ISOLATION AND MOLECULAR CHARACTERIZATION OF PECTINASE FROM *Penicillium chrysogenum* and *Aspergillus niger* GROWN ON SOME AGRO WASTES IN LAGOS, NIGERIA.

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DECLARATION

The Research work contained in this Thesis was undertaken entirely by me. No part of this study has been presented either in part or as a whole to any other institution or organization for the purpose of obtaining any degree or qualification.

OKAFOR, UZOMA ANTHONY.

DEDICATION

This work is first dedicated to God Almighty, in whom is hidden all the treasures of wisdom and knowledge, and then to the loving memory of my Dad, Chief Steven Ebozianam. Okafor (You live in me) and my late Father In-Law, Chief Joseph Babatunde Faremi (I missed you)

"We should be taught not to wait for inspiration to start a thing. Action always generates inspiration. Inspiration seldom generates action".

- Frank Tibolt

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List of Abbreviations

2DG	2 Deoxy D glucose
A.n	*Aspergillus niger*
CR	Catabolite Repression
FAO	Food and Agricultural organisation
FAOSTAT	Food and Agricultural Organisation Statistics
LAWMA	Lagos State Waste Management Authority
MSB-CC	Mineral Salt Broth – Corncob
MSB-Gluc	Mineral Salt Broth – Glucose
MSB-OP	Mineral Salt Broth – Orange peels
MSB-PA	Mineral Salt Broth – Pineapple peels.
MSB-SP	Mineral Salt Broth – Sugar cane pulp
MSB-Suc	Mineral Salt Broth – Sucrose.
MSB-WB	Mineral Salt Broth – Wheat bran.
MSW	Municipal Solid Waste
OR	Orange peels
PA	Pineapple peels
P.c	*Penicillium chrysogenum*
PGases	Polygalacturonase
PI	Potency Index
RAPD-PCR	Random Amplified Polymorphic DNA- Polymerase Chain Reaction
SC	Sugar cane pulps
SD	Sawdust

SEM	Standard error on mean
SmF	Submerged fermentation
SSF	Solid state fermentation
SUC	Sucrose
USEPA	United State Environmental Protection Agency
UV	Ultra violet
WB	Wheat bran

Definition of Terms and Acronyms

- **Agro waste**: Waste produced as a result of various agricultural operations.
- **Biomass**: Biological material from living or recently living organisms, most often referring to plants or plant-derived materials.
- **Biotechnology**: The use of living systems and organisms to develop or make useful products.
- **Catabolite repression:** The process by which microorganisms adapt quickly to a preferred rapidly metabolizable intermediate through the inhibition or repression of genes related to catabolism of less preferred intermediate.
- **Catabolite:** A product of catabolism.
- **Enzymes:** Large biological molecules responsible for the thousands of chemical interconversions that sustain life. They are highly specific bio-catalysts.
- **Fermentation**: A metabolic process that converts sugar to acids, gases and/or alcohol usually by microorganisms or oxygen-starved muscle cells.
- **Hyper production:** Excessive production of a substance.
- **Mutant:** An individual, organism, or new genetic character, arising or resulting from an instance of mutation
- **Mutation:** A change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parental type.
- **Pectinase:** A general term for enzymes such as pectolyase, pectozyme and polygalacturonase, which break down pectin, a polysaccharide substrate that is found in the cell walls of plants.
- **Polymorphism:** The occurrence of two or more clearly different phenotypes existing in the same population of a species
- **UV Radiation:** Electromagnetic radiation with a wavelength shorter than that of visible light, but longer than X-rays, that is, in the range between 400 nm and 1nm

- **Wastes:** Unwanted or undesired materials or substances that are discarded.

ABSTRACT

Waste generated by several agricultural activities has many alternative uses. Unfortunately, much of it is burnt in the open fields, resulting in environmental pollution.

In view of the economic and ecological importance of agro wastes, microfungi were isolated from decaying agro wastes samples and screened for pectinolytic activity using a plate assay method containing pectin alone. The effects of carbon, nitrogenous sources and pH on pectinase production by the strains with maximum pectinolytic activities were investigated. Hyper-production of pectinase by these strains using ultra-violet irradiation (UV) as mutagen was also investigated. The selected mutants of *Penicillium chrysogenum* and *Aspergillus niger* were characterized using catabolite repression. The genetic variability between the wild and mutant strains was investigated using Random Amplified Polymorphic DNA (RAPD) primers. The polygalacturonase (Pga) produced by the mutant strains were partially purified and the enzyme properties were investigated.

Five fungi strains were isolated they are*: Aspergillus niger, Aspergillus clavetus, Penicillium chrysogenum*, *Fusarium species* and *Trichoderma harzianum*. Pectinase production by these isolates showed that the highest pectinase activity index was observed in *Aspergillus niger* (A.n) followed by *Penicillium chrysogenum* (P.c). The growth profile of the A.n and P.c in various agro wastes studied showed that these fungi were able to grow in both simple sugar (metabolites) and various agro wastes. The production of pectinase by A.n and P.c in various agro wastes studied showed that wheat bran medium supported pectinase production better than other agro wastes used with a yield of 350.48 IU/ mg protein and 450 IU/ mg protein respectively with maximum yield at 48 h. The inclusion of various nitrogenous salts revealed that urea best supported the production of pectinase (195.75 IU/ mg protein) while polygalacturonase (Pga) production by the addition of urea (33.52 IU/ mg protein) was highly reduced. When P.c was used as the

fermenting organism, pectinase production was supported best by peptone 177.97 IU/ mg protein but its polygalacturonase activity was highly reduced (40.35 IU/ mg Protein). Pectinase and polygalacturonase optimization by P.c was best achieved when urea was used as nitrogen source, 124.26 IU/ mg protein for pectinase and 93.00 IU/ mg protein for polygalacturonase after 72 h post fermentation. The stimulation of polygalacturonase and pectinase in A.n using various agro wastes, revealed that sugar cane (73.40 IU/mg protein) and wheat bran (181.43 IU/mg protein) best sustained polygalacturonase and pectinase production respectively and when P.c was induced for both polygalacturonase and pectinase production, wheat bran best supported the production of both polygalacturonase and pectinase. The outcome of pH effect on pectinase production showed an optimal pH of 3.5 and 5.5 for A.n and P.c respectively.

Three 2 deoxy-D- glucose (2DG) UV mutants of *Aspergillus niger* and five 2DG UV mutants of *P. chrysogenum* were isolated post exposure of the strains to UV irradiation. The comparison of the pectinase production by the suspected mutants and wild strains revealed that; 2 DG UV M2 mutant of *Aspergillus niger* was selected as the best mutant with enzyme activities of 465% and 230% in solid state fermentation (SSF) and submerged fermentation (SmF) respectively, using wheat bran as the sole carbon source. *P. chrysogenum* 2DG-UV W1 gave a percentage enzyme activity of 218% in solid state fermentation, and 2DG-UV W2 gave a better pectinase yield (135%) in submerged fermentation over 2DG-UV W1 (122.58%). The glucose showed no catabolite repression (CR) on pectinase production abilities of both the 2DG UV M2 and 2DG UV W1. However, sucrose repressed pectinase production in both the wild and the 2DG UV W1 mutant strain of *P.chrysogenum*, while 2DG UV M2 of *A. niger* was able to produce pectinase. The results of RAPD-PCR on possible polymorphisms showed that there was considerable genetic variation between the wild and mutant strains of *A. niger* and *P.chrysogenum* using OPA 02, OPC 02, OPE 02 and OPA 04.

A purification fold of 7.94 and 4.7 were achieved for Pga of 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*) respectively. The molecular weight of Pga of 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*) were 105 and 95 kDa respectively. An optimal temperature of 40^{o}C was recorded for the Pga of both mutant strains, and the Pga retained 60% of its activity at 80^{o}C and an optimal pH of 4.5 and 5.0 for 2 DG UV M2 (*A.niger*) and 2 DG UV W1 *(P.chrysogenum*) respectively. The Vmax of 156.25 IU/ mg protein was obtained for both Pga of 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*) and Km of 1.27 and 2.6 g/l were obtained for 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*) respectively. It was also established that Mg^{2+} activated the Pga of both 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*).

This study showed that the natural process of biodegradation could be harnessed for the biotransformation of agro waste materials into a valuable resource. There is a good prospect for agro wastes as the sole carbon source for pectinase production and the use of UV irradiation in hyper-pectinase induction in wild mutants.

CHAPTER ONE

INTRODUCTION

1.1. Background of study

The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues. These materials have attracted considerable attention as an alternative feedstock and energy source, since they are abundantly available (Pedrolli *et al.*, 2008).

In Nigeria, agricultural and forest materials are dumped indiscriminately without considering the health and environmental implications (Agwu and Kalu, 2012). Over 80% of the annual average of 2.53 mega tons of municipal wastes collected for disposal in Lagos, Nigeria consists of cellulosic biomass (LAWMA, 2004). Agro-industrial wastes are generated during the industrial processing of agricultural or animal products. Those derived from agricultural activities include materials such as straw, stem, stalk, leaves, husk, shell, peel, lint, seed/stones, pulp or stubble from fruits, legumes or cereals (rice, wheat, corn, sorghum, barley etc.), bagasses generated from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many others (Mussatto and Teixeira, 2010). These wastes are generated in large amounts throughout the year, and are the most abundant renewable resources on earth. They are mainly composed of sugars, fibres, proteins, and minerals, which are compounds of industrial interest (Bhatia *et al.*, 2012). Due to the large availability of agricultural wastes and its rich composition in compounds that could be used in other processes, there is a great interest on the reuse of these wastes, both from economical and environmental viewpoints (Mussatto *et al.*, 2012). The economical aspect is based on the fact that such wastes may be used as low-cost raw materials for the production of other value-added compounds, with the expectancy of reducing the production costs of such products. The environmental concern is that most of the agro-industrial

wastes contain phenolic compounds and/or other compounds of toxic potential; which may cause deterioration of the environment when the waste is discharged to the nature (Martins *et al.,* 2011; Mussatto *et al.,* 2012). Improving the value of these wastes for economic and ecological gains has become a priority in most large biomass generating countries. The residual plant biomass can potentially be converted into various value-added products such as biofuel, chemicals, cheap carbon and energy sources for fermentation, improved animal feeds and human nutrients (FitzPatrick *et al.,* 2010; Zhu and Pan, 2010).

Conversion of agro wastes into such important economic products would alleviate food energy shortages, reduce pollution and transform the renewable organic matter into a valuable resource. Bioconversion of these wastes cannot be achieved without the presence of enzymes. Enzymes are among the most important products obtained for human needs via microbial sources. The utilization of the metabolic flexibility of microorganisms is advantageous in biological waste treatment; large number of microorganisms, such as bacteria, yeast and fungi, produce different types of enzymes such as cellulase, pectinase, ligninase, amylase and lipases (Sharma *et al.,* *2*011).

These enzymes also have significant potential applications in various industries including chemicals, fuel, food, beer and wine, animal feed, textile and laundry, pulp and paper and agriculture (Patil and Chaudhari, 2010).

Pectinases are one of the most important groups of enzymes used in the fruit and vegetable industry for increasing juice yield and juice clarification. The term pectinase refers to a mixture of specific enzymes that break down pectin by hydrolyzing the polymer backbone and its side chains (Favela-Torres *et al.,* 2006). The main chain of pectin is partially methyl-esterified-1, 4, D-galacturonan. Pectin is also known to contain other neutral sugars which are present in side chains.

Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations (Kaur *et al.,* 2004). These enzymes act on pectin, a class of complex polysaccharides found in the middle lamellae or primary cell walls of higher plants (Qureshi, *et al.,* 2012).

Among industrial applications of pectinases is the use of these enzymes as an animal feed supplements. This usage of pectinases for ruminants' feed production can reduce the feed viscosity, (which increases absorption of nutrients), liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers and reduces the amount of faeces (Murad *et al.,* 2011).

The search for viable pectinolytic fungi has led to the issue of strain development; the generation of mutant strains for industrial processes is a vital component of process biotechnology. The purpose of any industrial fermentation process is to produce in large quantities a given product. Under normal metabolic controls, the identified organism will produce the required enzyme, but in small, often insignificant amounts. To achieve commercial production, the organism must be made to overproduce the required product(s) (Lenihan *et al.,* 2010). Strain development refers to the process of improving the genetics of an organism so that it carries out a biotechnological process more effectively. This generates biological strains that can effectively produce desired enzymes or other proteins or carries out other desired specific functions. The aim of strain improvement is overproduction of the enzyme as the quantities produced by wild strains are usually too low.

In view of the utilization of agro wastes and industrial importance of pectinases, the present research was conducted with the objective of isolating, evaluating and improving the production of microbial pectinases by making use of different agro wastes and strain improvement using ultra violet (UV) radiation.

1.2. STATEMENT OF THE PROBLEM

Agricultural production generates large amounts of agricultural waste, some of it is recycled into production of organic fertilizers, while large amounts remain unused – and in many instances pose a disposal problem and ultimately leads to pollution. Agricultural output from Nigeria has seen phenomenal growth; this is due primarily to research in seeds and increased population (Adofu *et al.,* 2012). Large amounts of biomass waste are generated yearly as a result of improved agricultural output. These biomass wastes are usually incinerated, despite being an attractive technological option for waste management; combustion-based processes for agro wastes treatment are a subject of intense debate around the world. Uncontrolled burning in the fields is not only a hazardous disposal solution - it is also wasting useful energy. In the absence of effective controls, harmful pollutants may be emitted into the air, land and water which may influence human health and environment.

The demand for healthy food is growing not only in Nigeria, but all over the world. This kind of diet obviously includes the consumption of various fresh fruits, fruits juices. Fruit wastes generated from peeling fruits and during production of fruit juices are discarded and left to deteriorate, thus becoming threat to the environment. In the sugar industry, significant amounts of bagasse (the waste after extraction of sugar) is an equally excellent fuel, however bagasse are discarded indiscriminately, and feasted upon by natural flora of biodegradation becoming menace to the society. Also sawdust from wood mill industries, are burnt producing enormous amount of carbon dioxide which is a major cause of global warming.

Major food and industrial enzymes are imported mainly from the United Kingdom, United States, Japan, Holland, and Denmark (Gene watch UK, 2009). The importation of these industrial enzymes, are not cost effective, the durability and stability of imported enzymes are most times questionable. Pectinase is among these imported enzymes, it is one of the most

important industrial enzymes produced for industrial purposes; its production occupies about 10% of the overall manufacturing of enzyme preparations and they share about 25% of global sale in the food enzymes of the overall manufactured enzyme preparations.

Pectinolytic enzymes are widely used in the food industry for juice and wine production (Saeide *et al.,* 2012). It is imperative to look into the possibility of recycling or reusing agro wastes for industrial purposes and locally producing pectinase (a major industrial enzyme) by harnessing Nigeria's rich microbial flora and huge biomass. There is a need, therefore, to characterized viable organisms, capable of rapid growth and large extracellular enzyme production in Nigeria In addition, it is important to check the use of various agro wastes for cost-effective production of these enzymes.

1.3. AIM AND OBJECTIVES OF THE STUDY

Aim

The overall aim of this research work is to evaluate pectinase production and hyper production from microfungi using agro wastes as enzyme inducers.

OBJECTIVES OF THE STUDY

The specific objectives of the study are to:

1. Isolate microfungi from decaying agro wastes and investigate the potential value of agro wastes as a suitable source of nutrient (carbon) for enzyme induction.
2. Explore pectinase production by isolated microfungi and evaluate the effect of fermentation parameters on the enzyme activity.
3. Enhance pectinase production by the selected isolates (fungi) via strain improvement (UV mutation) and purified polygalacturonase.
4. Investigate the possible distinction between the mutants and wild strains of the microfungi using Randomly Amplified Polymorphic Deoxyribonucleic acid-Polymerase Chain Reaction (RAPD-PCR).

1.4. SIGNIFICANCE OF STUDY

This research is designed to evaluate and screen the fungi for pectinase production using agro wastes as an enzyme inducer in Nigeria. The significance of the research study are as follows:

1. Fungi are known to produce a large amount of extra-cellular enzymes during the process of biodegradation. These microfungi can be isolated from decaying agro wastes and then used as industrially important enzyme producers.
2. The use of agro wastes as enzyme inducers will increase the value of these wastes and provide a cheaper source of carbon (nutrients) in lieu of simple sugars (such as glucose, sucrose) for enzyme production.
3. Pectinase is one of the most important industrial enzymes with a wide range of applications; Pectinases share about 25% of global sale in the food enzymes of the overall manufactured enzyme preparations (Saeide *et al.*, 2012). These enzymes are generally imported from overseas and are very expensive. Sometimes they lose their activity before getting to the industries. This problem can be ameliorated only by locally producing enzymes for industrial purposes.

CHAPTER TWO

LITERATURE REVIEW

The advent of alternative energy sources (biofuels) in lieu of fossil fuels has increased the utilization of renewable biomass generated from agricultural and forest residues. The major components of these residues are cellulose, lignin and pectin. These biomass are receiving more attention as an alternative feedstock and energy source (since they are abundantly available) thereby reducing their impact as wastes constituting environmental hazards. Large amount of wastes are generated every year from the industrial processing of agricultural raw materials. Most of these wastes are used as animal feed or burnt as alternative for elimination. The presence of carbon sources, nutrients and moisture in these wastes provides conditions suitable for the growth of microorganisms, and this opens up great possibilities for their reuse as bioproducts resource.

Several microorganisms are capable of using these wastes as energy sources by producing a vast array of enzymes in different environmental conditions. The advantage of using microorganisms for the production of enzymes is that, these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield.

2.1. Biomass (Agro Wastes) Generation

Growth in human population, increasing urbanization and rising standards of living due to technological innovations have contributed to an increase in the quantity of a variety of solid wastes generated by industrial, mining, domestic and agricultural activities. An estimated 5 billion metric tons of agricultural biomass waste is produced annually. The thermal equivalent of approximately 1.2 billion tons of oil or 25% of current global production (UNEP, 2011).

The urban waste mainly consists of organic matter (46%), paper (6%), glass (0.7%), rags (3.2%), plastic (1%) and the rest is moisture (Mtui, 2009). Agricultural wastes (lignocellulosic wastes) refer to the plant biomass wastes that are composed of cellulose, hemicelluloses and lignin (Mtui, 2009). They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, peelings, cobs, stalks, nutshells, non food seeds, bagasse, domestic wastes (lignocelluloses garbage and sewage), food industry residues, municipal solid wastes (Rodríguez *et al.,* 2008). Table 2.1 illustrates the types and nature of solid wastes generated and their recycling potentials.

Table 2.1: Types and nature of solid wastes and their recycling and utilization potential

	TYPES OF SOLID WASTE	SOURCE DETAILS	RECYCLING AND UTILIZATION IN BUILDING APPLICATION
1	Agro waste (organic nature)	Bagasse, rice, wheat straw and husk, cotton stalk, sawdust wastes, peanut shell, banana stalk and jute, sisal and vegetable residues	Particle boards, Insulation boards, paper, roofing sheets, fuel, bricks, acids proof. Etc.
2.	Industrial wastes	Coal combustion residues, steel stag, bauxite red mud, construction debris	Cement, bricks, blocks, tiles, paint, aggregate, concrete, wood substitute products, ceramics products.
3.	Mining/Mineral waste	Coal washeries waste, mining overburden waste	Bricks, tiles, lightweight aggregates, fuel
4.	Non hazardous other process wastes	Waste gypsum, lime sludge, lime stone waste, marble processing residue, broken glass, ceramic and kiln dust.	Gypsum plaster, fibrous gypsum boards, bricks, blocks, cement, and hydraulic binder
5.	Hazardous wastes	Metallurgical residues, galvanizing wastes, tamery waste	Cement, brick, tiles, ceramic and board

Source: Rodríguez *et al.*, 2008

The lignocellulosic biomass represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Demirbas, 2008). This biomass is mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing lignocellulosic wastes (LCW) for the production and recovery of many value-added products. Among the main recovery products are enzymes, reducing sugars, furfural, ethanol, protein and amino acids, carbohydrates, lipids, organic acids, phenols, activated carbon, degradable plastic composites, cosmetics, biosorbent, resins, medicines, foods and feeds, methane, biopesticides, secondary metabolites, surfactants, fertilizer and other miscellaneous products (Demirbas, 2008; Mtui, 2009).

It is envisaged that the total solid wastes from municipal, agricultural, nonhazardous and hazardous wastes generated from different industrial processes in Nigeria seem to be even higher than the earlier reported data (Ukpong and Udofia, 2011). Already accumulated solid wastes and their increasing annual production are a major source of pollution. Ogwueleka (2009) observed that solid waste management in Nigeria is characterized by inefficient collection methods, insufficient coverage of the collection system and improper disposal. While Babayemi and Dauda (2009) decried the complete lack of efficient and modern technology for the management of waste.

One major way of turning agro wastes into great assets is by exploiting the natural forces of biodegradation. Production of enzymes from agro wastes could be important because they contain large amounts of cellulose, and hemicellulose, which could serve as inducers for the production of cellulase, hemicellulases such as xylanase and pectinases (Sharma *et al.,* 2011, Rodríguez-Fernández *et al.,* 2011; Abdel-Monem *et al.,* 2012). Microorganisms are widely accepted as the best agents for the production of enzymes from agro wastes. Though bacteria are known to

produce industrial enzymes, fungi are desired for the production of enzymes because their nature is generally regarded as safe (GRAS) (CRENOVO 2012)

2.2. Lignocellulosic Wastes

Natural lignocellulosic biomass consists of about 25 to 50% cellulose, 20 to 40% hemicelluloses, and 5 to 35 % lignin (Ioelovich and Morag, 2012). The major components of lignocellulosics; cellulose and hemicelluloses are hydrolyzed to soluble monomeric sugars (hexoses and pentoses) using cellulases and hemicellulases, respectively. The use of enzymes in the hydrolysis of cellulose is more effective than the use of inorganic catalysts, because enzymes are highly specific and can work at mild process conditions. In spite of these advantages, the use of enzymes in industrial processes is still limited by several factors, for example, most enzymes are relatively unstable at high temperatures, low enzyme yield, the costs of enzyme isolation and purification. (Verardi *et al.,* 2012).

The lignocellulosic biomass which represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2008). It is mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing lignocellulosic wastes (LCW) for the production and recovery of many value-added products (Foyle *et al.,* 2007). Among the main recovery products include enzymes, reducing sugars, furfural, ethanol, protein and amino acids, carbohydrates, lipids, organic acids, phenols, activated carbon, degradable plastic composites, cosmetics, biosorbent, resins, medicines, foods and feeds, methane, biopesticides, biopromoters, secondary metabolites, surfactants, fertilizer and other miscellaneous products (Galbe and Zacchi, 2007; Ubalua, 2007; Demirbas, 2008; Mtui, 2009).

A lot of emphasis has been given to screening of the agricultural wastes for release of sugars by hydrolysis of lignocellulosics which can be used for production of alcohol and other chemicals.

The hydrolysis of the carbohydrates in the lignocellulosic materials yields several products. For instance, xylan/xylose contained in hemicelluloses can be thermally transformed into furans, short chain organic acids (formic, acetic, and propionic acids), and Keto compounds (hydroxy-1-propanone, hydroxy-1-butanone, etc.) (Güllü, 2010; Bozell and Petersen, 2010).

Many fungal species such as *Trichoderma*, *Penicillium*, *Aspergillus* and *T. emersonii* are able to produce extracellular cellulases and hemicellulases. High temperature and low pH tolerant enzymes are preferred for the hydrolysis due to the fact that most current pretreatment strategies of lignocellulosic wastes rely on acid and heat (Mtui, 2009). Due to the promising thermostability and acidic tolerance of thermophilic fungal enzymes, they have good potential to be used for hydrolysis of lignocellulosic residues at industrial scales.

