophilic *Bacillus* sp. strain SMIA-2 isolated from the soil of Brazil produced amylase with an optimum temperature of 70 °C. In a study by Kim *et al* (1995) the optimum temperature of the amylase was observed to be 60 °C produced by the *Bacillus* Strain, GM890 and in a study by Fattah *et al* (2013) where the amylase produced from *Bacillus licheniformis* Isolate AI20 showed the highest activity between the range of 60-80 °C. In another study, it was observed that the strain *Bacillus* sp. WA21 produced amylase enzyme which had a lower optimum temperature of 55 °C (Asad *et al.*, 2011).

For cellulase enzyme activity by the four isolates using yam peels and cassava peels it was observed that the activity of cellulase was low from 30 °C to 50 °C. However, a drastic increased of activity was observed at 60 °C. At higher temperature of 70 °C. The activity started to decline. Therefore, the optimum temperature of cellulase activity in this study was at 60 °C. Thus, it can also be concluded that the cellulase is most active at moderate temperature as compared to cellulase work at different studies this work was in line with (Oyeleke *et al.*, 2012) who reported the temperature of 6 °C on the production of amylase by *Aspergillus niger* isolated from cassava peels.

For pectinase enzyme activity by the four isolates tested using yam peels and cassava peels as a substrate it was discovered that the activity of pectinase was low from 30°C to 40 °C. However, at 50 °C a tremendous increased in enzyme activity was observed, it was also observed that any temperature above 50 °C. Will results to the decrease in enzyme activity. Therefore, the optimum temperature of pectinase production in this

study was at 50 °C. Thus, it can be concluded that the pectinase activity in the study is most active at moderate temperature as compared to pectinase work at different studies. This particular pectinase was agreed with the work of Oyewole *et al.*, (2011) who worked on the production and optimization of pectinase and cellulase by *Aspergillus niger* isolated from soil sample.

Amylase activity enzyme was characterized to identify optimum pH by the four isolates using yam peels and cassava peels as a substrate it was observed that the amylase showed the highest activity at pH 6. From pH 4 to pH 5 the activity was moderate and gradually increasing but at pH 6 it increases drastically. However, the enzyme started showing lower activity as the pH moved to the alkaline range from 8 to 9. From this result, it can be concluded that the enzyme works best at a pH. 6 This was in line with the optimum pH found in the studies by Vaidya and Rathore (2015) who isolated the Bacillus strain APIB2 from a potato dump site in Madhya Pradesh, India and Vaseekaran *et al* (2010) who identified their strain as *Bacillus licheniformis*. Another research revealed the optimum pH of amylase produced by *Bacillus licheniformis* Isolate AI20 was within the range of 6 to 7.5 which is similar to that found in this study

(Fattah *et al.*, 2013). The optimum pH was detected to be7.5 in a study performed by Cordiero *et al* (2002) who worked with the *Bacillus* sp. strain SMIA-2. On the other hand, Asad *et al* (2011) analysed that for the bacterial strain *Bacillus* SP. WA21 the optimum pH of the amylase obtained was 6 which is less than that found in this study. A higher optimum pH of amylase was obtained within the range of pH 10.5 to 12 in an investigation by Kim *et al* (1995) who worked with the *Bacillus* Strain, GM890.

For cellulase activity the enzyme was characterized to determine the optimum pH by the four isolates using yam peels and cassava peels as a substrate it was observed that the cellulase the showed the highest enzyme activity at pH 6. From pH 4 to 5 the enzyme

activity increased gradually but at pH 6 it elevated tremendous However, the activity started to showed lower yield of Ezyme activity as the pH move to the alkaline range from 8 to 9 from this results it can be concluded that enzyme work best at pH 6 this was in line with the optimum pH found in the studies by Vaidya and Rathore (2015) who isolated the *Bacillus* strain APIB2 from a potato dump site in Madhya Pradesh, India and Vaseekaran *et al* (2010) who identified their strain as *Bacillus licheniformis*. Another research revealed the optimum pH of amylase produced by *Bacillus licheniformis* Isolate AI20 was within the range of 6 to 7.5 which is similar to that found in this study.

For Pectinase activity the enzyme was characterized to determine the optimum pH by the test isolates using yam peels and cassava peels as a substrate it was observed that the pectinase showed the highest enzyme activity at pH 7. From pH 4 to 6 the enzyme activity increased gradually, at pH 7 elevated drastically However, the activity started to showed lower yield of activity as to p. H move to the alkaline range from 8 to 9. This result showed that enzyme work best at neutral pH this result was not in agreement with optimum pH in the studies by Oyeleke *et al*., (2012) who work with cellulase and pectinase potentials of *Aspergillus niger* isolated from corn cob in Minna, Niger state

For the substrate concentration, the points of saturation were found to be within a range of 2.5% to 3.5%. From the results *Aspergillus niger* have the highest level of substrate concentration probably because of the fast rate of multiplication or because of the highest amounts of enzyme produced.

CHAPTER FIVE

CONCLUSION, RECOMMENDATION AND REFRENCES

5.1 Conclusion

Zynomonas mobilis, *Lactobacillus bulgaricus*, *Aspergillus niger* and *Saccharomyces cerevisiae* were isolated from spoilt orange, fermented milk, soil and fresh palm wine respectively.

The organisms were screened for amylase, cellulase and pectinase enzyme. The clear zone of inhibition observed, revealed that the test isolates were amylase, cellulase and pectinase producer.

The organisms were assayed at different incubation period. The highest amylase activity was obtained on the 4th day (8.5×10^{-2} mg/ml/sec.) by *Aspergillus niger* strain MN945947 using cassava peels as a substrate. 4×10^{-3}mg/ml/sec. Was recorded as the optimum cellulase activity on the 4th day by *Aspergillus niger* strain MN945947. The best pectinase activity was obtained on the 4th day (3.7×10^{-2} mg/ml/sec.) by *Aspergillus niger* strain MN945947 using yam peels as a substrate. The optimum pH for cellulase production was at pH 6 with the highest enzyme activity of 3.5×10^{-4} mg/ml/sec by *Aspergillus niger* using yam peels as a substrate,

pH 6 was recorded as the best enzyme activity (2.7×10^{-2} mg/ml/sec.) by *Aspergillus niger* using yam peels as a substrate. The best pH for pectinase activity was at pH 7 (2.6×10^{-2}mg/ml/sec.) by *Aspergillus niger*. The best temperature was at 50°C with the highest activity (8.5×10^{-2}mg/ml/sec.) by *Aspergillus niger* strain MN945947 using cassava peels as a substrate. The optimum temperature of cellulase enzyme activity was 60 °C. With the highest activity of ($1.7x10^{-4}$ mg/ml/sec.) using cassava peels as a substrate by *Aspergillus niger*. For pectinase production the optimum temperature of 50 °C gave the

highest pectinase activity of $1.5x10^{-2}$ mg/ml/sec. Substrate concentration for amylase cellulase and pectinase activity were also determined, points of saturation occurred between 2.5% to 3% in all the organisms tested *Aspergillus niger* have the highest concentration of substrate. This study revealed that all the test isolates using agro wastes as a substrate have the ability to produce amylase, cellulase and pectinase, hence it may be considered as a source for the production of industrial amylase, cellulase and pectinase.

5.2 Recommendations

i. Serious work should be done on the abundant of agricultural wastes that are generated annually in Nigeria which could be utilized for the production of amylase, cellulase and pectinase this will help address the problem of wastes disposal in the environment and conserve the foreign exchange used to procure them.

ii. However, further research work is necessary for extensive chemical characterized further to determine the thermostability and effect of different metal ions and different substrates.

iii. Moreover, genetic modification can be done for the improvement of the strain, the crude amylase can be purified and also the encoded sequence of amylase can be determined. With all this information in hand, in the near future it is hoped that the demand of commercial amylase, cellulase and pectinase in the industries will be fulfilled.

iv. Also, new industries can be established with a sole focus on the commercial production of amylase which is hoped to overthrow the need of expensive commercial amylase import processes and use of harmful chemicals. Instead, it is anticipated that Nigeria will soon be able to export amylase to other countries and help improve the economic condition of the country.

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APPENDIX

Molecular characterization of the test organisms

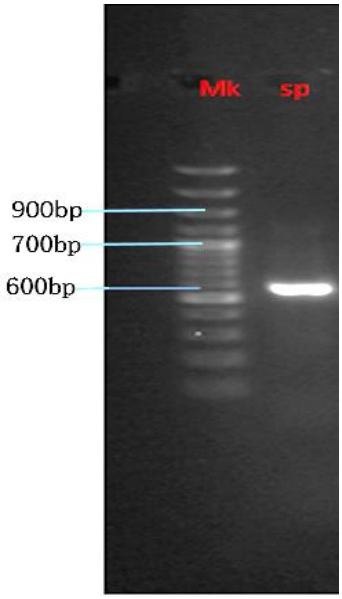

Plate 4.5 Gel electrophoresis micrograph of amplified product.

Sequencing results shows that the sample is 99% identical to *Saccharomyces cerevisiae* strain CBS 1171)

AAAAAAATTTAAAAATTTTGAAAATGGATTTTTTTTTTTTGGGCAAGAGCATGAGA
GCTTTTACTGGGCAAGAAGACAAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAA
GTGCGCGGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCAAACGGTGAGAGA
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TTTTCATATCTTTGCAACTTTTTCTTTGGGCATTCGAGCAATCGGGGCCCAGAGGTA
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TGAGCGTCATTTCCTTCTCAAACATTCTTTTTGAGTGAGTGATACTCTTTGGAGTTA
ACTTGAAATTGCTGGCCTTTTCATTGGATGTTTTTTTTCCAAAGGAGGTTTCTCTGCG
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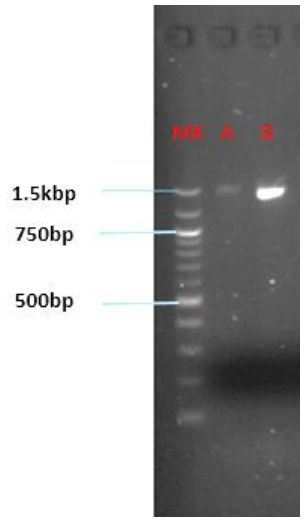

Plate 4.6 Gel electrophoresis micrograph of amplified product.