2.2.1. Composition of Agro wastes

The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues, the major components of which are cellulose, starch, lignin, xylan and pectin (Ioelovich and Morag, 2012). Cellulose represents the major constituent of cell wall polysaccharides and consists of a linear polymer of β-1,4-linked D-glucose residues. The cellulose polymers are present as ordered structures (fibers), and their main function is to ensure the rigidity of the plant cell wall. Hemicelluloses are more heterogeneous polysaccharides and are the second most abundant organic structure in the plant cell wall. Figure 2.1 shows the primary cell wall of flowering plants.

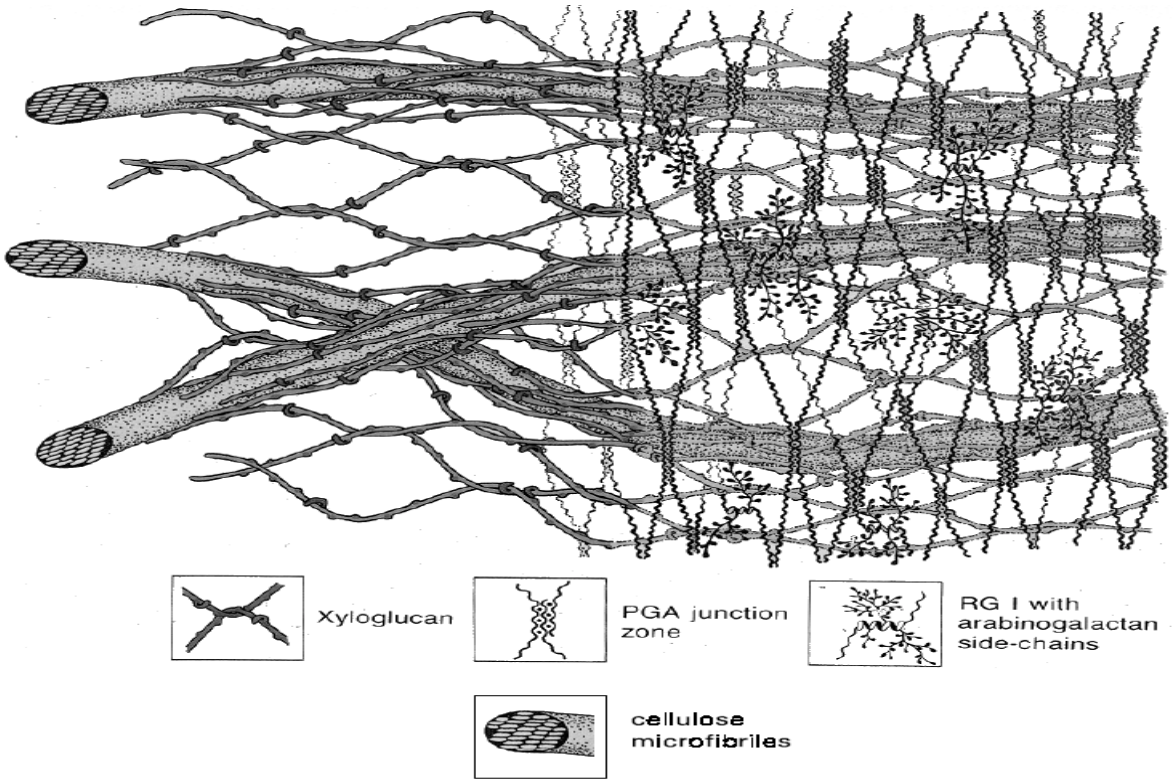


Figure 2.1: The primary cell wall of most flowering plants. The cellulose microfibriles are interlaced with xyloglucan polymers, and this framework is embedded in a matrix of pectic polysaccharides, polygalacturonic acid and rhamnogalacturonan. (Adapted from Carpita and Gibeaut, 1993).

Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells (Pedrolli *et al.*, 2009). Pectin is composed of six covalently linked substructures that are arranged to form a backbone substituted with side chains. Three pectic structural elements (homogalacturonan, rhamnogalacturonan-I and substituted galacturonans have been isolated from primary cell walls and were structurally characterized (Pedrolli *et al.*, 2009) (Figure 2.2). The

architecture of pectin is dependent on the plant species and tissue, resulting in strong variations in the ratio of these different polysaccharides as well as their level of substitution (Willats *et al.*, 2006; Pedrolli *et al.*, 2009). The possible cross links between the different polysaccharide constituents are still not fully elucidated and several different models have been proposed to describe pectin architecture (Coenen *et al.*, 2007). In addition, some proteins are known to be associated to pectin, such as extensin in sugar beet pectin (Nunez *et al.*, 2009).

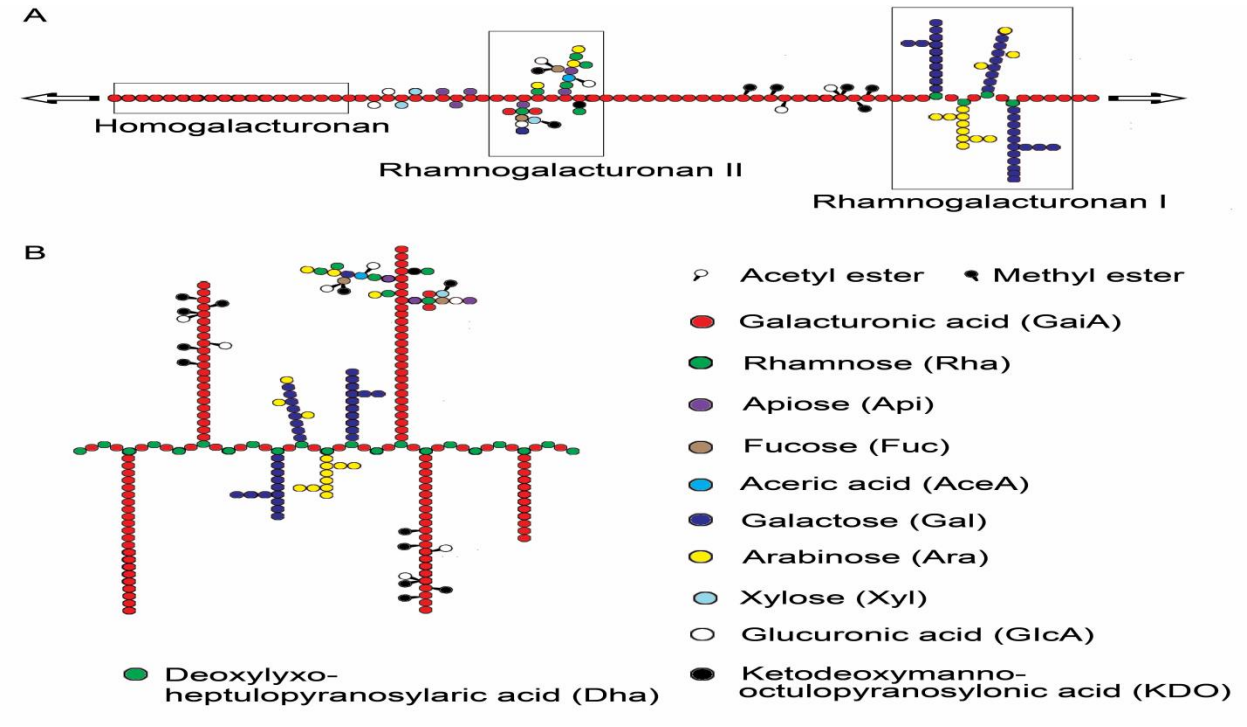


Figure 2.2: The basic structure of pectin. Schematic representations of the conventional (A) and recently proposed alternative (B) structures of pectin. The polymers shown here are intended only to illustrate some of the major domains found in most pectins rather than definitive structures. (Willats *et al.*, 2006).

Pectin is an important and versatile additive in several food types because of its hydrocolloid character that makes it a gelling, stabilizing or thickening agent. The emulsification properties of sugar beet pectin due to a high content of acetyl groups and proteins make it an interesting

components for microencapsulation (Drusch, 2007). As a dietary fibre naturally present in plant products, it also provides nutritional benefits in human diet.

Derivatives of pectic polymers and oligosaccharides from pectin were shown to have positive effects on human health. These included immuno-regulatory effects in the intestine, lowering the blood cholesterol level and slowing down the absorption of glucose in the serum of diabetic and obese patients (Glinsky and Raz, 2009; Holck *et al.,* 2011). A modified form of citrus pectin appears to be effective for a range of cancers at all stages of development and was patented as anti-cancer agent (Glinsky and Raz, 2009). Moreover, pectin oligosaccharides proved to be good probiotic compounds (Holck *et al.,* 2011).

2.3. Hydrolysis of Pectin

Enzymatic hydrolysis of pectin has advantages over chemical hydrolysis as enzymes target specific linkages of the pectin molecules while chemical methods are less specific (Battaglia *et al.,* 2011), this is especially important when partial hydrolysis is required to produce specific fragments. To meet the demand for specific pectin-derived oligosaccharides, specialized mixtures of pectinolytic enzymes are required that cleave only those linkages necessary to produce these oligosaccharides, but do not further hydrolyse them (Pedrolli *et al.,* 2009).

Most of the current commercial pectinolytic enzyme mixtures are produced by filamentous fungi. These organisms are very efficient in the degradation of plant cell wall polysaccharides and use a broad set of enzymes to convert them into monomeric sugars that can be taken up as nutrients. However, the composition of these enzyme sets differs significantly between fungal species and this is also observed for the subset of pectinolytic enzymes. For instance, Rhizopus spp. mainly degrades the homogalacturonan part of pectin, while Aspergilli produce enzymes to hydrolyse all pectic structural elements (Battaglia *et al.,* 2011). A better understanding of the correlation

between the make-up of these enzyme sets and hydrolysis of different structural elements of pectin is likely to result in novel strategies to produce tailor-made pectinase preparations for the production of specific pectin-oligosaccharides.

In recent Biotechnology, microbial derived pectinases have industrial exploitation and market potential. Pectinase are enzymes breaking pectic substances by hydrolysis of pectin and breakdown of complex polysaccharides in plant tissues into simpler molecules with extra ordinary specificity, catalytic power and substrate specificity (Chaudhri and Suneetha, 2012). Pectinases are produced from a wide variety of microorganisms including bacteria, fungi, actinomycetes, yeast (Suneetha and Khan, 2010). They are classified into polysaccharide hydrolases, polysaccharide lysases and carbohydrate esterases. The production of pectinase from microorganisms involves screening and characterization of microorganisms, while the downstream processes depend on the purity required. Pectinases have wide applications; they find use in fruit clarification, textile and food industries and water treatment. (Chaudhri and Suneetha, 2012). Pectins are complex polysaccharides consisting of partially methyl esterified α-(1,4) linked homogalacturonic acid backbone and branched neutral sugar side chains (Chaudhri and Suneetha, 2012).

2.4 Pectinolytic Enzymes

Pectinases are a group of enzymes that catalyse pectic substances through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions (Neagu *et al.,* 2012). The well-known pectinolytic enzymes are homogalacturonan degrading enzymes. Figure 2.3 shows the mode of action of the most studied pectinases.

2.4.1. Pectin Methyl Esterases (PME)

Pectin methyl esterase or pectinesterase (EC 3.1.1.11) catalyzes the deesterification of the methoxyl group of pectin forming pectic acid and methanol. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a non-esterified galacturonate unit. It acts before polygalacturonases and pectate lyases which need non-esterified substrates (Jayani *et al.,* 2005).

2.4.2. Polymethylgalacturonases (PMG)

Polymethylgalacturonase catalyzes the hydrolytic cleavage of α-1,4-glycosidic bonds in pectin backbone, preferentially highly esterified pectin, forming 6-methyl-D-galacturonate (Pedrolli *et al.,* 2009).

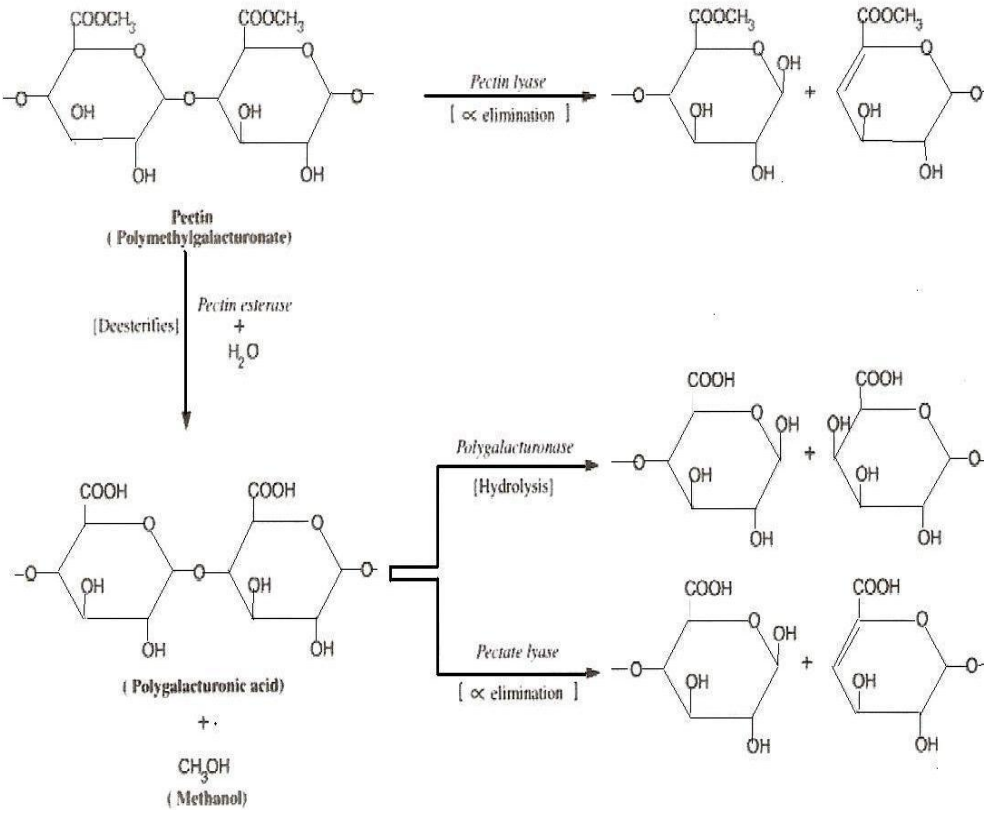


Figure 2.3: The mode of action of the most studied pectinases (Pedrolli *et al.,* 2009)

2.4.3. Polygalacturonases (PG)

Polygalacturonase catalyzes the hydrolysis of α-1,4-glycosidic linkages in polygalacturonic acid producing D-galacturonate. PMG and PG can act in an endo- or exo- mode. Endo-PG (EC 3.2.1.15) and endo-PMG catalyze the random cleavage of substrate, while, exo-PG (EC 3.2.1.67) and exo-PMG catalyze the hydrolytic cleavage at substrate nonreducing end producing monogalacturonate or digalacturonate in some cases (Pedrolli *et al.,* 2009). Hydrolases are produced mainly by fungi, and are more active on acid or neutral medium at temperatures between 40 ºC and 60 ºC (Zhang *et al.*, 2009).

2.4.4. Pectate Lyases (PGL)

Pectate lyase cleaves glycosidic linkages preferentially on polygalacturonic acid forming unsaturated product (Δ-4,5-D-galacturonate) through transelimination reaction. PGL has an absolute requirement of Ca^{2+} ions. Hence it is strongly inhibited by chelating agents as EDTA (Soriano *et al.*, 2006; Zhang *et al.*, 2009). Pectate lyases are classified as endo-PGL (EC 4.2.2.2) which acts towards substrate in a random way, and exo-PGL (EC 4.2.2.9) that catalyze substrate cleavage from non-reducing end (Pedrolli *et al.,* 2009; de Lima Damásio *et al.,* 2011).

2.4.5. Pectin Lyases (PL)

Pectin lyase catalyzes the random cleavage of pectin, preferentially high esterified pectin, producing unsaturated methyloligogalacturonates through transelimination of glycosidic linkages. PLs do not have an absolute requirement of calcium ion (Ca^{2+}) but they are stimulated by this and other cations (Zhang *et al.*, 2009). Up until now, all described pectin lyases are endo-PLs (EC 4.2.2.10). Pectin lyases are the only known pectinase capable of degrading pectin polymers directly via a β-elimination mechanism that results in the formation of 4,5-unsaturated oligogalacturonides without methanol production (Yadav, *et al.*, 2009). This is very important

because methanol's toxicity and unpleasant, volatile off flavors are a concern for the paper, food and textile industries (Taragano and Pelosof 1999; Mantovani *et al.,* 2005).

2.5. HYPER PRODUCTION OF ENZYMES (STRAIN IMPROVEMENT)

One of the key issues in fungal strain breeding is the generation of improved producer strains in terms of enzyme yield. Classical mutagenesis is a common tool that has been successfully applied for many microorganisms, including fungi, to improve the production of various industrial enzymes (e.g. glucoamylase, lipase and cellulase) (Mehdi *et al.,* 2009).

In the past, this has traditionally been achieved by a combination of classical mutagenesis during which the fungus was exposed to different mutagens such as X-rays, gamma-rays, UV rays, or chemicals, including N-methyl-N′-nitro-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS), in combination with different screening procedures to isolate cellulase-overexpressing strains (Seiboth *et al.,* 2011).

Many fungal strains have been subjected to extensive mutagenic studies due to their ability to secrete large amounts of ligninocellulosic-degrading enzymes. Kovács *et al.,* (2008) had shown that mutant of wild-type *Trichoderma atroviride* (F-1505) produces the most cellulase among 150 wild-type *Trichoderma*. Moreover, *T. atroviride* mutants were created by mutagenesis using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NTG) as well as UV-light. These *T. atroviride* mutants (e.g. *T. Atroviride* TUB F-1724) produce high levels of extracellular cellulases as well as β-glucosidase when grown on pretreated willow.

Cellulase and xylanase activities in *P. verruculosum* 28K mutants were improved about 3-fold using four cycles of UV mutagenesis. The enzyme production was further improved by 2- to 3-fold in a two-stage fermentation process using wheat bran, yeast extract medium and microcrystalline cellulose as the inducer (Solov'eva *et al.,* 2005). Silvia *et al.,* (2001) reported the

analysis of 18 strains of filamentous fungi, with the purpose of obtaining enzymes for textile fibers degumming. The strains were evaluated for production of pectinolytic enzymes under several growth conditions (culture medium and growth temperature). Among the tested strains, *P. chrysogenum* IFO 4626 (Q 176) showed the best performance. However, caution has to be taken during strain improvement by mutagenesis. Seidl, (2008) reported that the best *T. reesei* mutant (RUT-C30) lacks an 85 Kb genomic fragment and is consequently missing 29 genes which include transcription factors, metabolic enzymes and transport proteins. There is also possibility for repression /and activation of other gene (both wanted and unwanted genes).

Site-directed mutagenesis (SDM) plays a central role in the characterization and improvement of enzymes including their putative catalytic and binding residues. The application of SDM revealed that Glu 116 and 200 are the catalytic nucleophile and acid–base residues in *Hypocrea jecorina* (anamorph *T. reesei*) Cel12A, respectively (Okada *et al.,* 2000). In the study, mutant enzymes were produced where Glu was replaced by Asp or Gln at each position (E116D/Q and E200D/Q). The specific activity of these mutants was reduced by more than 98%, suggesting the critical role of these two residues in the catalytic function of the enzyme (Okada *et al.,* 2000). In another study, the thermostable endo-1,4-β-xylanase (XynII) mutants from *T. reesei* were further mutated to resist inactivation at high pH by using SDM. All mutants were resistant to thermal inactivation at alkaline pH. For example, thermotolerance for one mutant (P9) at pH 9 was increased approximately 4–5°C, resulting in better activity in sulphate pulp bleaching compared to the reference (Fenel *et al.,* 2006). Also, the catalytic efficiency and optimum pH of *T. reesei* endo-β-1,4-glucanase II were improved by saturation mutagenesis followed by random mutagenesis and two rounds of DNA shuffling. The pH optimum of the variant (Q139R/L218H/W276R/N342T) was shifted from 4.8 to 6.2, while the enzyme activity was improved more than 4.5-fold (Qin *et al.,* 2008). Moreover, the stability of *T. reesei* endo-1,4-

β-xylanases II (XynII) was increased by engineering a disulfide bridge at its N-terminal region. In fact, two amino acids (Thr-2 and Thr-28) in the enzyme were substituted by cysteine (T2C:T28C mutant) resulting in a 15°C increase in thermostability (Fenel *et al.,* 2004).

2.5.1. Co-culturing

Fungal co-culturing offers a means to improve hydrolysis of lignocellulosic residues, and also enhances product utilization which minimizes the need for additional enzymes in the bioconversion process. In the case of cellulose degradation, for example, all three enzymatic components (Endoglucanse-EG, Cellobiose hydrolase-CBH and β-glucosidase) have to be present in large amounts. However, none of the fungal strains, including the best mutants, are able to produce high levels of the enzymes at the same time. *T. reesei* for example produces CBH and EG in high quantities whereas its β-glucosidase activity was low (Stockton *et al.,* 1991). *A. niger* however, produces large amounts of β-glucosidase, but has limited EG components (Kumar *et al.,* 2008). In addition, hemicellulose hydrolysis must also be considered when lignocellulosic residues are subjected to biomass conversion. However, this will be determined by the pretreatment methods. Specifically in an alkali pretreatment method, a part of lignin will be removed and thus hemicellulose has to be degraded by the use of hemicellulases, whereas in acid-catalyzed pretreatment, the hemicellulose layer will be hydrolyzed (Hahn *et al.,* 2006). Again, some fungal strains have been shown to work more efficiently on cellulosic residues whereas others produce more hemicellulolytic enzymes and efficiently hydrolyze hemicellulosic portions (Howard *et al.,* 2003). Conversion of both cellulosic and hemicellulosic hydrolytic products in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. In fact, in nature, lignocellulosic residues are degraded by multiple co-existing lignocellulolytic microorganisms. Mixed fungal cultures have many advantages compared to their monocultures, including improving productivity, adaptability and substrate utilization.

Many studies have been conducted on the production of pectinases from various microorganisms internationally. But a few works have been published about cost-effective production of enzymes using agro wastes. There is also dearth of information on microbial strains capable of producing high yield of the enzymes in Nigerian. The difficulties to obtaining the appropriate agro wastes (substrate of enzyme induction) might be the biggest problem to developing such studies, a suitable substrate should be cost-effective and support the growth of the microorganism.

This study, reports the production of pectinases by a newly isolated strain of micro fungi from agro wastes s, purification of the pectinase produced and hyper production of the enzyme via strain improving using UV mutation. Keeping in view the economic importance of pectinases in the industry, the problems associated with the importation of enzymes and the disposal of agro wastes and food processing industry waste, the present study was undertaken with the set objectives.

CHAPTER THREE

MATERIALS AND METHODS

This research work involved microbiological, biochemical and other specialized forms of analyses. A wide array of equipment, glass wares, chemicals and reagents were used in the course of the research work.

3.1. Collection and Treatment of Agro waste Materials

Agro wastes materials used for the analyses were prepared as follows:

(i) Corncob

Fresh maize (*Zea mays*) was bought from Oshodi market, Lagos, Nigeria (6°33'23.2"N 3°21'02.9"E). The corncobs were obtained by removing the maize grains. The materials were cut into small pieces and oven dried for 7 h to reduce the moisture content to enhance grind. Grinding was done using Marlex grinder. Fine powder was obtained by passing the ground materials through a “fine mesh" sieve of about 0.5mm pore size.

(ii) Sawdust

Sawdust of abora wood (*Mitragyna ciliata*) was collected from Saw-Mills at Yaba, Lagos, Nigeria (6°30'29.6"N 3°22'13.1"E). This was dried, milled using the Marlex grinder and passed through the sieve to obtain the fine powder used for the studies.

(iii) Sugarcane pulp

Mature sugarcane stem (*Saccharum offinarum*) was purchased from Oshodi market in Lagos, Nigeria (6°33'23.2"N 3°21'02.9"E). Fibrous pulp of the sugarcane was obtained by crushing and washing the pulp repeatedly in water to remove all residual sugars. The pulp was sun-dried for 3-5 h, milled using the Marlex Excella Mixer Grinder (Mumbai, India) and passed through the sieve to obtain the fine powder used for the analyses.

(iv) Wheat Bran

Wheat shaft and wastes were collected from Mushin market in Lagos, Nigeria (6°33'23.2"N 3°21'02.9"E). The wheat bran were sieved and milled using local grinding machine, the bran was further sieved via a fine sieve mucilin cloth to obtain fine particles.

(v) Pineapple peel

Peels of pineapple were sourced and collected from hawkers and from the old Yaba market in Lagos, Nigeria (6°30'29.6"N 3°22'13.1"E). The peels were washed properly and sun dried, then washed again and oven dried at 65 ^{o}C for 24 h, the well dried peels were milled using the Marlex grinder and passed through the sieve to obtain the fine powder used for the analyses.

(vi) Orange peels

Peels of oranges were sourced and collected from hawkers and from the old Yaba market in Lagos Nigeria (6°30'29.6"N 3°22'13.1"E). The peels were washed properly and sun dried, then washed again and oven dried at 45^{o}C for 3-4 h, the well dried peels were milled using the Marlex Excella Mixer Grinder (Mumbai, India) and passed through the sieve to obtain the fine powder used for the analyses.

3.2. Proximate Analysis of Agro waste Samples

Proximate analysis or composition is usually carried out with representative samples. Hence a subsample of the thorough mixed, ground powder of various agro wastes was used. The ash, crude fibre, and moisture content were determined as described by AOAC (2005).

3.2.1. Lipid Content

Two grammes of the dried sample (Agro wastes sample) were weighed into an extraction thimble. The sample in the thimble was placed in a 100ml beaker and dried in mechanical convention oven (100^{0}C) for seven hours.

The thimble and the contents were removed from the oven into the Soxhlet apparatus .The sample was extracted with 10 ml of absolute ethanol for about seven hours at a condensation rate of about 240 drops per minutes. After the extraction, the extract was transferred into 52 g evaporating dish. The dish was placed in a fume chamber and the fan was switched on in order to evaporate the solvent. The dish and the content were dried in an oven (100^{o}C for 25 minutes), cooled and the weight was obtained.

Calculation:

$$\textbf{\% Crude Fat} = \frac{(X-Y)}{Z} \times 100$$

Where

x = weight (g) of dish and content after drying

y = weight (g) of empty evaporating dish

z = weight (g) of sample taken for analysis

3.2.2. Determination of Nitrogen and Crude Protein

The estimation of the protein content of the agro wastes were analyzed using the Kjeldahl method; which involves digestion, distillation and titration.

Digestion: Agro wastes sample powder (0.5g) was placed in a Pyrex digestion tube with 0.1g of catalyst (Copper sulfate) added and 1g of sodium sulphate and 20 ml of concentrated H_2SO_4 was carefully added to the mixture in the Kjeldahl flask. Some glass beads were then added. The tube was placed in a digestion rack and heated on a heating mantle in the fume cupboard.

The mixture was heated for 2 hours until a pale green solution was observed. This was cooled and diluted to 250ml with NH_3- free distilled water.

Distillation:

A total of 100ml of the reaction mixture was transferred into a Kjeldahl distillation flask. 50ml of 40% NaOH was added through a dropping funnel. A conical flask containing 10ml of boric acid was put at the delivery end of the condenser (receiver). The flask was heated gently in order to distill the content into the conical flask containing boric acid. The distillation process was stopped when the volume of the liquid in the Kjeldahl flask had been reduced to half.

Titration: The ammonium borate produced was titrated against 0.1N HCl until the color changed from bluish green to pink.

Calculation:

$$\% \text{ Nitrogen} = \frac{\text{Titre value X 1.40 X Total volume of sample}}{\text{100 mg X Sample weight X Aliquot Volume Distilled}} \text{ X 100}$$

3.2.3: Ash Content

The ash content is an expression of minerals or inorganic residues of a biological material. It may not give the absolute values or content of inorganic matter because there may be volatilisation of some minerals during ashing.

A dry clean crucible was weighed (24 g). A 2.5 g of dried agro wastes sample was weighed into the crucible; the sample was dried in an oven (100^{o}C) for 3 hours and then removed from the oven. The crucible and the contents were placed into a muffle furnace at 560^{o}C for 7 hours until grayish-white residue was obtained which indicates the destruction of organic carbonaceous portion of the sample.

The hot crucible was removed from furnace using tongs and the ash obtained was moistened with some drops of water to expose any unashed carbon present. It was cooled completely in a desiccator.

The ash content was weighed and determined.

$$\% \text{ Ash} = \frac{X-Y}{Z} * 100$$

Where

x = weight (g) of dish and content after drying

y = weight (g) of empty dish

z = weight (g) of sample taken for analysis

3.2.4. Moisture Content

The true moisture content of biological material is not easy to obtain as part of the water may be highly bound to the material. However, it is possible to determine the unbound or integrated water component in the structure of the sample.

Agro wastes sample powder (3 g) was weighed into an empty dish. The sample in the dish was dried in an oven at 100^{o}C for about 17 hours. The sample was removed from the oven after drying. The dish was placed in a desiccator and cooled to room temperature.

The weight of the content was determined.

$$\% \text{ Moisture} = \frac{\text{Loss of Weight X 100}}{\text{Weight of Sample}}$$

3.2.5. Determination of Crude Fibre

The sample (3.0015 g) was weighed into 250 ml conical flask. 10ml of petroleum ether (B.P 60^{o}C) was poured into the flask and stirred. The sample was allowed to settle and the ether was decanted. It was dried in freely circulating air. 200ml of 1.25% H_2S0_4 was carefully added to the dried sample with a dispenser. Six drops of 2% silicon in CCl_4 solvent was added. The mixture was boiled gently

for 30 minutes under the cold-finger condenser, and then filtered with filter paper. At room temperature, 200ml of 1.25% NaOH was measured and it was used to wash the sample back into the conical flask and boiled for 30 minutes under the cold-finger condenser.

All the insoluble matter was then transferred to the sintered crucible, washed with boiling water, 1% HCl and alcohol, then oven dried at 105^{o}C for about 10 minutes. The sample was ashed in a muffle furnace at 560^{0}C for about 1 hour, Cooled in a desiccator and weighed.

$$\% \text{ Crude Fibre} = \frac{y - a}{x} \times 100$$

Where

a = weight (g) of ash

x = weight (g) of sample used for analysis

y = weight (g) of insoluble matter

3.2.6. Carbohydrate Determination

Percentage of carbohydrates was obtained by subtracting the percentage of other components from one hundred percent.

% CHO= (100 - % ash+ % moisture + % lipid +% protein + % crude fibre).

3.3. Media Preparations.

Different media (both solid and liquid) were prepared for the various analyses. The solid media contained agar which was absent in the liquid media (broth). Each of the media was prepared as described below.

For solid media; the medium was sterilized by autoclaving at 121^{o}C for 15 mins. The cooled medium was distributed into sterile Petri dishes (about 10 - 15 ml/dish) and allowed to gel. While for liquid media; medium was distributed into 330 ml sterile bottles (Fermentation tank) and autoclaved at 121^{O}C for 15 minutes allowed to cool before inoculation with appropriate fungi.

The composition of the media include:

(a) Potato Dextrose Agar (PDA).

Thirty-nine grams (39.0 g) of Potato dextrose agar was dissolved in distilled water and made up to a liter with sterile distilled water. This medium, which supports the growth of most fungi, contains per liter: Potato Infusion (from 200 g potatoes); Glucose, 20.0 g; and Agar, 15.0 g. The medium was supplemented with 0.05% (w/v) Ampicillin to inhibit bacterial growth. Its pH was adjusted to 5.5.

(b) Czapek-Dox Agar (CDA).

Czapek-dox agar (CDA) consists of (per liter of distilled water): Sucrose, 30.0 g; $NaNO_3$, 2.0 g; KH_2PO_4,1.0 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g and Agar (No.2), 20.0 g. The media was supplemented with (1.0 mL per liter) of trace solution containing ZnSO4, 1.0 g and CuSO4. $5H_2O$, 0.5 g. 0.05% (w/v) Ampicillin was added to inhibit bacterial growth.

3.3.1. Basal Mineral Salt Agar (MSA-PEC)

The basal media were used principally for screening the wood-wastes to isolate the pectinolytic fungi. The mineral salt agar media, which was a modification of the medium described by Phutela *et al., (*2005), contained (gram per liter of distilled water): Pectin, 10.0; Peptone, 3.0; Yeast Extract, 2.0; KCl, 0.5; $MgSO_4$. 7H2O, 0.5;$MnSO_4.5H_2O$, 0.01; $(NH_4)_2SO_4$, 2.0; supplemented with 1 ml mineral salt solution (composition g/100 ml $CuSO_4.5H_2O$,0.04 $FeSO_4$, 0.08; Na_2MoO_4, 0.08; $ZnSO_4$, 0.8; $Na_2B_4O_7$, 0.004, $MnSO_4$, 0.008), and 20.0 of agar, Ampicillin (100 mg/mL) was added to inhibit bacterial growth. The pH of each medium was adjusted to 5.6 using dilute HCl.

3.4. Isolation of Fungi

3.4.1. Microbial Source

Decomposing agro-wastes were collected from dump-sites at Alamutu fruit market Idi-Oro, Agege Motor road, Lagos, Nigeria. Stock culture of the decomposing fruit-waste was prepared by introducing 1.0 g of the sample aseptically into 9.0 ml of sterile distilled water in a sterile tube and shaking vigorously. The stock culture was used for the isolation of fungi.

3.4.2. Isolation of Pectinolytic Fungi

Freshly prepared stock culture of the decomposing fruit-wastes was inoculated on agar media Mineral salt agar containing-Pectin media (MSA-PEC) plates using sterile wire loop. It was incubated at 37^oC for 72 hr in order to determine the types of microorganisms present in the waste. Isolates were subcultured to obtain pure isolates.

3.4.3. Screening of Pectinolytic Microfungi

Mineral Salt Agar – Pectin (MSA-PEC) was used to select the best pectinolytic microfungi. Each pure isolate was screened for pectinase production using a modified plate method of Phutela *et al.,*(2005). The cleared zone formed around the isolates was determined by flooding the plates with Potassium Iodide-Iodine solution (1.0g Iodine, 5.0g KI and 330ml H_2O), for a period of 30 min and

the extent of cleared zone formed indicated the hydrolysis of pectin in media plate; which was measured as the ratio of the diameter of clearance (DC^1) to the diameter of the plate (Dp). The isolates with highest pectinolytic activity were used for the rest of the study.

3.5. Analysis of Growth Rate and Pectinase Production by the Pectinolytic Fungi

3.5.1. Analysis of Growth Rate

The growths of the selected microfungi were monitored in mineral salt broth containing glucose, sucrose and agro wastes. The growth was assessed based on turbidity measurement at an optical density (OD) of 530 nm for a period of 168 h (Nwodo *et al.,* 2007a).

The media used included:

Mineral Salt Broth – Corncob (MSB-CC).

This basal liquid medium (pH 5.6) contained corncob as sole carbon source

Mineral Salt Broth – Glucose (MSB-Gluc).

This basal liquid medium (pH 5.6) contained glucose as sole carbon source.

Mineral Salt Broth – Orange peels (MSB-OP).

This basal liquid medium (pH 5.6) contained orange peels as sole carbon source.

Mineral Salt Broth – Pineapple peels (MSB-PA).

This basal liquid medium (pH 5.6) contained pineapple peels as sole carbon source.

Mineral Salt Broth – Sawdust (MSB-SD).

This basal liquid medium (pH 5.6) contained sugarcane pulp as sole carbon source.

Mineral Salt Broth – Sucrose (MSB-Suc).

This basal liquid medium (pH 5.6) contained crystalline sucrose as sole carbon source.

Mineral Salt Broth – Sugarcane pulp (MSB-SP).

This basal liquid medium (pH 5.6) contained sugarcane pulp as sole carbon source.

Mineral Salt Broth – Wheat bran (MSB-WB).

This basal liquid medium (pH 5.6) contained wheat bran as sole carbon source.

3.5.2. Pectinase Production Studies

The production of pectinase using various agro-wastes (Pineapple peel, Orange peels, sawdust, sugarcane pulps and wheat bran) as sole carbon source was followed for 72 h using a modified Czapek-Dox medium.

The composition of the media include (per liter of distilled water): $NaNO_3$, 3.0 g; KCl, 0.5 g; KH_2PO_4, 1.0 g; $MnSO_4.7H_2O$ 0.5 g; FeSO4. $7H_2O$, 0.01 g; and a carbon source (Crystalline pectin or any other agro wastes material), 10.0 g. The media was supplemented with 1.0 ml (per liter) of trace solution containing $ZnSO_4$, 1.0 g and $CuSO_4$. $5H_2O$, 0.5 g. The modified Czapek-Dox Liquid Media included:

Modified Czapek-Dox Broth – Corncob (CDB_M-CC).

This medium contained corncob as sole carbon source.

Modified Czapek-Dox Broth – Orange peel (CDB_M-OP).

This medium contained milled orange peels as sole carbon source.

Modified Czapek-Dox Broth – Pectin (CDB_M-PEC).

This medium contained crystalline pectin as sole carbon source.

Modified Czapek-Dox Broth – Pineapple peels (CDB_M-PA).

This medium contained pineapple peels as sole carbon source.

Modified Czapek-Dox Broth – Sawdust (CDB_M-SD).

This medium contained sawdust as sole carbon source.

Modified Czapek-Dox Broth – Sugarcane Pulp (CDB_M-SP).

This medium contained sugarcane pulp as sole carbon source.

Modified Czapek-Dox Broth – Wheat bran (CDB_M-WB).

This medium contained orange peels as sole carbon source.

3.5.3. Effect of Physico-Chemical Parameters on Pectinase Production.

3.5.3.1. Nitrogenous sources:

The effect of nitrogen sources on the pectinase production of the pectinolytic fungi was studied by substituting $NaNO_3$ in the Czapek-Dox medium with the following nitrogenous salt; Urea, Peptone, Yeast extract, $(NH_4)_2SO_4$ and Soy meal while using pectin as sole carbon source. Dilutions of selected microfungi (1ml of 10^6) was used to inoculate the above media; the media were incubated at 37^{o}C for 72 h. The media were centrifuged at 4000 X g for 20 min and the clear extract was used as crude enzyme.

3.5.3.2. pH:

The effect of pH of Czapek-Dox media on pectinase production by the pectinolytic fungi at pH range of 3.5 to 8.5 was evaluated. The media were incubated at 37^{o}C for 72 h. The media were centrifuged at 4000 g for 20 min, and the clear extract was used as crude enzyme.

3.6. Hyper production of Pectinase Using UV Mutagenesis.

The pectinolytic fungi were subjected to UV radiation to generate mutants which were evaluated for hyperproduction of pectinase compared to the wild-type pectinolytic fungi using modified method of Santiago *et al.*,(2006). The steps taken involved the determination of the UV irradiation survival rate (time of exposure required for 80% of selected microfungi spores to be inactivated by UV irradiation). Four day old spores of the wild strain of selected pectinolytic microfungi were harvested from the stock culture media and counted using Neuber chamber, the concentration was adjusted to 1 X 10^6 spore/ml. The UV radiation was placed at 15 cm above the surface of the spores suspension. Radiation was delivered using UV lamp (13 W germicidal lamp) at an interval of 5 min, 4 ml of spore suspension was taken and kept in dark for 2 h and diluted to 10^3 spore/ml, 1ml

of this spore was used to inoculate the sterilized mineral salt media (MSM), pH 5.5 containing (g/l) Pectin, 10.0, 2-Deoxy glucose 0.001, KH_2PO_4 1.3, Na_2HPO_4 0.60, $(NH_4)_2SO_4$ 0.75, Urea 0.25, $MgSO_4$ 0.34, $CaCl_2$ 0.3 and Agar 20, then incubated at 30 ^{0}C for 48 h followed by colony count. Survival curves were plotted from colony counts on MSM plates.

3.6.1. Selection of Hyper Producing Mutants

The wild and the mutant strains were inoculated separately in MSBM (without agar) pH 5.5 containing (g/l) Pectin, 10.0, 2 Deoxy glucose 0.001, KH_2PO_4 1.3, Na_2HPO_4 0.60, $(NH_4)_2SO_4$ 0.75, Urea 0.25, $MgSO_4$ 0.34, $CaCl_2$ 0.3. Incubated at 30 ^{o}C for 72 h. The contents of the flasks were harvested after 72 h by centrifugation at 4000g for 10mins. The resultant clear extract was used as crude enzyme. The hyper-producing mutant was selected using the modified Cup-Plate method of Kastner *et al.,* 2007.

3.7. Characterization of Hyper-Producing Mutants

3.7.1. Effect of Catabolite Repression

The selection of major mutant strains for industrial production of enzyme, one key factor monitored is the effect of catabolite repression (CR). This study was carried out in SmF tanks containing pectin alone or in pectin plus varying concentrations of glucose (1, 5, and 10 g/l) and sucrose (1, 5, and 10 g/l). The wild and suspected mutant strains were inoculated into these media to test for the effect of CR on pectinase production. The contents of the flasks were harvested after 72 h by centrifugation at 4000g for 10mins. The resultant clear extract was used as crude enzyme.

3.8. Molecular Characterization of Fungi Strains

3.8.1. DNA Isolation Procedure:

Mycelia cultures were harvested from wheat broth grown for 24 to 36h in 100ml bottles containing 30ml of culture (Per litre: 2.5 g wheat, 0.2 g Yeast Extract, 0.14 g $(NH_4)_2S0_4$, 0.20 g KH_2P0_4 and 0.02 g $MgS0_4$). The mycelia mat was collected by filtration /decantation through Whatman paper, washed successfully with cold sterile water and cold acetone (10 ml acetone per gram wet weight of mycelia; 1:10, w/v) on a Buchner funnel. The shrunken mycelial mat was held for 3 h under cold acetone (1:10, w/v) at –20 ^{o}C. The mat was transferred to fresh cold acetone (1:10, w/v) and stored for a further 3h at –20 ^{o}C. The dehydrated mycelia so obtained were desicated under vacuum (to remove acetone vapors) for 20 min. The dried sample was either directly used or stored at –20^{o}C for future use.

About 200-500 mg of acetone dried mycelium material was added to 1.5 ml microcentrifuge tubes. 500 µl of a lysis solution containing 2 g of glass beads (1.0mm/0.5mm/0.1mm) for crushing of cell walls were also added. The tubes were then vortexed and homogenized for 10 min at maximum speed. The tubes were centrifuged (Eppendorf microcentrifuge) for 10 min at 11,000 g. After centrifugation, the supernatants were decanted into new tubes and the extraction procedure was repeated. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample; the samples were then vortexed briefly, and centrifuged for 5 min in a microcentrifuge. The aqueous layer was transferred to a new tube and extracted again with an equal volume of chloroform: isoamyl alcohol (24:1). The tubes were mixed vigorously and centrifuged for 5 min at 10,000 g. The supernatants were transferred to the new Eppendorf tubes, and 2.5 volumes of isopropanol were added for precipitation of DNA. The tubes were incubated in a refrigerator for 1 hour, and centrifuged at 4°C for 10 min at 14,000 g. The pellets were washed twice with cold 70% ethanol, air-dried, and then resuspended in sterile double deionised water. The samples were treated

with RNase (DNase free) (Jena Bioscience Jena, Germany). The subsequent DNA yields and quality were assessed by standard electrophoresis through a 1% (w/v) ethidium bromide-stained agarose gel.

The A_{260} / A_{280} ratios were used to determine DNA concentration and purity. This was done with a Nano Drop spectrophotometer ND1000 (Thermo fisher scientific. MA. USA). The subsequent DNA yields and quality were assessed by standard electrophoresis through a 1% (W/V) ethidium bromide-stained agarose gel.

3.9. Random Amplified Polymorphomic DNA Polymerase Chain Reaction (RAPD-PCR) Analysis:

The ten 10-mer random primers used in this study were selected from the Operon Kits and are listed in Table 3.1.

PCR reactions were carried out in a volume of 50 µl containing 5 µl of 10 x buffer, 50 mM of $MgCl_2$, 1 µl of 2 mM dNTPS, 1 µl of 10 µ M of each primer, 1 µl of 5U/mL Taq-polymerase, 1 µl of 10 ng template DNA and 41 µl of sterile double distilled water.

DNA amplification was performed in master cycler gradient machine programmed for 45 cycles. The cycling parameter used was one cycle of initial denaturation 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. This was then followed by a final extension of 72°C for 5 minutes.

Amplified samples were analyzed by electrophoresis in 1.8 % agarose gels. The RAPD patterns were visualized with a UV trans-illuminator and photographed using Kodak 13 mega pixel (Polaroid Camera MP4 land Camera).

The genetic distance from RAPD data for each RAPD primer was calculated using the equation:

$$D_{xy} = 1 - \frac{N_{xy}}{N_x + N_y - N_{xy}}$$

Where:

D_{xy} = the genetic distance between mutant (x) and wild (y) strains

N_{xy} = number of bands shared by the mutant and wild strains

N_x = number of bands in mutant (x)

N_y = number of bands in wild (y)

Table 3.1: List of RAPD primers used in this study

Primer	sequence 3' TO 5'
OPA-02	TGC CGA GAT G
OPA-04	AAT CGG GGT G
OPB-06	TGC TCT GCC C
OPB-08	GTC CAC ACG G
OPC-04	CCG CAT CTA C
OPC-10	TGT CTG GGT G
OPD-06	ACC TGA ACG G
OPD-14	CTT CCC CAA G
OPE-01	CCC AAG GTC C
OPE-02	GGT GCG GGA A

3.10. Partial Purification of the Pectinase (EC: 3.2.1.15)

Partial purification of the crude enzyme of the selected microfungi was achieved by the combination of the conventional purification methods. The crude enzyme was prepared using SSF for a period of 72 h.

3.10.1. Pectinase Production

SSF was carried out in 5 (250 ml) flat bottom shallow glass containers; using wheat bran medium containing: Wheat bran 30 g, and 20 ml of mineral salt medium containing (in g/l): $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.5 and $FeSO_4 \cdot 7H_2O$ 0.0005. The pH of the medium was adjusted to 5.0. After sterilization at 121 °C for 15 min, flasks were cooled, inoculated (1·105 spores/mL) and incubated at 30 °C for a period of 72 h. The contents of the flasks were harvested by adding 100 ml of 50 mM Na citrate buffer pH 5 and centrifuged at 5000 g for 10 mins, the clear filtrate was used as crude enzyme. The clear filtrates were pooled and freeze dried at 4 ^{o}C, to about 120 ml.

3.10.2. Ammonium Sulphate Precipitation

The freeze dried fractions (100 ml) of the crude enzyme was precipitated using 80% $(NH_4)_2SO_4$ with gentle agitation until final dissolution of the salt. The mixture was centrifuge at 1000 X g for 20 mins. The precipitate was reconstituted in 50 mM Na Citrate buffer pH 5 and dialyzed over-night at 4^{o}C against the same buffer.

3.10.3. Gel Filtration

Gel chromatography was carried out using Sephadex A25-120 and Sephadex G120 (Sigma). Ten grams (10 g) of the powder form of the gel was suspended in Tris-HCl buffer pH 8.3, to equilibrate and allowed to swell for 10 h. After swelling it was packed in a chromatography column of 1.5 × 70 cm. The column was eluted several times with 0.05 M citrate-phosphate buffer (pH 6.0). The void volume was determined with blue dextran.

Ten millilitres of the ammonium sulphate-dialysate enzyme concentrate was applied to the column and eluted with buffer. Fractions of 1 ml/min per tube were collected. The protein content was determined with UV spectrophotometer at 280 nm and polygalacturonase activity was assayed using Miller (1959) method. The enzyme peaks were pooled together and further subjected to gel filtration using Sephadex G120 using the same buffer as described above.

3.11: Properties of Pectinase (EC: 3.2.1.15)

The optimum temperature and pH values of the pectinase enzyme (EC: 3.2.1.15) were determined by measuring pectinase activity using polygalacturonic acid as substrate at various temperature and pH values. The effects of substrate concentration and metallic ions on pectinase activity were also determined. Maximum velocity (V max) and Michaelis Menten (*Km*) constant were obtained from the reciprocal plot of the effect of substrate concentration (Lineweaver –Burk plot).

3.11.1. Effect of pH and Temperature on Enzyme Activities

The enzyme extract was pre-incubated at different temperatures ranging from 30-80ºC for 30 mins and pectinase activity was assayed. The effect of pH on PG activities was studied between pH 3.0-9.0 using citrate/phosphate (pH 3.0-7.2) and Tris-HCl (pH 7.2-9.0) buffers (50 mM).

3.11.2. Effect of Substrate Concentration

The effect of various concentrations of polygalacturonic acid (0.1%-1%) on the enzyme activity was studied at optimum temperature and pH. The V max. and *Km* of the enzyme were obtained using the reciprocal plot (Line-weaver Burk-plot).

3.11.3. Effect of Metal Ions

The effect of cations on the enzyme activity was determined by incubating the enzyme-substrate mixture containing 2.0 mM salts of respective cations under standard assay conditions for a period of 30 mins. The salts include; $MgSO_4$, $MnSO_4$, $CuSO_4$ $CaSO_4$, and $FeSO_4$. $7H_2O$. The effect of the

metal chelator, EDTA on the enzyme activity was also determined by adding 10.0 mM of EDTA to the enzyme-substrate mixture.

3.11.4. Protein Molecular Weight Determination:

The Molecular weight of the partially purified enzyme was determined using the method of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as applied by Khairnar *et al.*, (2009).

SDS-PAGE.

The enzyme preparations were subjected to native polyacrylamide electrophoresis using the Hoefer SE 600 Ruby electrophoresis system. Tris-glycine buffer was used as the electrophoresis buffer. Approximately 50 µl of prepared sample was introduced into disc gel. Each sample was run in duplicate. Electrophoresis was initiated by applying a voltage of 60 Volts for 15 mins while the samples enter the gel and then voltage was increased to about 120 volts for another 45 mins in electrophoresis buffer. Gels were submerged in Coomassie Blue staining solution overnight while shaking slowly on a laboratory shaker (Chiltern). The bulk of stain was removed by replacing Coomassie Blue stain solution with Coomassie Blue destaining solution I, slowly shaken for 30 minutes. The Coomassie Blue destaining solution I was replaced with Coomassie Blue destaining solution II, shaken slowly until gel background became+ clear. Coomassie Blue destaining solution II may require changing during this step.

3.12. Pectinase Assay

Assays for crude pectinase and Polygalacturonase activities were performed using 0.5% citrus pectin (60% methylation) and polygalacturonic acid respectively (Sigma) in 50 mM sodium acetate buffer, pH 5.5. The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude enzyme. The mixture was incubated in a water bath at 40 ^{o}C for 30 mins . The reducing sugar released was measured by the 3,5- dinitrosalicylic acid (DNSA) method (Miller, 1959) as applied by Sharma, *et*

al., (2011) in which the reaction was stopped by adding 3 ml of DNSA acid reagent and boiled. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm with galacturonic acid as the standard. One unit (U) of pectinase activity is defined as the amount of enzyme that releases 1 μmol galacturonic acid/min/ml under the above mentioned conditions.

3.13. Protein Assay

The protein content of the culture supernatant was determined by the colorimetric method described by Lowry *et al.,* (1951), as applied by Nwodo *et al.,* (2007a). The protein concentration of the elution fractions were determined by optical density measurement at 280nm using Bovine serum albumin as protein standard.

3.14. Result Presentation

The data of the study were obtained in triplicates, unless otherwise stated. The results were expressed as Mean± SEM (Standard error on mean) using Statistical Package for the Social Science 15 for windows (SPSS 15.0).

CHAPTER FOUR:

RESULTS

4.1. Proximate Analysis of Agro Waste Materials

The proximate compositions of the agro waste materials (sawdust, sugarcane pulp, wheat bran, Corn cob, orange peels and Pineapple peels) are shown in Table 4.1. Generally, the materials contained low levels of moisture, ash and fat. High percentage crude protein content of 15.8 ± 0.7, 12.3 ± 0.4 and 13.6±0.7 were obtained for Wheat bran, Sugarcane pulp and Pineapple peels respectively. Percentage crude fibre contents of 61.0 ± 3.4, 42.1 ± 2.9, 42.6 ± 1.2 and 27.4 ± 1.5 were recorded for Sawdust, Sugarcane pulp Orange peel and Wheat bran respectively. The percentage carbohydrate was high in corn cob (49.4±1.5) Pineapple peels (36.3±2.2) and Wheat bran (35.1±2.3).

Table 4.1: Results of the proximate composition of the agro wastes

Agro wastes	Moisture (%)	Ash (%)	Fat (%)	Crude Protein (%)	Crude Fiber (%)	Carbohydrate (%)
Sawdust	6.1±0.6	1.1±0.1	6.4±0.4	3.1±0.2	61.0±3.4	22.3±1.8
Sugarcane pulp	13.5±0.8	1.7±0.1	9.0±0.5	12.3±0.4	42.1±2.9	21.2±2.4
Wheat Bran	10.9±0.8	5.1±0.3	5.7±0.6	15.8±0.7	27.4±1.5	35.1±2.3
Corn cob	3.9±0.9	3.2±0.4	7.2±0.2	3.5±0.4	32.8±1.8	49.4±1.5
Orange peels	3.2±0.4	7.5±1.2	5.6±0.5	8.4±0.6	42.6±1.2	32.7±1.1
Pineapple peels	4.3±0.5	6.5±0.9	7.3±0.7	13.6±0.7	32±1.9	36.3±2.2

4.2. Pectinolytic Microfungi Spectrum of Decomposing Agro Wastes

Different colonies of fungi and yeast appeared on the agar media (MSA-PEC) innoculated with the stock culture of decomposing fruit wastes after 72 h (plate 4.1). A total of five microfungi including one pathogenic Aspergillus; *Aspergillus clavatus* were isolated (plate 4.2). The other isolates were *Aspergillus niger* (plate 4.3), *Penicillium chrysogenum* (plate 4.4), *Trichoderma harzianum*. (plate 4.5), and *Fusarium species* (plate 4.6). The colonies characteristics, morphological features and economical importance of the isolates are illustrated in Table 4.2.

Plate 4.1: Plate shows mixed cultured of fungi isolated from decomposing agro wastes source 72 h post inoculation.

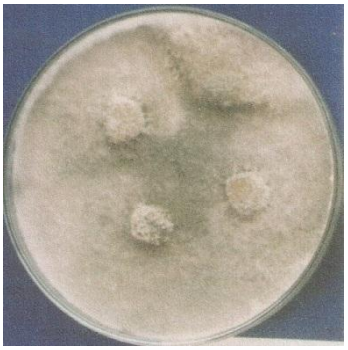
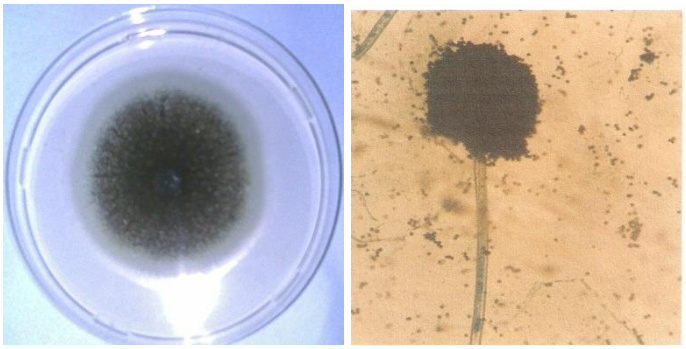
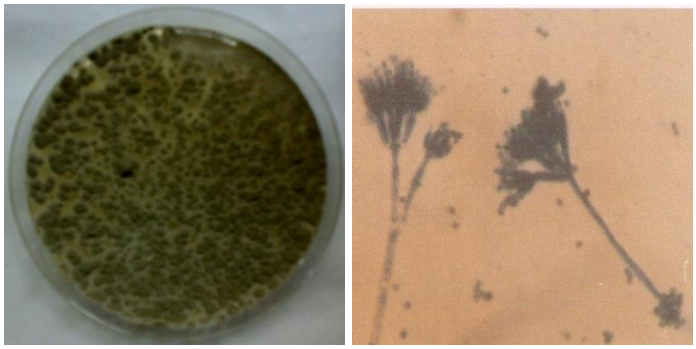


Plate 4. 2: Plate shows pure colony of *Aspergillus clavatus* on PDA



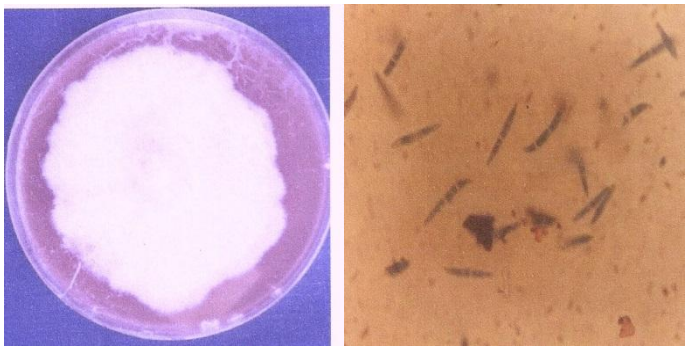
A **B**

Plate 4.3: Plate shows pure colony of *Aspergilus niger* on PDA (A) and micrograph of the spores (B) (X400).



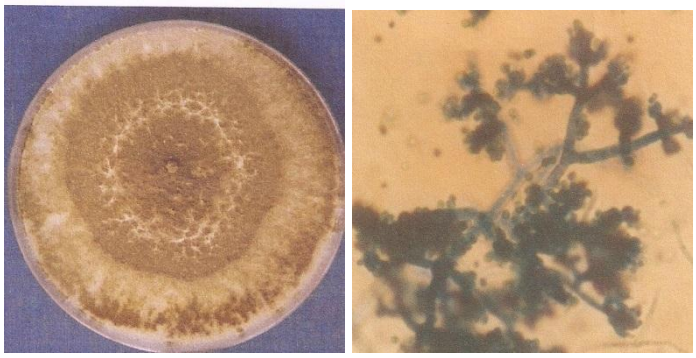
A **B**

Plate 4.4: Plate shows pure colony of *Penicillium chrysogenum* (A) on PDA and micrograph of the spores (B) (X400).



A **B**

Plate 4.5: Plate shows pure colony of *Fusarium sp.* on PDA (A) and micrograph of the spores (B) (X400)



A **B**

Plate 4.6: Plate shows pure colony of *Trichoderma harzianum* on PDA (A) and micrograph of the spores(B) (X400)

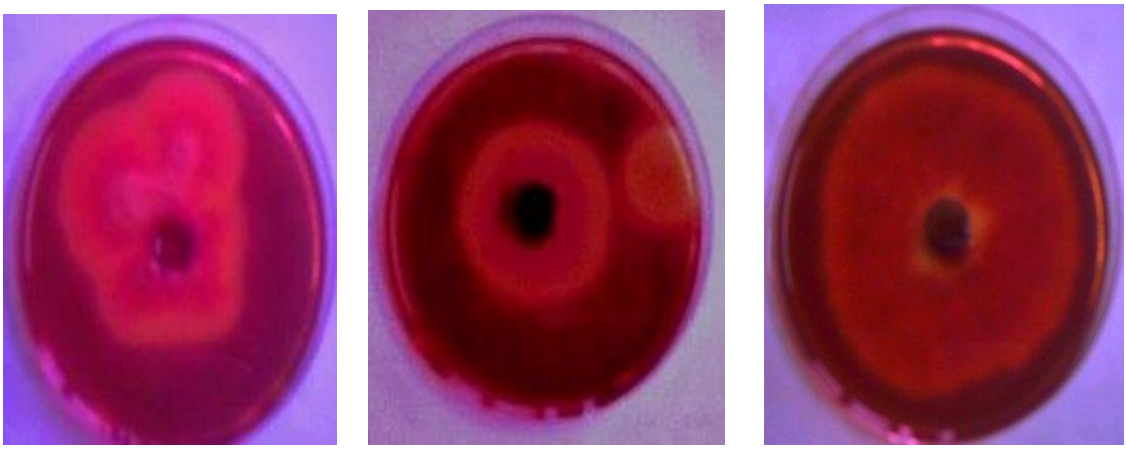
Table 4.2: Morphological Characteristics of pectinolytic fungi

ISOLATES	CHARACTERISTICS OF COLONY	MORPHOLOGICAL FEATURES
Aspergillus niger	Dark brown turns dark black, when old	Conidial heads are biseriate, large, globose, dark brown. Becomes radiate with the phialides borne on metulae
Penicillium chrysogenum	Greenish, turn gray when old	Septate hyaline hyphea, branched conidiophores and having a brush like conidial heads
Aspergillus clavatus	Grayish with long hyphae turns dark gray when old	Elongate, club-shaped vesicle at the apex of the tall, stout conidiophore
Fusarium sp.	White woolly mycelia turns pinkish	Hyphae are septate with micro conidia that are hyaline, curved and stout.
Trichoderma harzianum.	Gray turns dark gray-brown when old	Septate hyaline hyphea, with branched conidiophores with clustered conidial head

4.3. Screening of Pectinolytic Microfungi (Plate Assay Method):

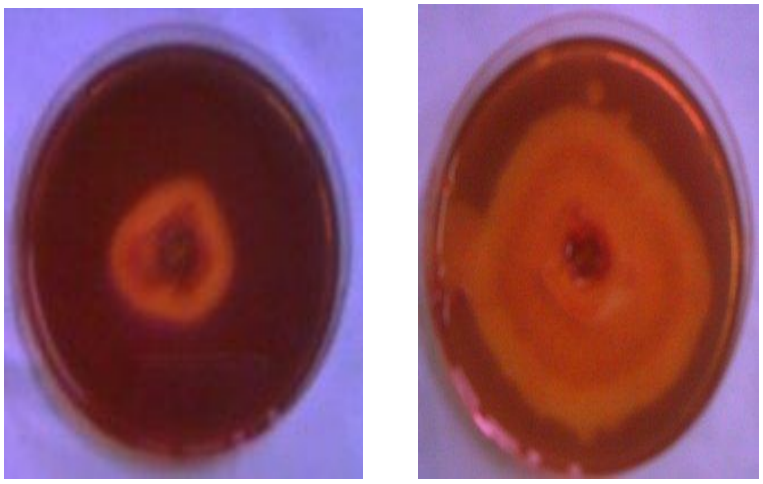
Results in Figure 4.1 show that the maximal pectinolytic activity (potency index-PI) was observed in isolate *Aspergillus niger* (PI.value-0.978) closely followed by *Penicillium chrysogenum* (PI. Value 0.957) and *Trichoderma harzianum.*(0.802). Minimum enzyme activity was observed in *A.clavatus and Fusarium sp.* (0.142 and 0.126 respectively).

A **B** **C**



Trichordema sp. *Aspergillus clavatus.* *Aspergillus niger*

D **E**



Fusarium sp. *Penicillium chrysogenum*

Figure 4.1: Preliminary screening of isolates for pectinase production using the plate assay method. PI. = Potency index is defined as the ratio of the diameter of clearance of pectin in the plate to the diameter of the plate.

4.4. Analysis of Growth Profile

A. niger and *P. chrysogenum* were selected as the best pectinolytic strains based on large clearance zones (over 60%).The growth profile of *A. niger* and *P. chrysogenum* were monitored for a period of 168 h at an interval of 24 h in all the agro wastes containing media. Changes in turbidity of culture media of the isolates in mineral salt broth containing various agro wastes were monitored. The growth profile was compared to the growth of the fungus in Czapek dox (with sucrose as the sole carbon source) and the glucose containing medium. It was observed that the fungi grew best in glucose than in the other agro wastes considered. Conversely the growth in wheat bran medium shows a promising agro wastes that can substitute glucose in both fungi cultivation Figures 4.2 and 4.3

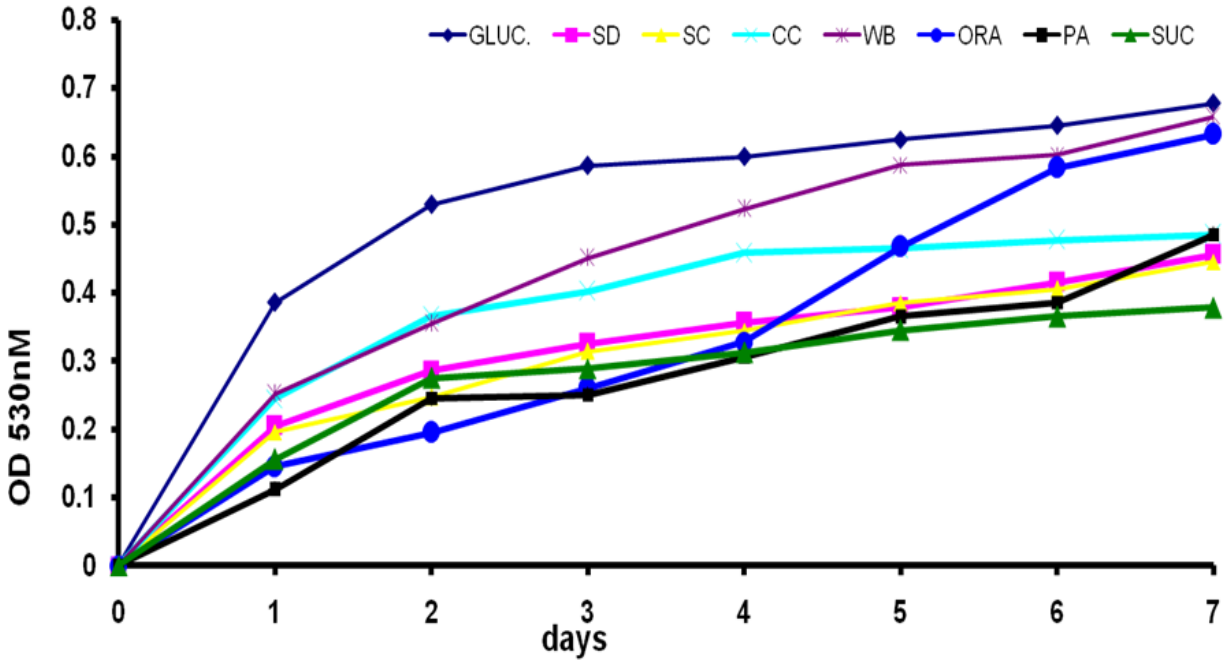


Figure 4. 2: The growth profile of *A. niger* in mineral salt broth containing glucose-Glu, Sucrose-Suc, Corn cob-CC, Sawdust-SD, Sugarcane Pulp -SP, Wheat bran-WB, Pine apple peels-PA, and Orange peels-ORA.

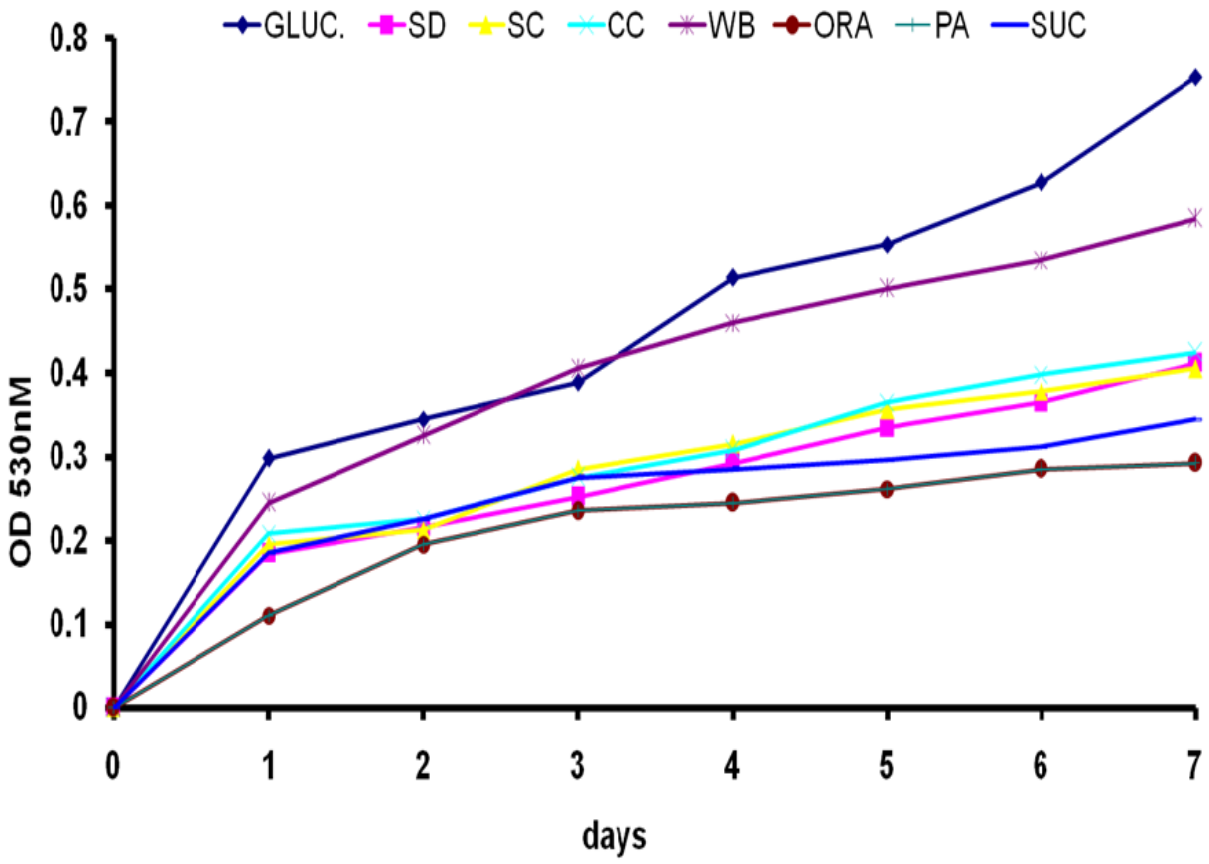


Figure 4.3: The growth profile of *P. chrysogenum in* mineral salt media containing glucose-Glu, Sucrose-Suc, Corn cob-CC, Sawdust-SD, Sugarcane Pulp -SP, Wheat bran-WB, Pine apple peels-PA, and Orange peels-ORA).

4.5. Pectinase Production Studies

It was observed that *Aspergillus niger* was able to produce pectinase in all agro wastes studied. However, the medium containing wheat bran as sole carbon source gave an optimum enzyme yield of 60.48 IU/ mg protein which is quite higher than the remaining agro wastes (figure 4.4). The specific activities of the pectinase are quite low in glucose, corncob, pineapple and sucrose containing media with a value of 4.05, 3.18, 3.34 and 7.89 IU/ mg protein respectively when *P.chrysogenum was used as the fermenter* (Figure 4.5).

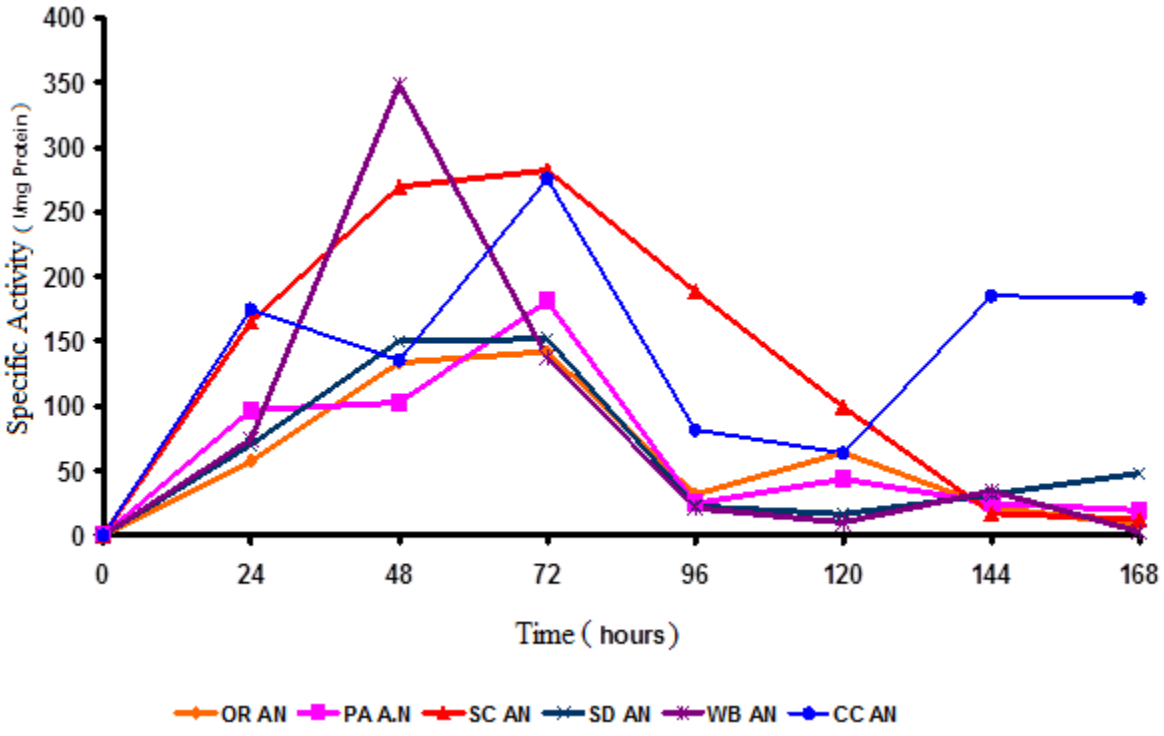


Figure 4.4: Specific Activities of pectinase produced by *Aspergillus niger* in culture media containing various Agro-wastes. NB: Corn cob-CC, Sawdust-SD, Sugarcane Pulp -SP, Wheat bran-WB, Pine apple peels-PA, and Orange peels-OR.

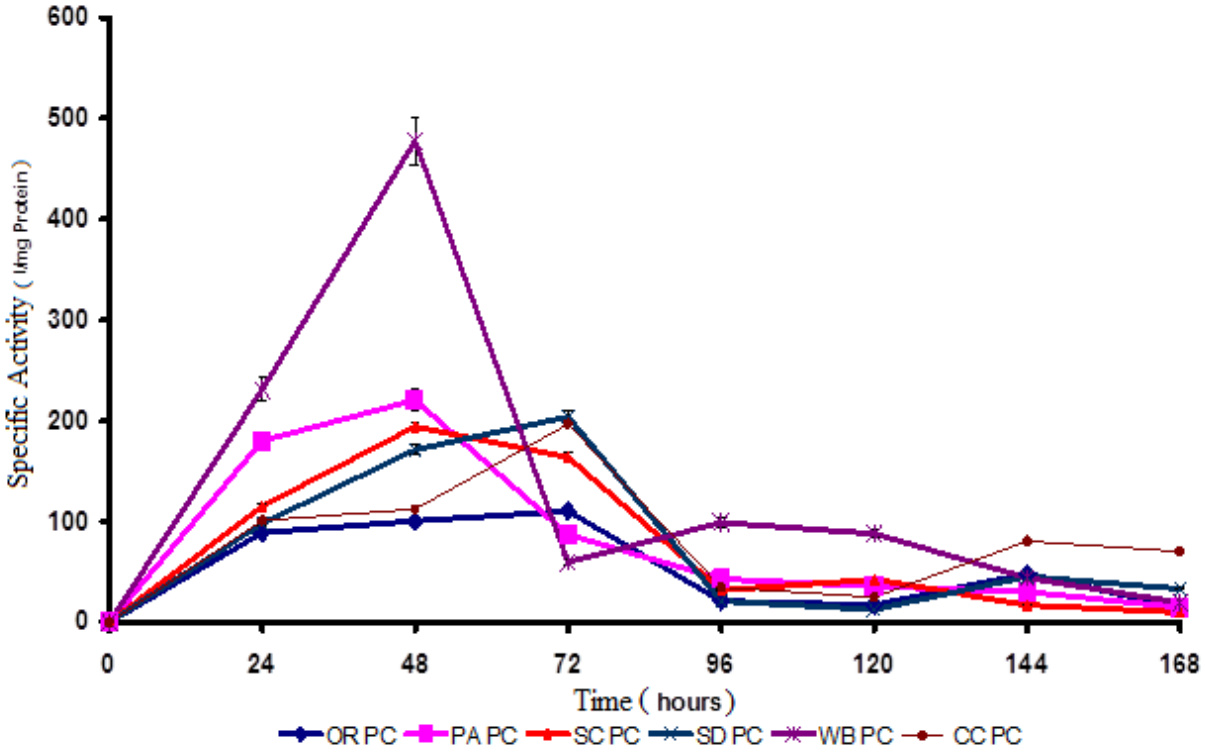


Figure 4.5: Specific Activities of pectinase produced by *Penicillium chrysogenum* in culture media containing various Agro-wastes. NB: Corn cob-CC, Sawdust-SD, Sugarcane Pulp -SP, Wheat bran-WB, Pine apple peels-PA, and Orange peels-OR

4.6. Effect of Physicochemical Parameters on Pectinase Production

4.6.1. Effect of Nitrogenous Sources

Figures 4.6 and 4.7 show the effects of the different nitrogenous salts and proteinous sources as sources of nitrogen on the production of pectinase by *A. niger* and *P. chrysogenum.*

It was observed that after 72 h of fermentation by *A. niger*, urea, best supported the production of pectinase (195.75 IU/ mg protein) while its polygalacturonase production by the addition of urea (33.52 IU/ mg Protein) was highly reduced. The result also showed that soy beans extract (SOY) gave a pectinase and polygalacturonase yield of 179.89 and 100.78 IU/ mg protein respectively, while peptone, and NH_2SO_4 also yielded pectinase activity of 187.21 and 183.91 IU/ mg Protein, however, the yield of polygalacturonase in media containing urea and NH_2SO_4 were low (34.64 and 75.48 IU/ mg protein respectively).

When *P. chrysogenum* was used as the fermentation organism, pectinase production was supported best by peptone 177.97 IU/ mg protein but its polygalacturonase activity was highly reduced (40.35 IU/ mg Protein). Pectinase and polygalacturonase optimization by *Penicillium chrysogenum* was best achieved when urea was used as nitrogen source, 124.26 IU/ mg protein for pectinase and 93.00 IU/ mg protein for polygalacturonase were obtained after 72 h post fermentation.

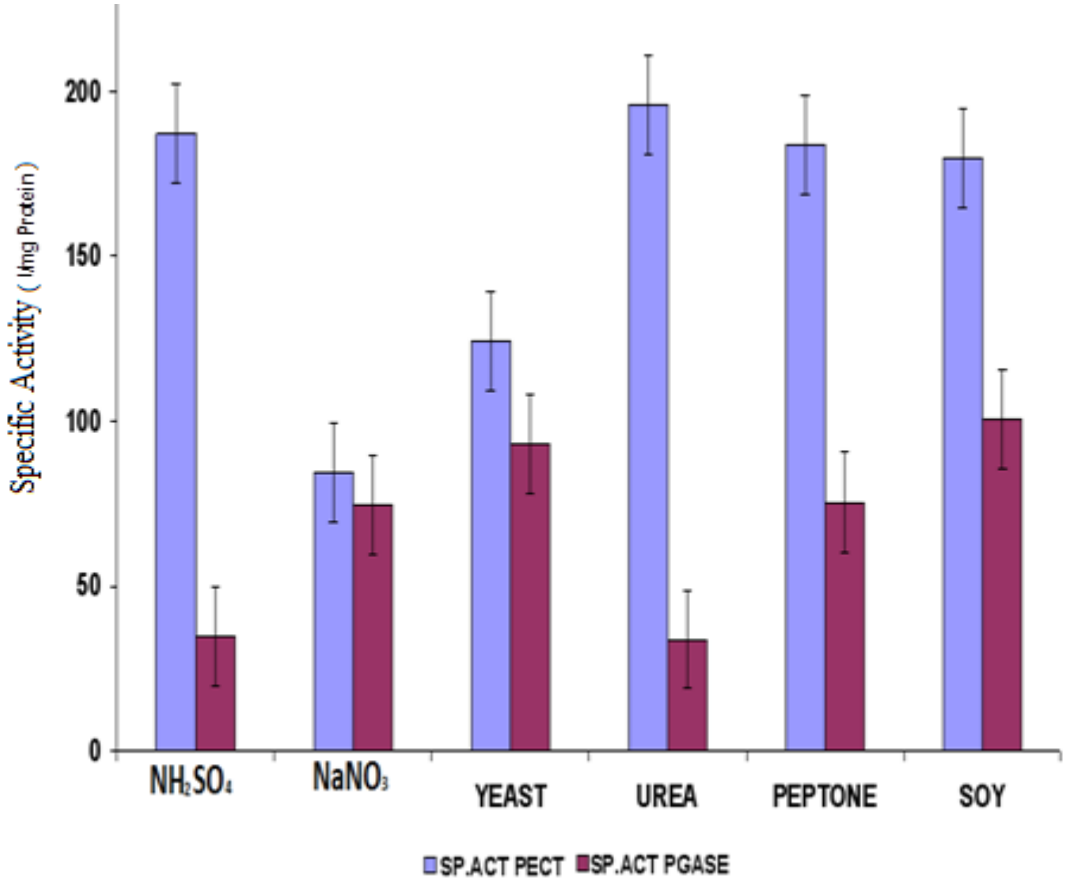


Figure 4. 6: Effect of nitrogenous sources on pectinase and polygalacturonase production by *Aspergillus niger.* (Data represent mean ± SEM)

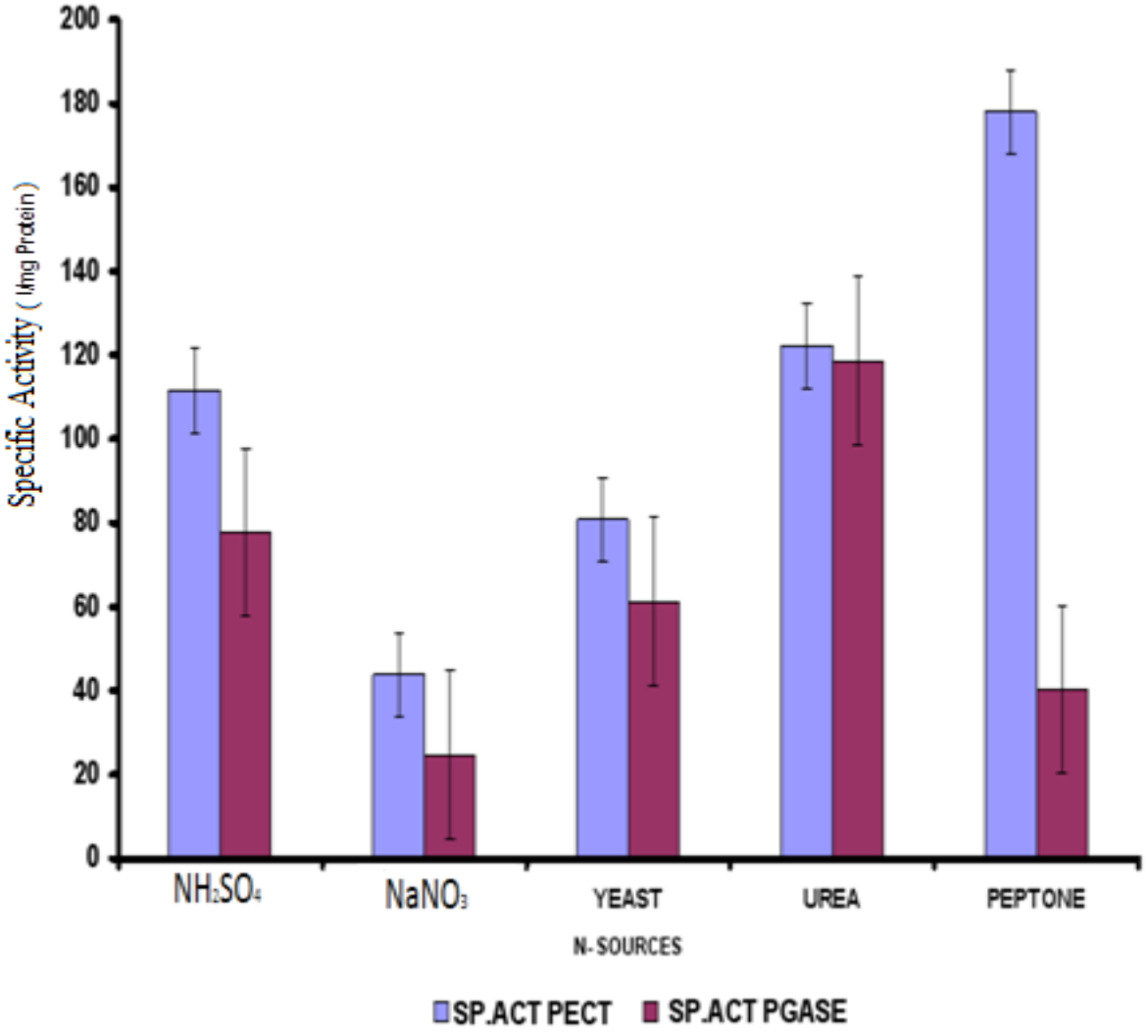


Figure 4.7: Effect of nitrogenous sources on pectinase and polygalacturonase production by *Penicillium chrysogenum.* (Data represent mean ± SEM)

4.6.2. Effect of Carbon Sources

The effect of various carbon sources on pectinase and polygalacturonase activities by the fungi isolates are presented in Figure 4.8 and 4.9 respectively. *A. niger* gave the highest pectinase activity in wheat bran containing medium of 181.43 IU/ mg protein, while the highest polygalacturonase activity was recorded in sugarcane pulp with a value of 73.40 IU/ mg protein. The two enzymes were highly repressed by corn cob and pineapple peels. Also glucose was found to induce polygalacturonase (63.27 IU/ mg protein) better than other agro wastes studied except sugar cane pulps while sucrose gave a minimal polygalacturonase activity of 8.58 IU/ mg protein and pectinase activity of 39.8 IU/ mg protein. However when the effect of various carbon sources on pectinase and polygalacturonase were studied using *P. chrysogenum* as the enzyme producer (Figure 9), it was observed that there was no significant different between the yield of the enzymes – polygalacturonase (62.25 IU/mg protein) and pectinase (64.35 IU/mg protein) when wheat bran was the source of carbon. Also *P. chrysogenum* shows affinity for saw dust for the productions of polygalacturonase (50.24 IU/mg protein) while the pectinase yield was 33.22 IU/ mg protein. The used of simple sugar- glucose and sucrose showed that they are better inducers of polygalacturonase (32.77 and 47.19 IU/ mg protein for glucose and sucrose respectively) than pectinase (19.83 and 10.07 IU/ mg protein for glucose and sucrose respectively).

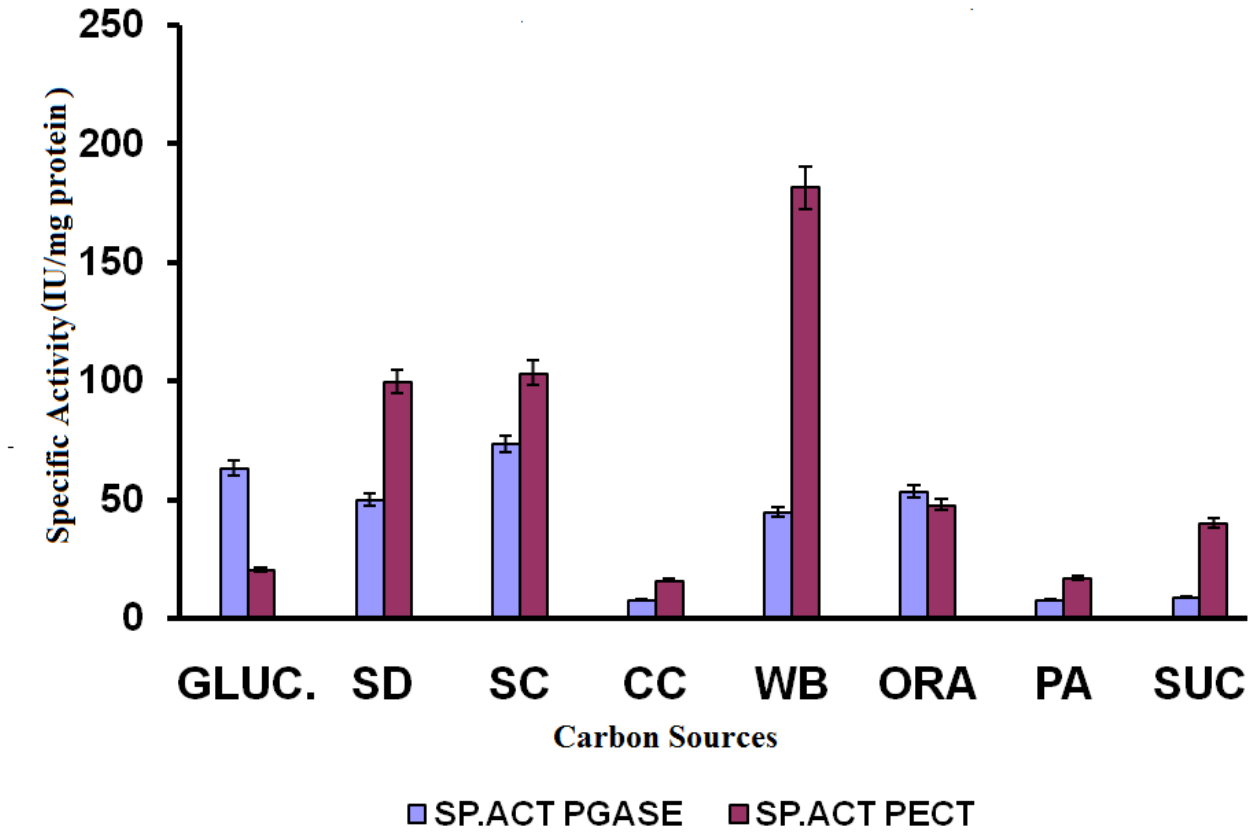


Figure 4.8: Effect of Carbon sources on pectinase and polygalacturonase production by *Aspergillus niger*. (Data represent mean ± SEM).

NB:

Glucose- GLUC,

Corn cob-CC,

Sawdust-SD,

Sugarcane Pulp -SP,

Wheat bran-WB,

Pine apple peels-PA,

Orange peels-OR.

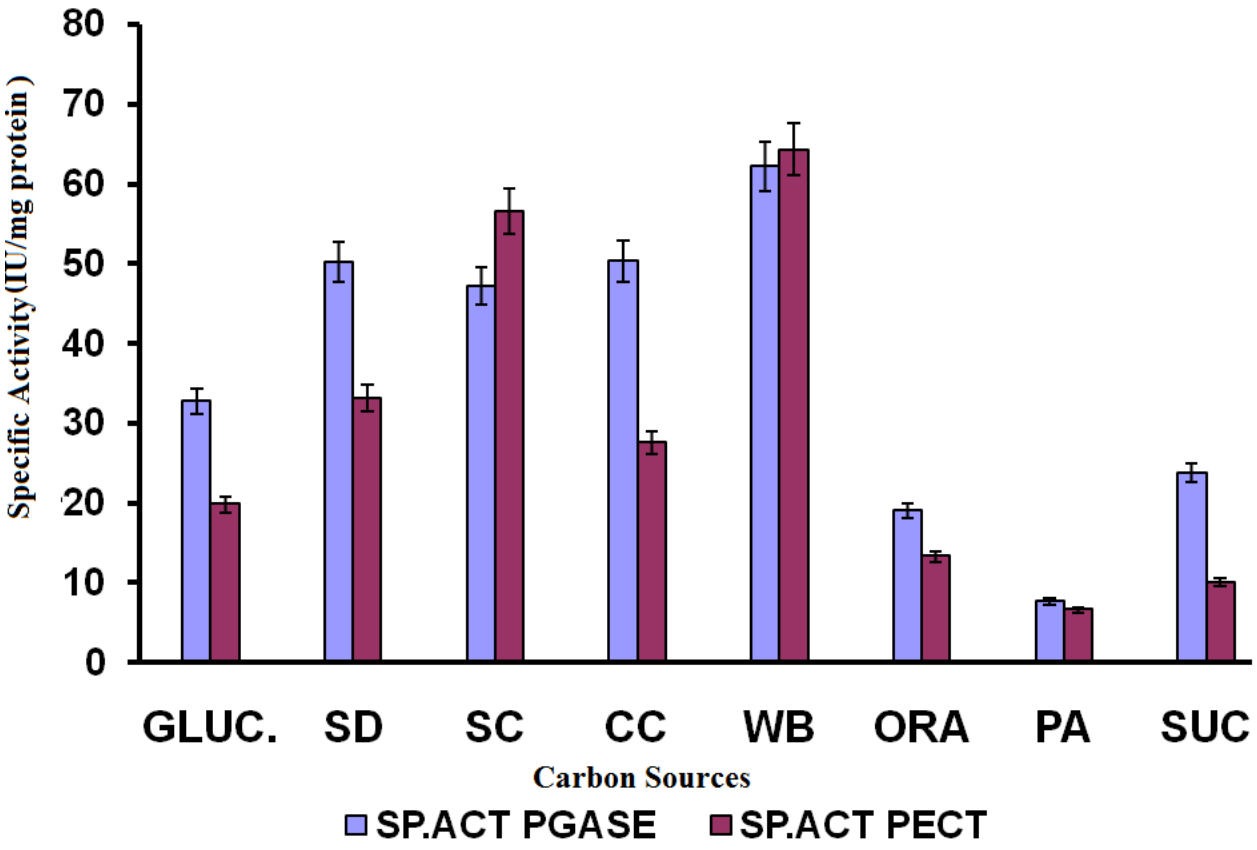


Figure 4.9: Effect of Carbon sources on pectinase and polygalacturonase production by *Penicillium chrysogenum*. (Data represent mean ± SEM)

NB:

Corn cob-CC,

Sawdust-SD,

Sugarcane Pulp -SP,

Wheat bran-WB,

Pine apple peels-PA,

Orange peels-OR

4.6.3. Effect of pH

The result showed enzyme activity at all the pH studied. At pH 3.5, a specific activity of 458.64 IU/ mg protein was observed while the least enzyme activity was noted at pH 6.5 with an activity of 19.46 IU/ mg protein when *Aspergillus niger was* used (Figure 4.10). The optima pH on pectinase production by *Penicillium chrysogenum* was observed at pH 5.5 and 6 (294.72 and 295.20IU/ mg protein respectively), It was also noted that pH 3.5 yielded an activity of 251.90 IU/ mg protein. pH 6.5 did not support pectinase production by this strain; the enzyme yield at this pH gave the lowest enzyme activity (Figure 4.11).

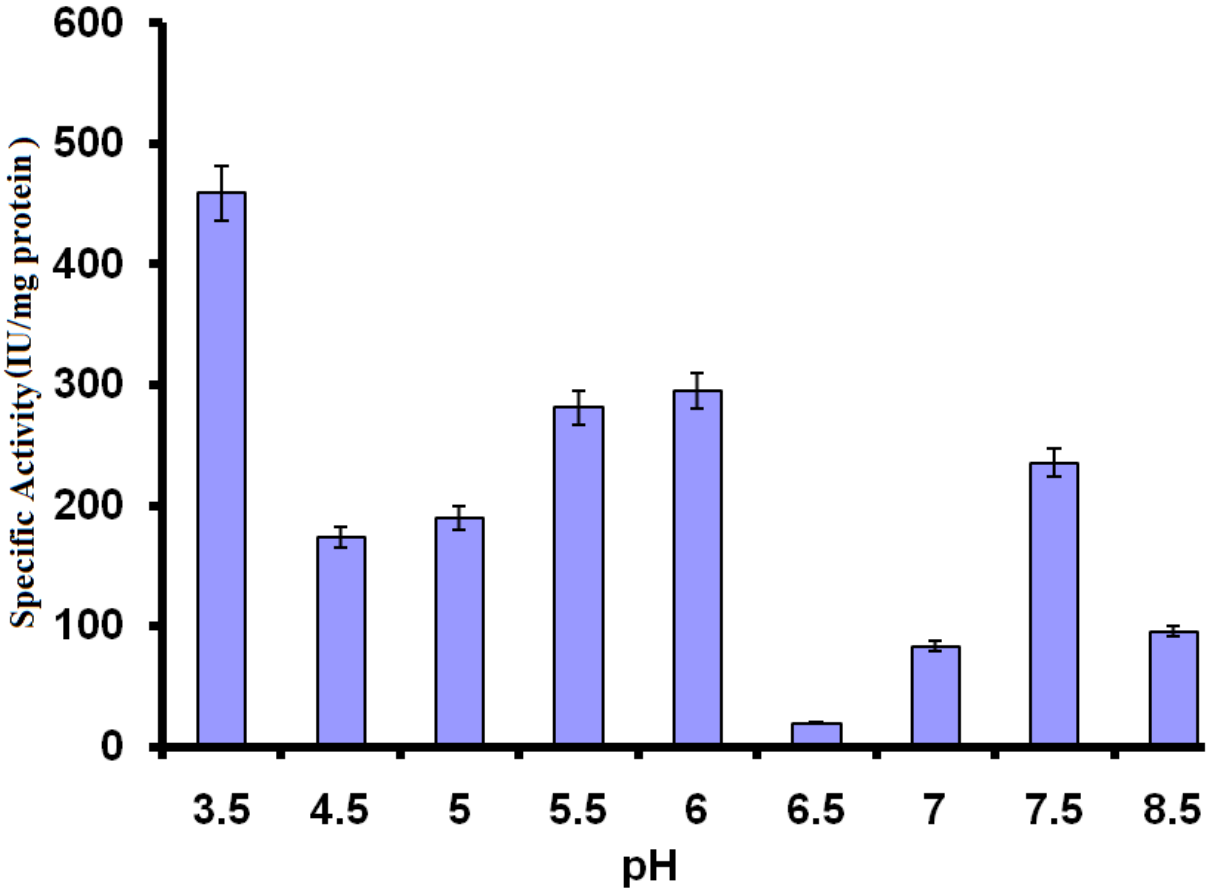


Figure 4.10: Effect of pH on pectinase production by *A. niger*. Pectinase activity was determined after 72 h of fermentation on wheat bran medium (Data represent mean ± SEM)

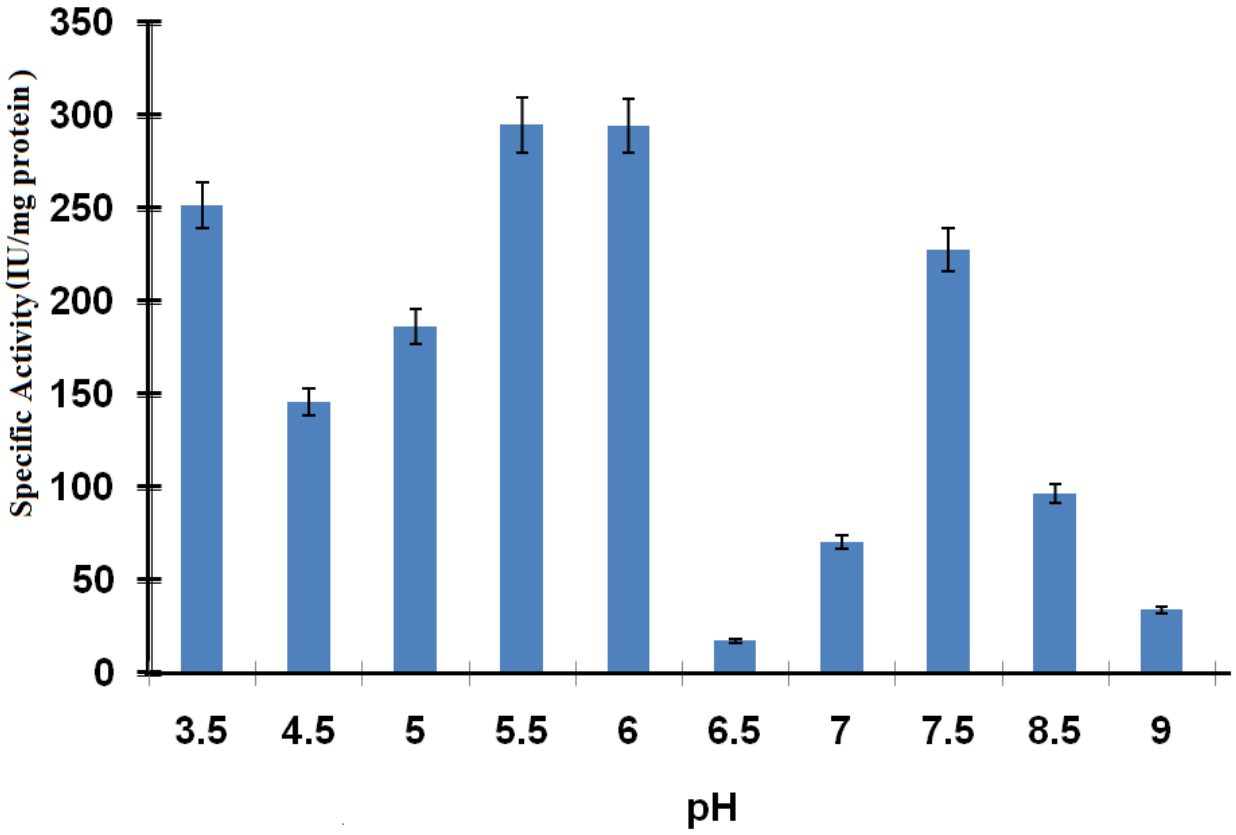


Figure 4.11: Effect of pH on pectinase production by *P. chrysogenum*. Pectinase activity was determined after 72 h of fermentation on wheat bran medium. (Data represent mean ± SEM)

4.7. Hyper production of Pectinase Using UV Mutagenesis

4.7.1. Survival Curve Determination

The optimization of pectinase production using UV irradiation, revealed a 97% death (3% survival) of *A.niger* (colony count) after an exposure time of 30 min (Figure 4.12) in MSB-Pectin containing 2 Deoxy d glucose (2DG) plates. Three colonies which were resistant to 2DG were isolated and further screened for hyper-pectinase activity (Figure 4.13). While the percentage of survival of *P.chrysogenum* at 30 min was 1% (colony count), and a survival of 5% at 10 min exposure to UV radiation (Figure 4.14). Five suspected mutants of *P. chrysogenum* were screened and used for further studies.

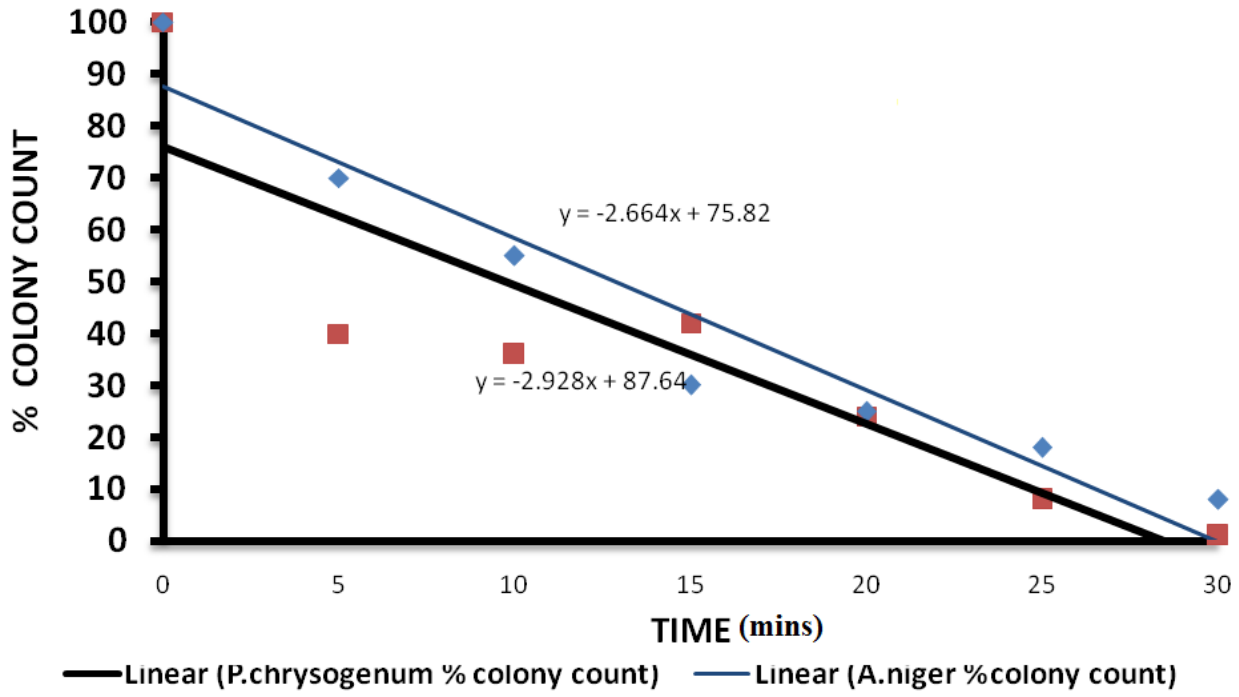


Figure 4.12: The survival curve of post UV radiation of spores of *A.niger* and *P.chrysogenum*.

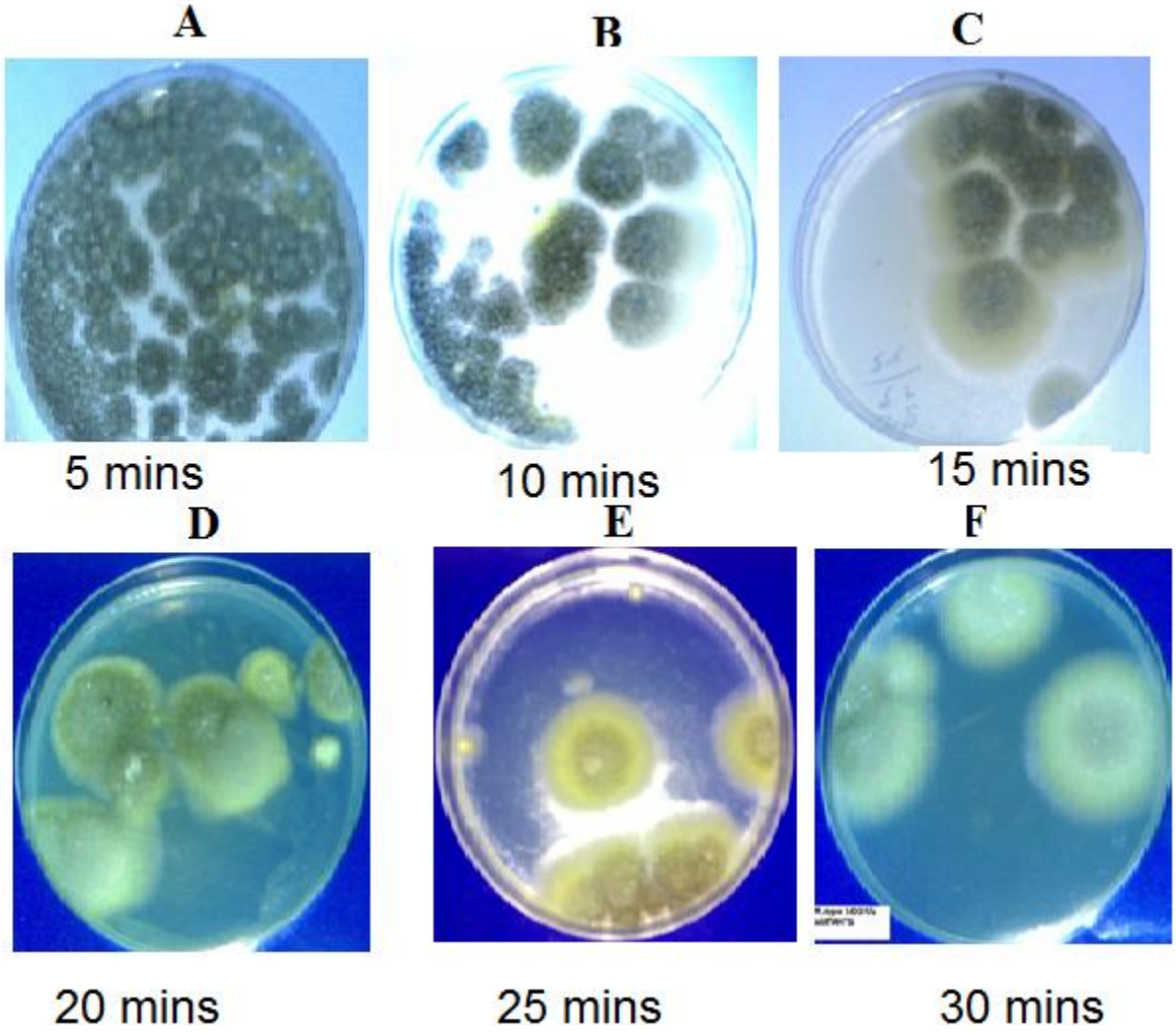


Figure 4.13: MSB-Pectin+ 2 Deoxy d glucose Plates showing colonies of *A. niger* that survived the UV radiation after 96 h post irradiation.

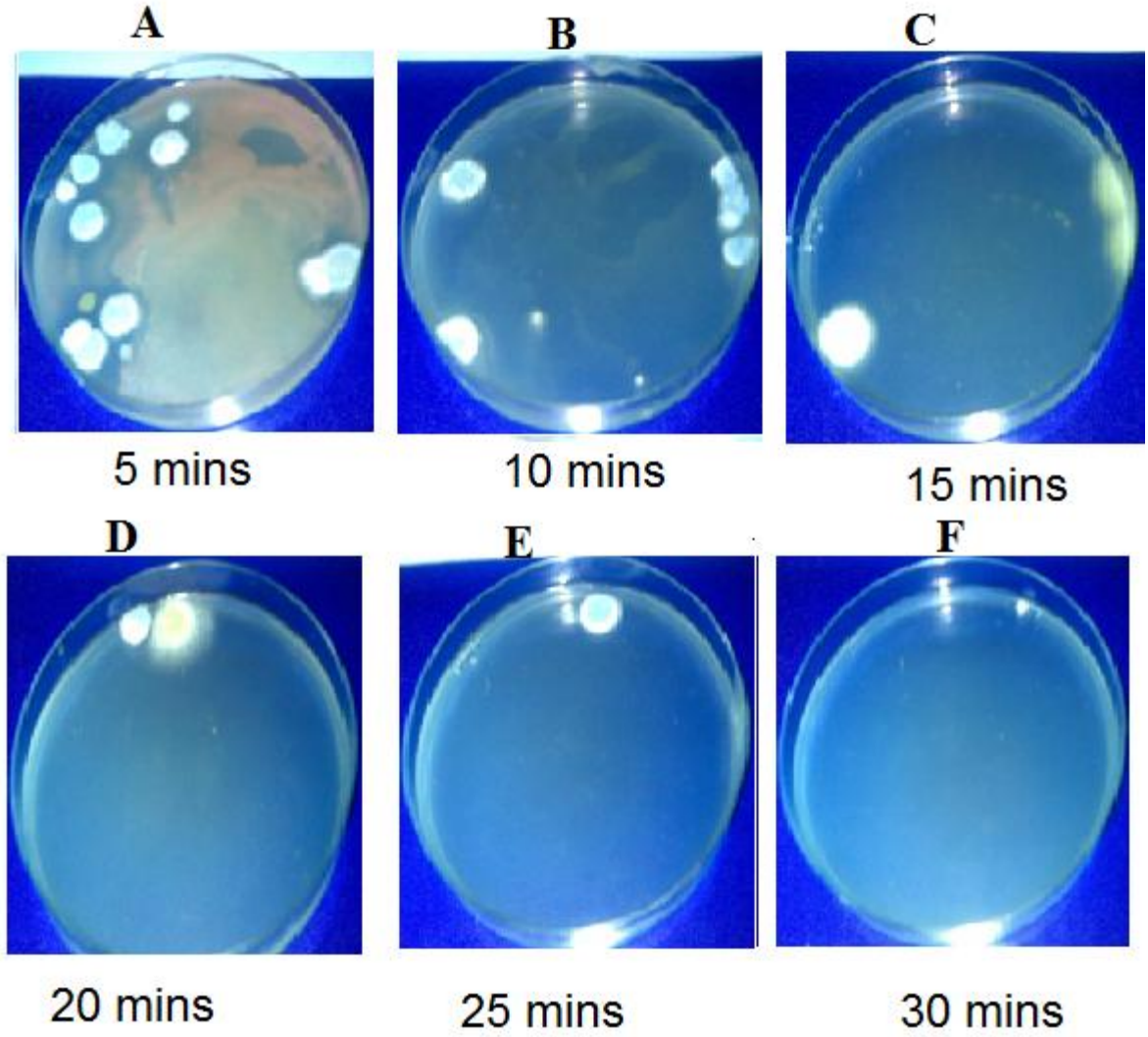


Figure 4.14: MSB-Pectin+ 2 Deoxy d glucose Plates showing colonies of *P.chrysogenum* that survived the UV radiation after 96 h post irradiation.

4.7.2. Selection of Hyper Producing Mutants

The result on selection of hyper producing pectinase mutants of the *A.niger* and *P.chrysogenum* was based on the diameter of the cleared zone by the pectinase extract of suspected hyper producing mutants and the wild strains cultured in mineral salt media containing pectin (MSM-PECT) supplemented with 0.001g of 2DG are presented in figures 4.15 and 4.16.

Table 2 depicts the pectinase activity indices and percentage yield of the mutants compared to the wild strain of the organism. The mutant designated 2DG-UV M2 of *A.niger* produced the highest pectinase yield (140% increase) using cup-plate screening method, while 2DG-UV W1 of *P.chrysogenum* gave a percentage yield of 123% over the wild strain.

When these organisms (both wild and mutant) were sub cultured in wheat bran (MSB-WB) using SSF and SmF, the yield of pectinase by the selected mutants was also higher than the wild strains. *A.niger* 2DG-UV M2 gave a pectinase activity of 465% and 230% higher pectinase activity than the wild strain in SSF and SmF respectively (Figures 4.17 and 4.18). While *P.chrysogenum* 2DG-UV W1, gave a percentage enzyme activity of 218% in solid state fermentation, (Figure 4.19) and 2DG-UV W2 gave a higher pectinase yield (135%) in submerged fermentation over 2DG-UV W1 (122.58%) (Figure 4.20).

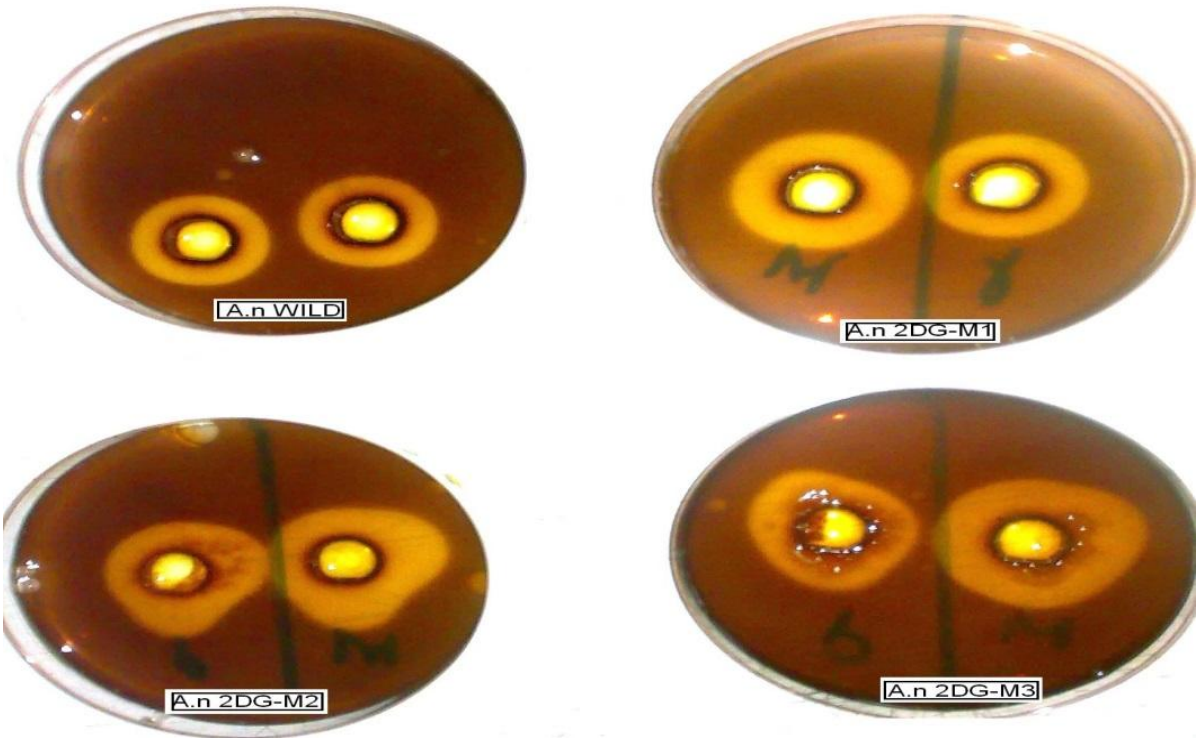


Figure 4.15: Pectinase cup-Plate screening of suspected mutants of *A.niger* for pectinase production.

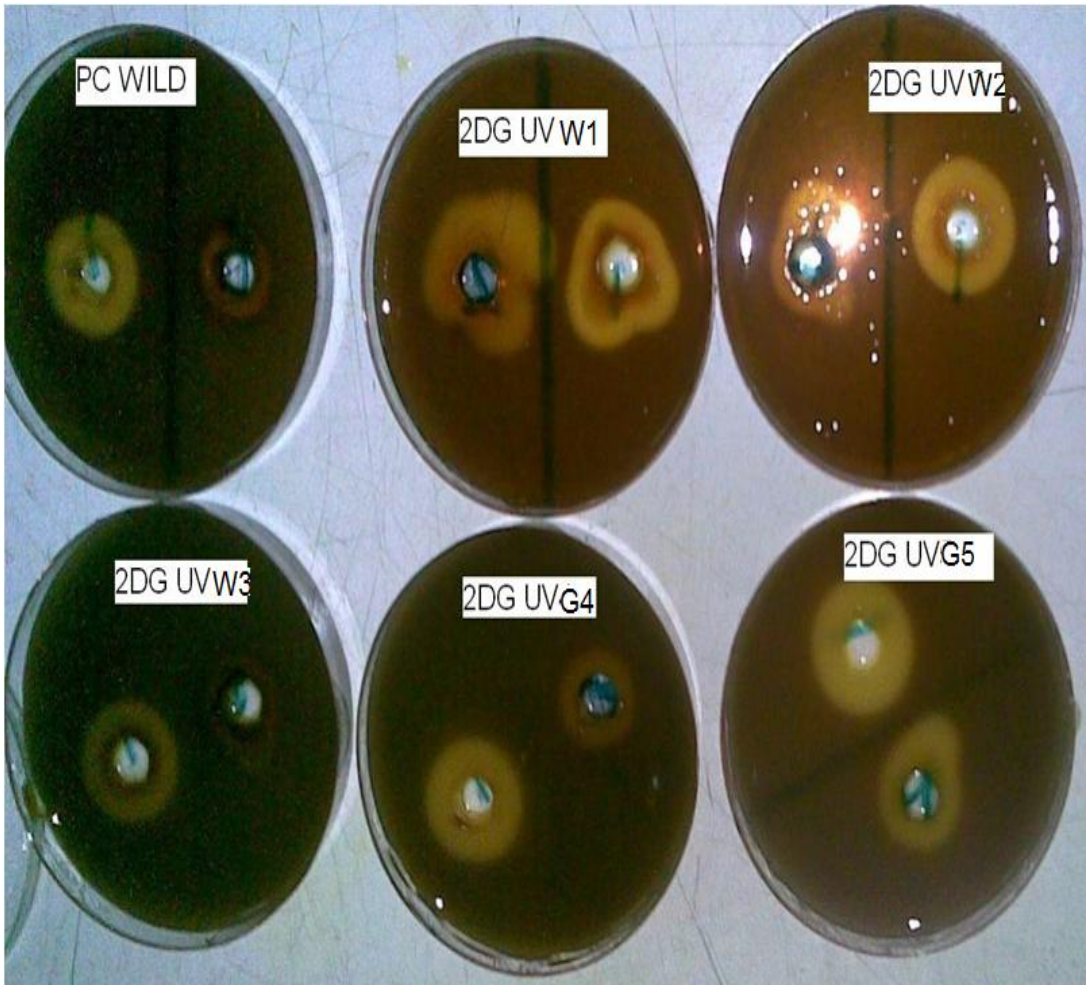


Figure 4.16: Pectinase cup-Plate screening of suspected mutants of *P.chrysogenum* for pectinase production.

Table 4.3: Pectinase activity index and % activity index of wild strain of the *A.niger* and *P.chrysogenum* produced from wheat bran containing medium. (Data represent mean ± SEM)

STRAINS	ENZYME ACTIVITY INDEX	% ACTIVITY INDEX
***A.niger* WILD**	2.71±0.06	100
2DG-UV M1	2.04±0.01	75.28
2DG-UV M2	3.80±0.02	140.22
2DG-UV M1	3.09±0.03	114.02
***P.chrysogenum* WILD**	1.50±0.12	100
2DG-UV W1	1.85±0.10	123
2DG-UV W2	1.70±0.11	113
2DG-UV W3	1.60±0.20	106
2DG-UV G1	1.50±0.13	100
2DG-UV G2	1.50 ±0.12	100

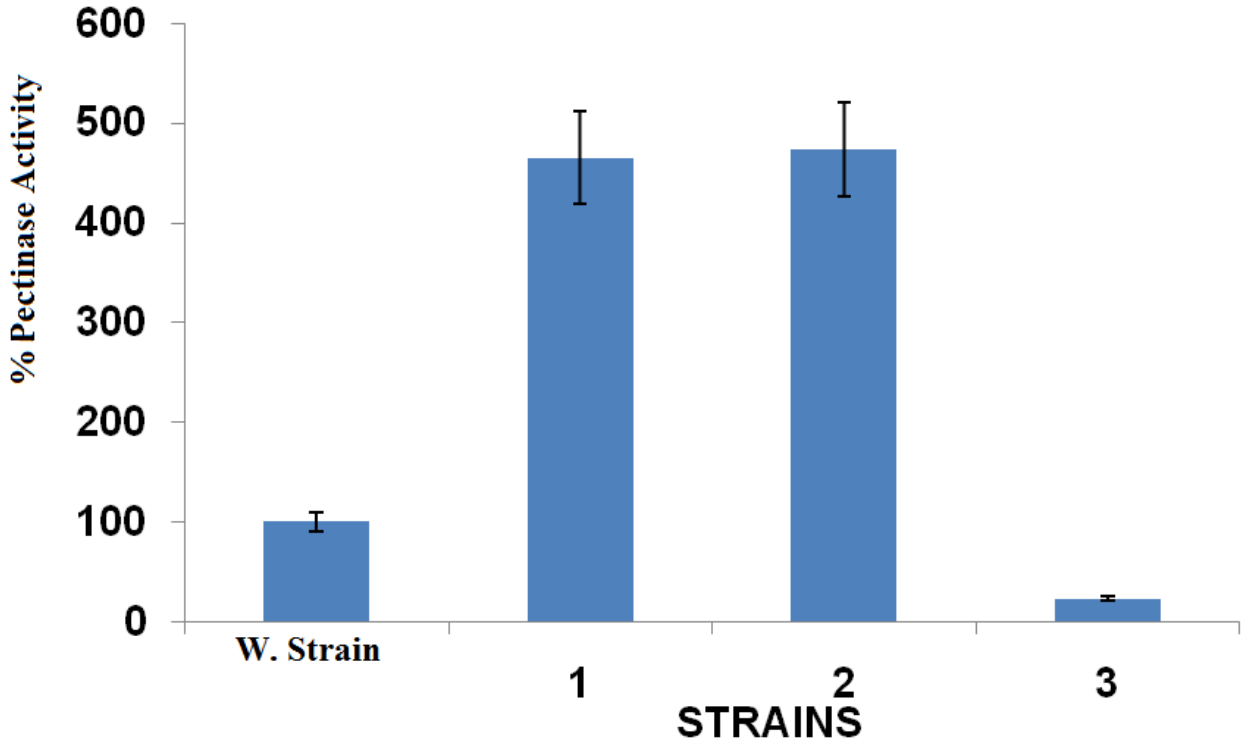


Figure 4.17: Screening of isolated 2DG UV mutants of *Aspergillus niger* for pectinase production in wheat bran containing medium (MSB-WB) using solid state fermentation (SSF). NB: W. Strain: Wild strain, Mutant Strains- 1: 2DG-UV M1, 2:2DG-UV M2, 3:2DG-UV M3. (Data represent mean ± SEM)

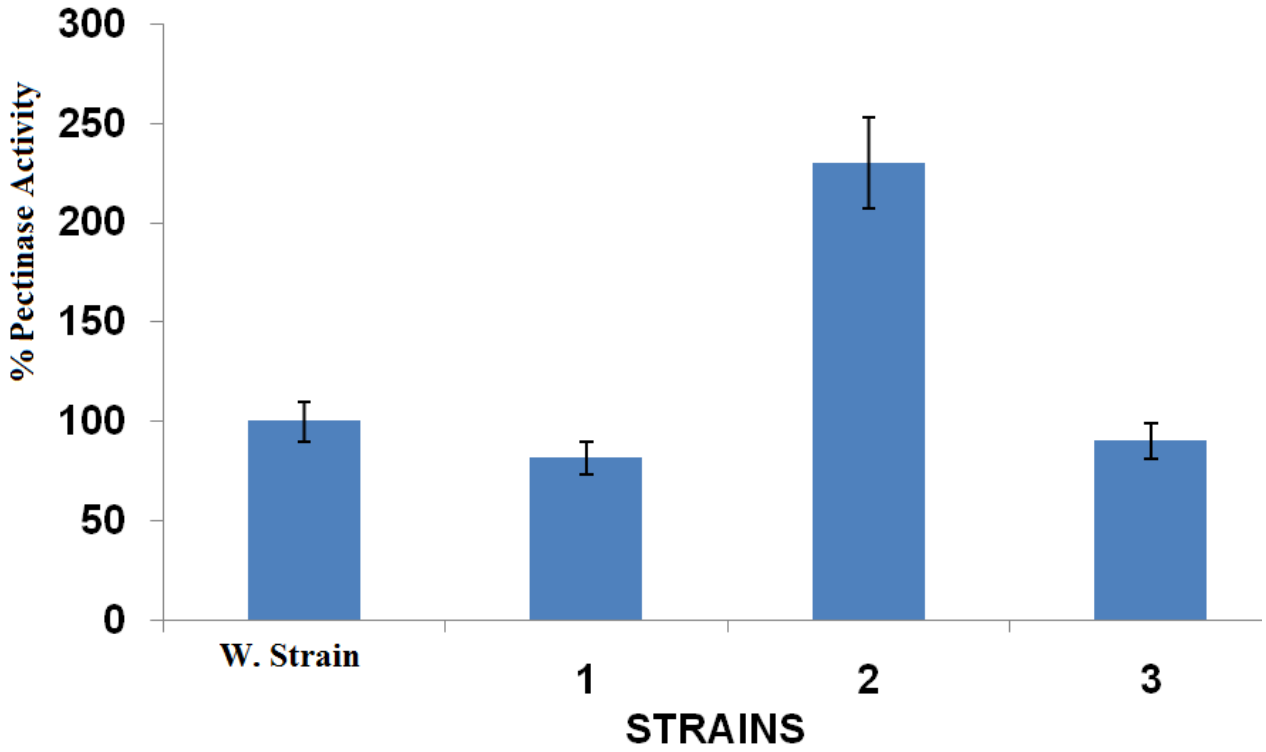


Figure 4.18: Screening of isolated 2DG UV mutants of *Aspergillus niger* for pectinase production in wheat bran containing medium (MSB-WB) using submerged fermentation (SmF). NB: W.STRAIN: Wild strain, Mutant Strain- 1: 2DG-UV M1, 2:2DG-UV M2, 3:2DG-UV M3. (Data represent mean ± SEM)

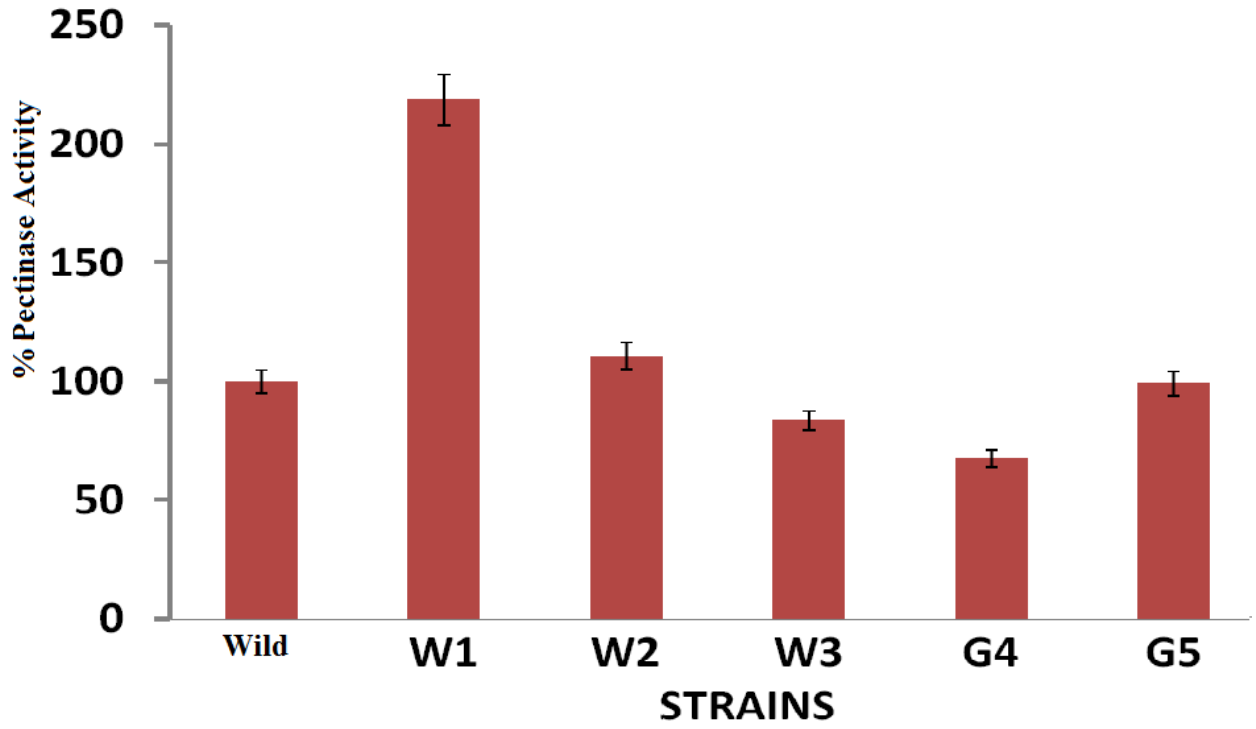


Figure 4.19: Screening of isolated 2DG UV mutants of *P.chrysogenum* for pectinase production in wheat bran containing medium (MSB-WB) using solid state fermentation (SSF). NB: WILD: Wild strains, Mutants strains-W1: 2DG-UV W1, W2: 2DG-UV W2, W3: 2DG-UV W3, G4: 2DG-UV G4, G5: 2DG-UV G5. (Data represent mean ± SEM)

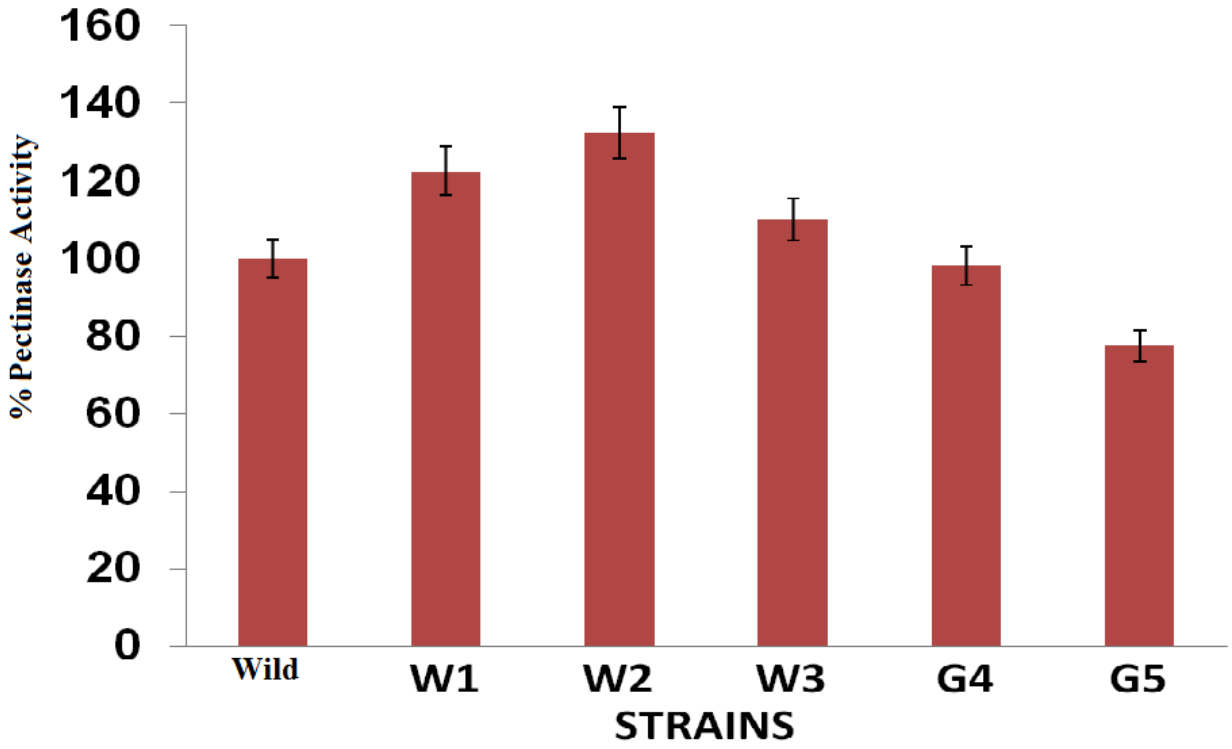


Figure 4.20: Screening of isolated 2DG UV mutants of *P.chrysogenum* for pectinase production in wheat bran containing medium (MSB-WB) using submerged fermentation (SmF). NB: WILD: Wild strains, Mutants strains-W1: 2DG-UV W1, W2: 2DG-UV W2, W3: 2DG-UV W3, G4: 2DG-UV G4, G5: 2DG-UV G5. (Data represent mean ± SEM)

4.8. Characterization of Hyper-Producing Mutant

4.8.1. Comparison of Growth and Pectinase Production of Wild and Mutant Strains.

The growth (mycelia dry weight) of both the wild and the mutant strain of *A. niger* (2DG-UV M2) and *P. chrysogenum* (2DG-UV W2) and the their extracellular enzyme pectinase production under solid state fermentation using pectin, sugar cane pulps and wheat bran were measured for each strain. Table 4.4 showed the comparison of wild strain and mutant 2DG-UV M2 of *A. niger* during growth and pectinase production in pectin, sugarcane pulps and wheat bran mineral salt media studied using the solid state fermentation (SSF). The result showed that pectinase yield was increased by 213, 276 and 376 percent in MS-sugar cane, pectin and wheat bran respectively by the 2DG-UV M2 of *A. niger* over the wild strain in SSF. In Table 4.5, pectinase production yield by *P. chrysogenum* was found to be best activated in solid state fermentation by MSC-PECT, (432.68%), while MSC-SC gave a repressed pectinase yield (88%) as compared to the wild strain. MSC-WB also enhanced the yield of pectinase by the mutant strain with a percentage pectinase activity of 256.84%.

Table 4.4: Growth and pectinase production by wild and 2DG-UV M2 of *Aspergillus niger* in MS-PECT, MS-WB and MS-SC in solid state fermentation.

Strains	Substrates	Mycelia Dry Weight (mg/ml)	Total Protein (mg/ml)	Pectinase Activity (IU/ml)	Specific Activity (IU/mg protein)	% Activity
	Pectin	**0.53±0.02**	**1.68±0.02**	**44.50±0.21**	**26.5**	**100**
Wild	**Sugarcane pulps**	**0.49±0.04**	**1.51±0.05**	**40.45±0.01**	**26.79**	**100**
	Wheat Bran	**0.53±0.01**	**1.69±0.01**	**41.23±0.02**	**24.40**	**100**
	Pectin	**0.45±0.05**	**1.44±0.09**	**105.42±0.01**	**73.21**	**276**
2DG-UV M2	***Sugarcane pulps***	**0.47±0.02**	**1.63±0.04**	**93.12±0.03**	**57.13**	**213**
	Wheat Bran	**0.53±0.01**	**1.68±0.0**	**152.65±0.02**	**90.86**	**372**

Mean ±SEM

Table 4.5: Growth and pectinase production by wild and 2DG-UV W1 of *Penicillium chrysogenum* in MS-PECT, MS-WB and MS-SC in submerged fermentation.

Strains	Substrates	Mycelia Dry Weight (mg/ml)	Total Protein (mg/ml)	Pectinase Activity (IU/ml)	Specific Activity (IU/mg protein)	% Activity
	Pectin	**0.45±0.012**	**1.78±0.02**	**34.50±0.02**	**19.40**	**100**
Wild	**Sugarcane pulps**	**0.40±0.014**	**1.63±0.03**	**46.24±0.02**	**28.37**	**100**
	Wheat Bran	**0.58±0.012**	**1.59±0.01**	**51.23±0.02**	**32.22**	**100**
	Pectin	**0.35±0.005**	**1.34±0.06**	**112.52±0.18**	**83.94**	**432.68**
2DG-UV W1	***Sugarcane pulps***	**0.38±0.002**	**1.73±0.04**	**43.12±0.01**	**24.92**	**88.02**
	Wheat Bran	**0.49±0.021**	**1.98±0.01**	**165±0.02**	**83.33**	**258.64.**

Mean ±SEM

4.8.2. Effect of Catabolite Repression (CR)

4.8.2.1. Glucose

Figure 4.21 shows the effect of CR on the pectinase production by the wild and mutant strains of *A.niger*. The wild strain positively responded to CR effect as the concentration of glucose increased, however the mutant strain 2 DG-UV M2 showed no effect of CR on pectinase production. The highest pectinase yield was observed at 5 gl^{-1} of glucose (399 %) while the pectinase production yield reduced at 10 gl^{-1} glucose to 270%. However when the effect of CR (using glucose) was tested on both the wild and selected mutant strain (2 DG-UV W1) of *P.chrysogenum*, it was observed (Figure 4.22) that there was moderate effect of CR on the wild, while 2 DG-UV W1 strain produce pectinase as the concentration of glucose increased. The pectinase production under the influence of CR by wild strain of *P.chrysogenum*, was reduced to 57% and 73% at concentration of 1 and 5 gl^{-1} of glucose respectively, while at 10 g glucose concentration, the maximum effect of CR was established. For the 2DG-UV W1 (mutant strain of *P.chrysogenum*), there was relative increased in pectinase production with slight decrease as the concentration of glucose increases. At 1 gl^{-1} glucose concentration the percentage pectinase activity was 150% while at 5 and 10g glucose concentration the percentage activities were 142 and 141% respectively.

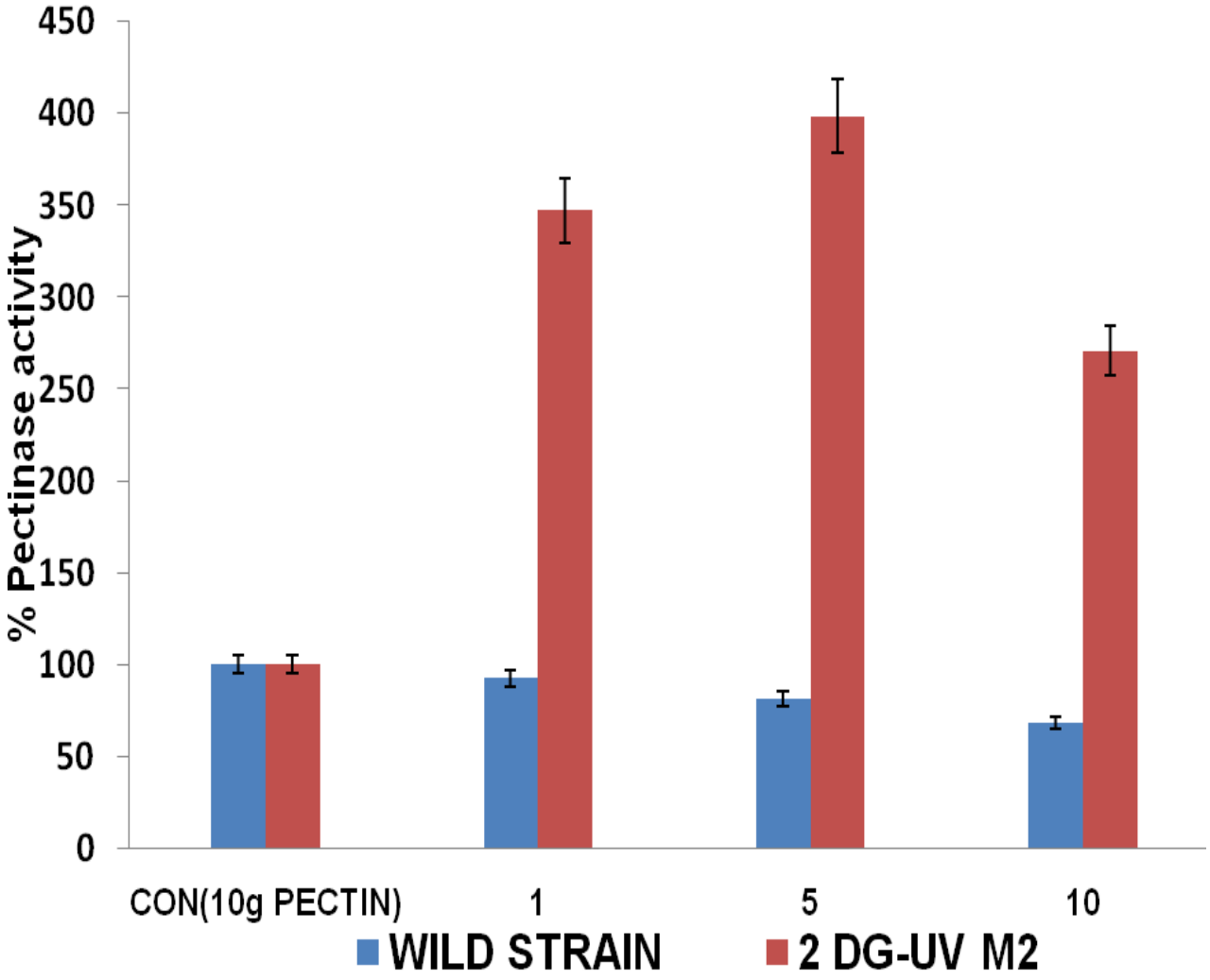


Figure 4.21: Comparison of the effect of catabolite repression (glucose) on pectinase activity produced by both wild and 2DG-UV M2 strains of *A. niger*.

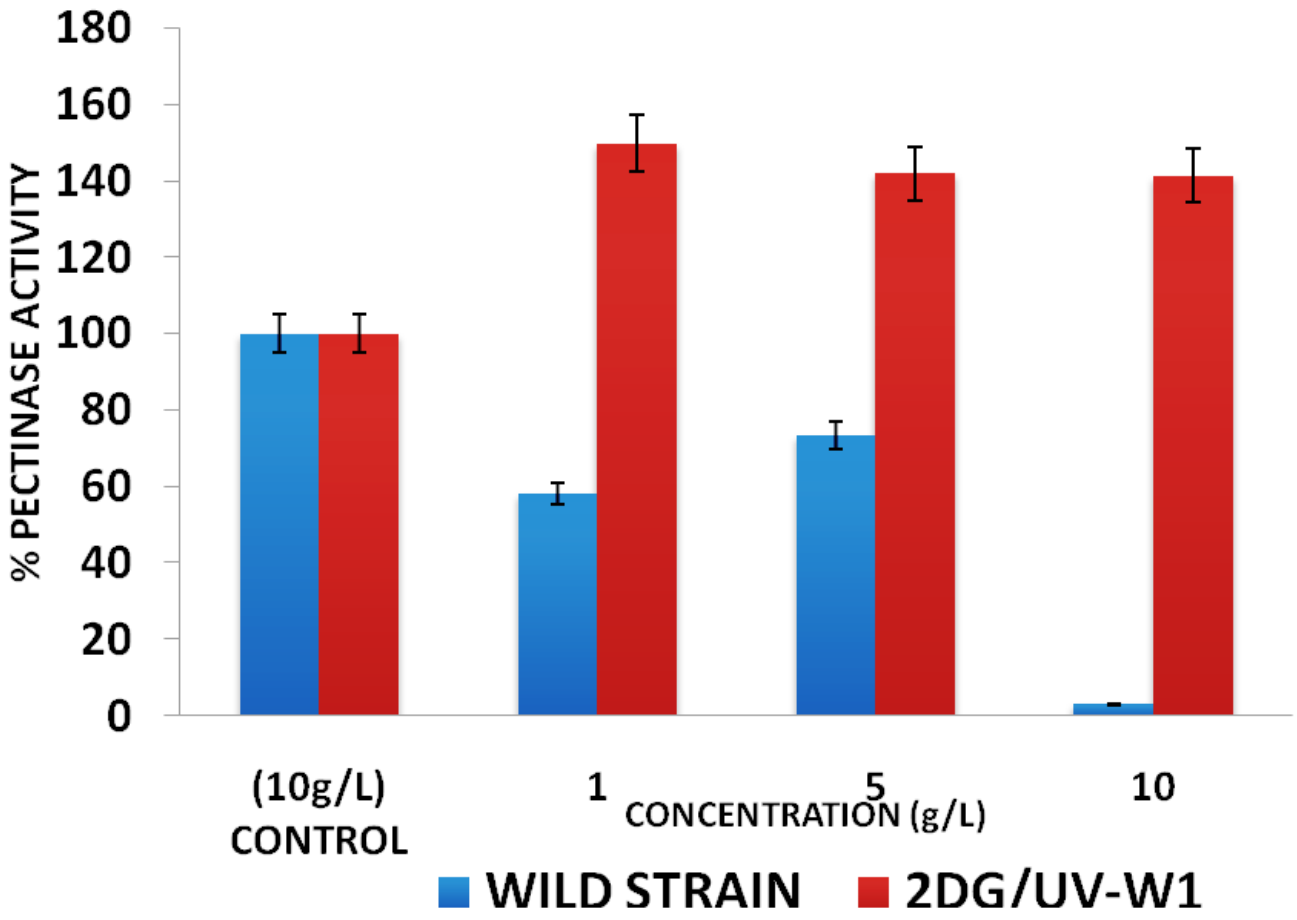


Figure 4.22: Comparison of the effect of catabolite repression (glucose) on pectinase activity produced by both wild and 2DG-UV W1 strains of *P. chrysogenum*.

4.8.2.1. Sucrose

Figure 4.23, shows that there is an overall CR effect on the wild strain of *A.niger* with percentage pectinase activities reduced as the concentration of sucrose increased. However, 2DV-UV M2 strain (mutant) showed no repression of pectinase production by sucrose. At 1g/l of sucrose, the percentage pectinase yield was 286% while the pectinase activity was highly reduced at 5g/l sucrose. Figure 4.24 shows the effect of CR (using sucrose as the repressor) on both wild strain and mutant strain of *P.chrysogenum.* The effect of CR on both the wild and the 2 DG-UV W1 was relatively high compared to the percentage yield when pectin alone was used as the inducer.

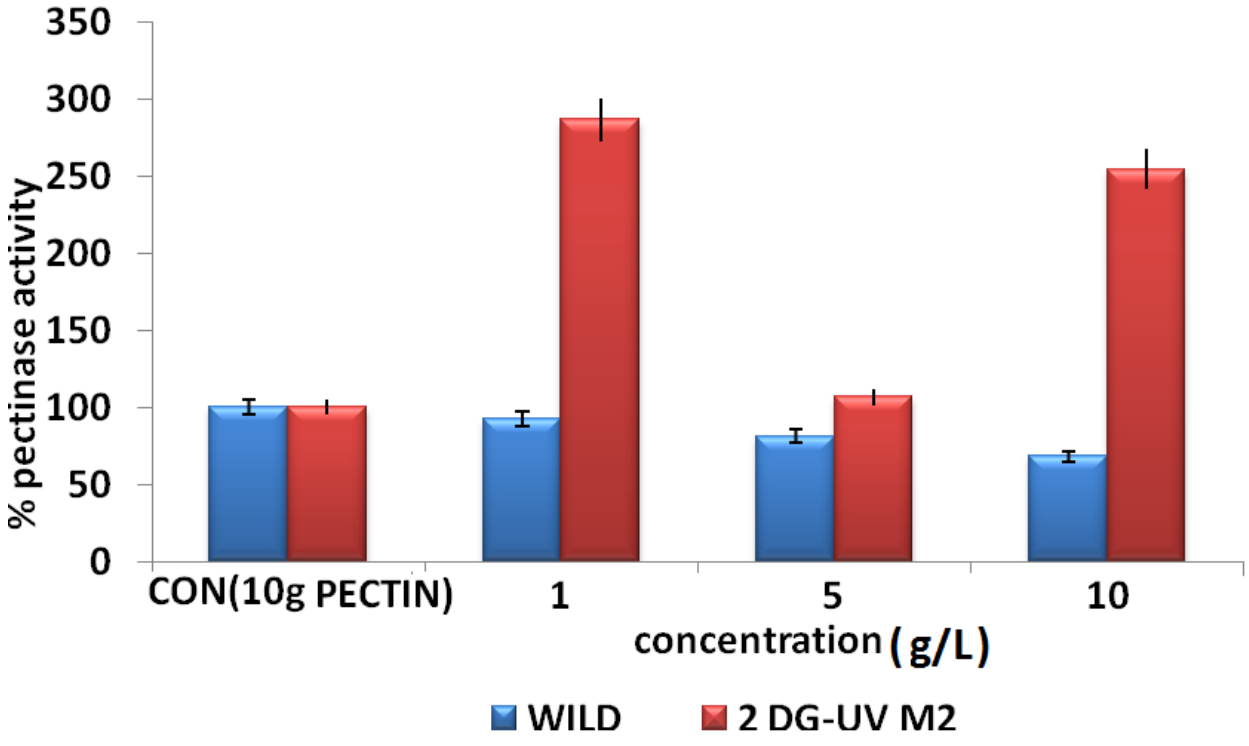


Figure 4.23: The effect of sucrose supplementation on pectinase production by wild and 2DG-UV M2 of *A.niger.*

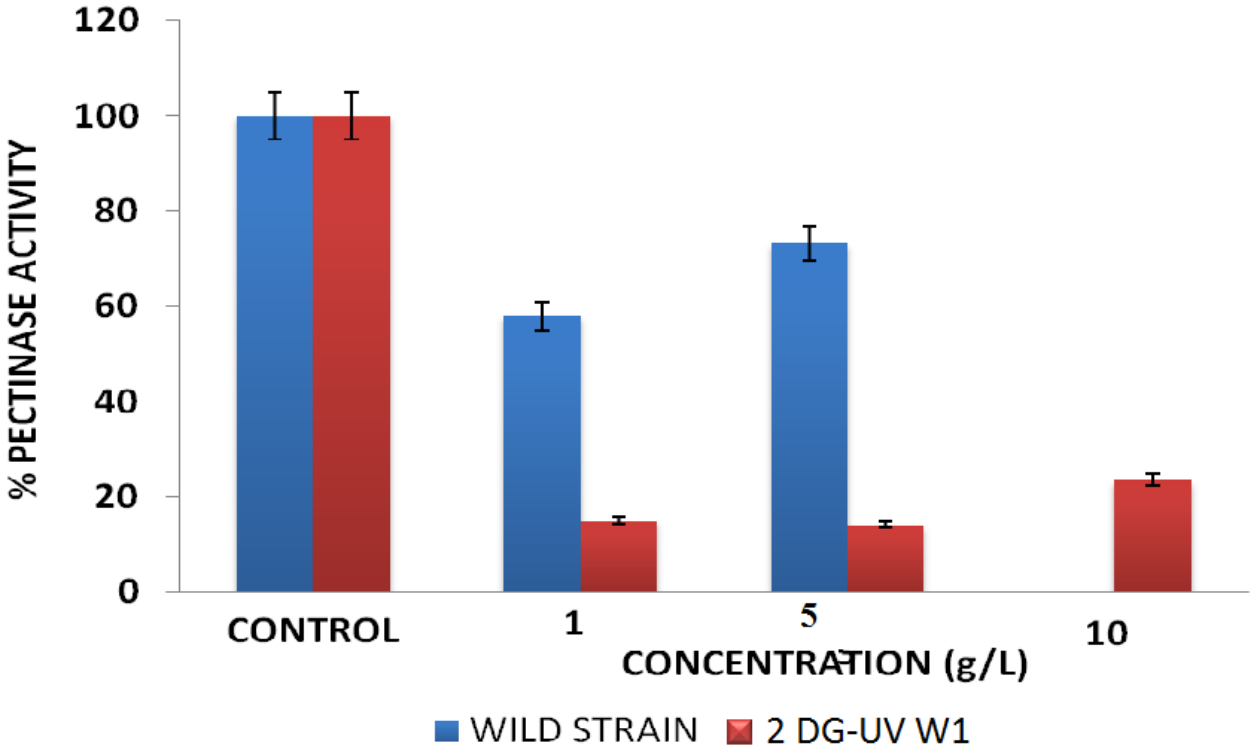


Figure 4.24: The effect of sucrose supplementation on pectinase production by wild and 2DG-UV W1 of *P.chrysogenum*.

4.9. Molecular Characterization of the Genomic DNA of the Wild and Mutant Isolates

Figure 4.25 showed the electrophoresed agarose gel of the DNA of the

4.9.1. Random Amplified Polymorphic DNA (RAPD) Profile

The RAPD-PCR analysis of the total genomic DNAs of *A.niger* (both wild and mutant strains) and *P.chrysogenum* (both wild and mutant strains) revealed amplification by OPA 02 and 04, OPC 02, and OPE 02.

Figure 4.26 shows genomic DNA variability in both wild and mutant strains of *A.niger* using RAPD primers; OPA 02, 04, OPC 02, and OPE 02. Figure 4.27 shows possible polymorphism in wild and mutant strains of *P.chrysogenum* using RAPD OPA 02 and 04. The rest of the RAPD primers revealed no visible amplification. Tables 4.6 and 4.7 show the percentage variables between the wild and mutants of *A.niger* and *P.chrysogenum* respectively.

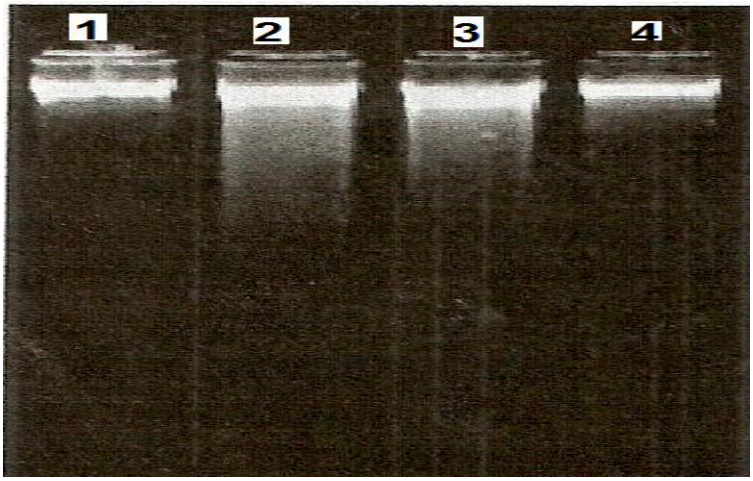


Figure 4.25: Electrophoregram of DNAs isolated from pectinolytic fungi. Lane 1: *Penicillium chrysogenum* wild, 2: *Aspergillus niger* wild, 3: *Aspergillus niger* mutant (2DG-UV M2) 4: *Penicillium chrysogenum* mutant (2DG-UV W1),

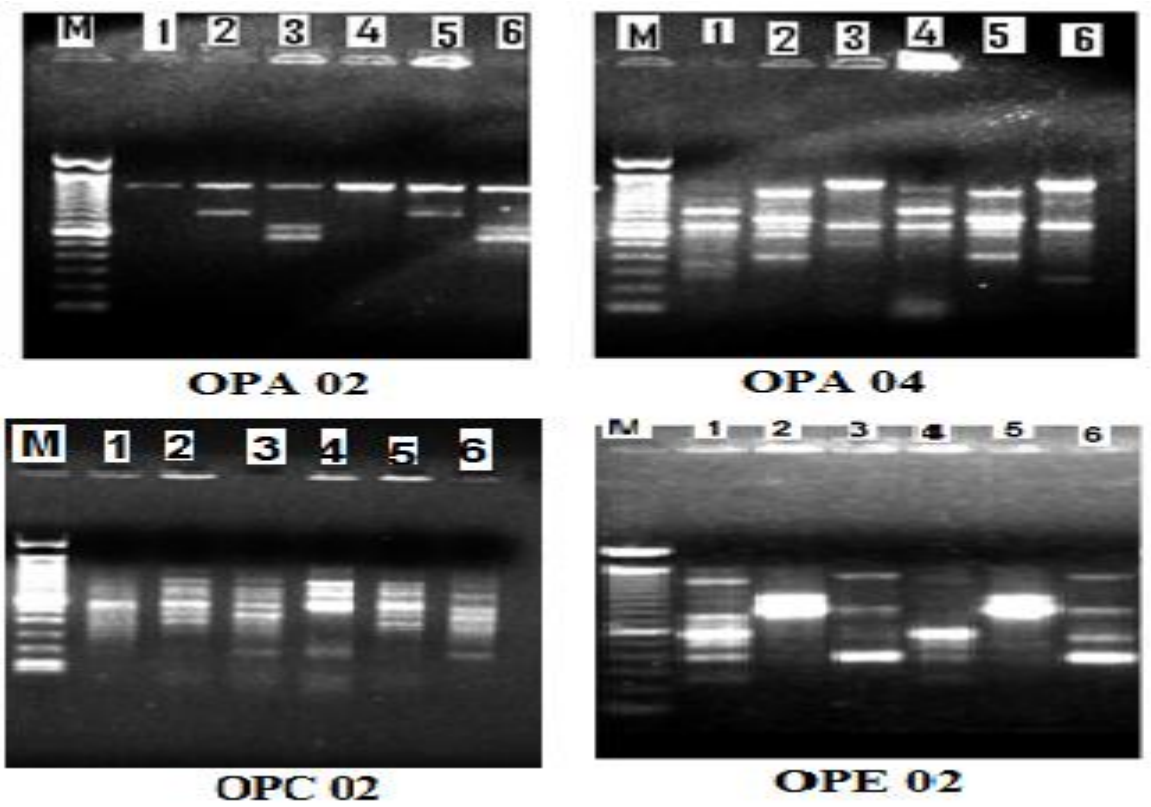


Figure 4.26: Electrophoregram of RAPD amplified products of *A.niger* (AN) of both wild and mutant strains. Lane: M: Molecular weight marker (1kb), 1 and 4: *A.niger* isolated from decaying sawdust, 2 and 5: *A.niger* (mutant 2DG-UV M2), 3 and 6: *A.niger* (wild).

Table 4.6: Percentage variable between wild and mutant strains of *A.niger* using RAPD primers

PRIMER	DISSIMILAR (%)	SIMILAR (%)
OPA 02	33	67
OPA 04	43	57
OPC 02	67	33
OPE 02	20	80

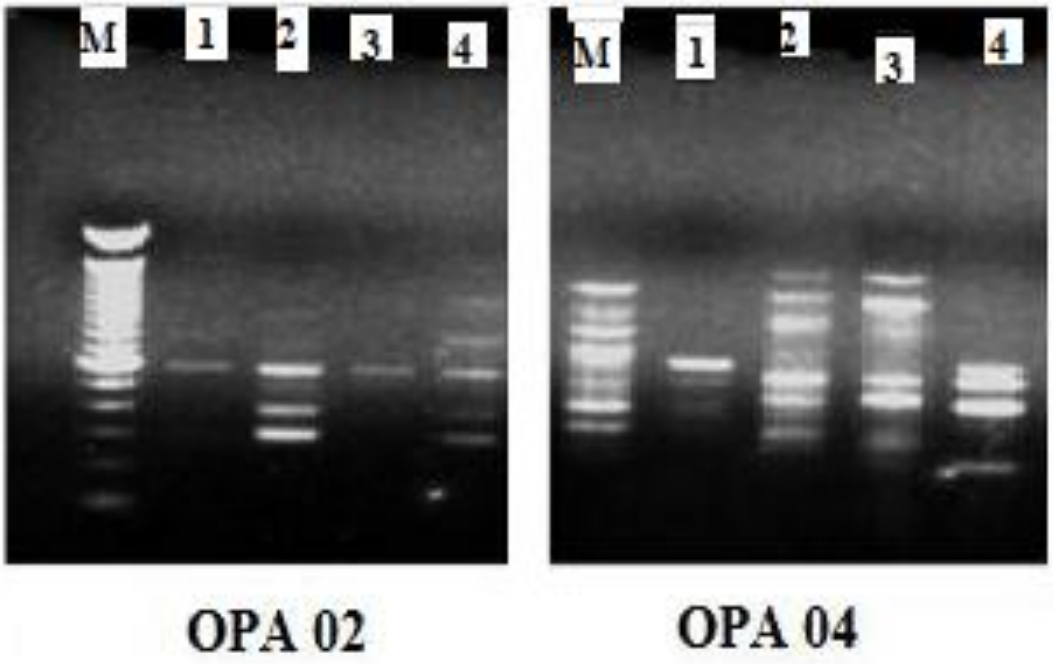


Figure 4.27: Electrophoregram of RAPD amplified products of *P.chrysogenum* of both wild and mutant strains. Lane: M: Molecular weight marker (1kb), 1 and 3: *P.chrysogenum* (wild), 2 and 4: *P.chrysogenum* (mutant 2DG-UV W1).

Table 4.7: Percentage variable between wild and mutant strains of *P. chrysogenum* using RAPD primers

PRIMER	DISSIMILAR (%)	SIMILAR (%)
OPA 02	33	67
OPA 04	37.5	62.5

4.10. Partial Purification of the Polygalacturonase (EC: 3.2.1.15)

Table 4.8A and B depict the purification steps for the polygalacturonase of *A.niger* and *P.chrysogenum.* The polygalacturonase of *A.niger* and *P.chrysogenum* were purified to about 7.9 and 4.7 purification fold respectively. Enzyme yield of 17% and 23% were obtained respectively for the polygalacturonase enzymes of *A.niger* and *P.chrysogenum* through gel filtration (Sephadex G120). The DEAE Sephadex A25-120 chromatograph profiles of the polygalacturonase of 2 DG UV M2 and 2 DG UV W1 of *A.niger* and *P.chrysogenum* were respectively shown in Figures 4.28 and 4.29. Four enzyme peaks were obtained for 2 DG UV M2 *A.niger* while three enzyme peaks were obtained for 2 DG UV W1 *P.chrysogenum.* These peaks were combined and subjected to gel chromatography using Sephadex G120. Figures 4.30 and 4.31 show the chromatograph of the polygalacturonase of *A.niger* and *P.chrysogenum.* One enzyme peak was obtained for each polygalacturonase enzyme of *A.niger* and *P.chrysogenum*.

Table 4.8A: Purification of the polygalacturonase (EC 3.2.1.15) of *A. niger.*

Organism	Purification steps	Total activity (unit)	Total protein (mg)	Sp.activity (unit/mg protein)	Enzyme yield (%)	Purification fold
2 DG/UV-M2 A.niger	Crude Enzyme	1324	2546	0.52	100	1
	$(NH_4)_2SO_4$ (70-80%)	1150	1282	0.90	86.85	1.7
	DEAE-Sephadex A25-120	557	376	1.75	42.0	3.4
	Sephadex G120	227	55	4.13	17.14	7.94

Table 4.8B: Purification of the polygalacturonase (EC 3.2.1.15) of *P. chrysogenum*.

Organism	Purification steps	Total activity (unit)	Total protein (mg)	Sp.activity (unit/mg protein)	Enzyme yield (%)	Purification fold
2 DG/UV-W1 P.chrysogenum	Crude Enzyme	1443	2387	0.60	100	1
	$(NH_4)_2SO_4$ (70-80%)	1321	1182	1.12	91.5	1.87
	DEAE-Sephadex A25-120	652	336	1.94	45.2	3.23
	Sephadex G120	341	121	2.82	23.6	4.70