Agarose gel confirming the positive amplification of bacteria 16s rRNA gene region using the 16s prokaryotic universal primers. A 1.5kbp amplification indicates a positive amplification. Loading arrangement molecular marker (MK) sample A (A) and sample B (B).

Sequence of sample A; *Lactobacillus delbrueckii* strain MN945906

GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCT
GAATTCAAAGATTCCTTCGGGATGATTTGTTGGACGCTAGCGGCGGATGGG
TGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAAC
AGGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGG
CGGCGTAAGCTGTCACTTTAGGATGAGCCCGCGGCGCATTAGCTAGTTGGT
GGGGTAAAGGCCTACCAAGGCAATGATGCGTAGCCGAGTTGAGAGACTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGT
GAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAG
GCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGAAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAG
GAGAGTGGAATTCCATGTGTAGCGGTGTAATGCGTAGATATATGGAAGAAC
ACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAA
GCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGA
TGAGCGCTAGGTGTTGGGGACTTTCCGGTCCTCAGTGCCGCAGCAAACGCA
TTAAGCGCTCCGCCTGGGGAGTACGACCGCAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAGCG

AAGAACCTTACCAGGTCTTGACATCCTGTGCTACACCTAGAGATAGGTGGTT
CCCTTCGGGGACGCAGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGC
CATCATTAAGTTGGGCACTCTAAAGAGACTGCCGGTGACACCGGAGGAAGG
TGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGC
TACATGGGCAGTACAACGAGAAGCGAACCCGCGAGGGTAAGCGGATCTCTT
AAAGCTGTTCTCAGTTCGGACTCAGGCTGAACTCGCCTGCACGAAGCTGGA
ATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCATGGAAGTCTGCAATGCCCAAAGTCGGTG
GGATAACCTTTATAGAGTCAGCCGCCTAAGGCAGGGCAGATGACTGGGGTG
AAGTCGTAACAAGGTAGCCGTAGGGGCAT

Sequence of sample B; *Zymomonas mobilis* strain MN945907

TGGCCCAGAACGAACGCGGGGGGCATCCTTAACACATGCAAGTCGAACGAA
GGCTTCGGCCTTAGTGGCGCACGGGTGCGTAACGCGTGGGAATCTGCCTTC
AGGTACGGAATAACTAGGGGAAACTCGAGCTAATACCGTATGACATCGAGA
GATCAAAGATTTATCGCCTGAAGATGAGCCCGCGTTGGATTAGCTAGTTGGT
AGGGTAAAGCTTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATC
AGCCACACTGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGGGAAACCCTGATCCAGCAATGCCGCGTGAGTGA
AGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGATGATAATGACAGTA
CCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG
AGGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGTACGTAGGCGGT
TTAATAAGTCAGGGGTGAAAGCCCAGAGCTCAACTCTGGAACTGCCTTTGA
GACTGTTAGACTAGAACATAGAAGAGGTAAGTGGAATTCCGAGTGTAGAGG
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CTGATCGCGGAAAGTGGAGACACATTCTTTCAGTTCGGCTGGATCAGAGAC
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GATTGTTCTCTGCAACTCGAGAGCATGAAGGCGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCAGGCCTTGTACACACCGCCCGTC
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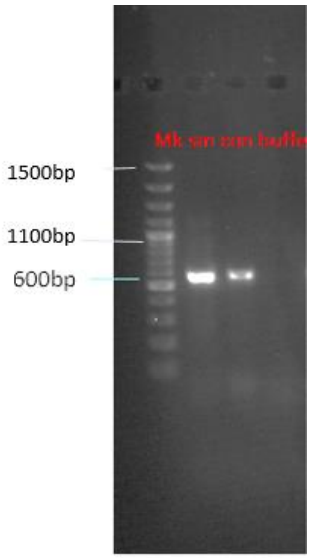

Plate 4.7 Gel electrophoresis micrograph of amplified product.

Agarose gel confirming the positive amplification of fungi ITS gene region using the ITS universal primers. A 600pb amplification indicates a positive amplification. Loading arrangement molecular marker (MK) fungi isolate (Sm) fungi positive control (con) and buffer control.

99% identical to *Aspergillus niger* strain MN945947

TAAACCTGCGGAAGGATCATTACCGTGCGGGTCCTTTGGGCCCAACCTCCCA
TCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGC
CGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAC
GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAA
AACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT
TGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGG
GGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATG
GGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCA
ACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACAAA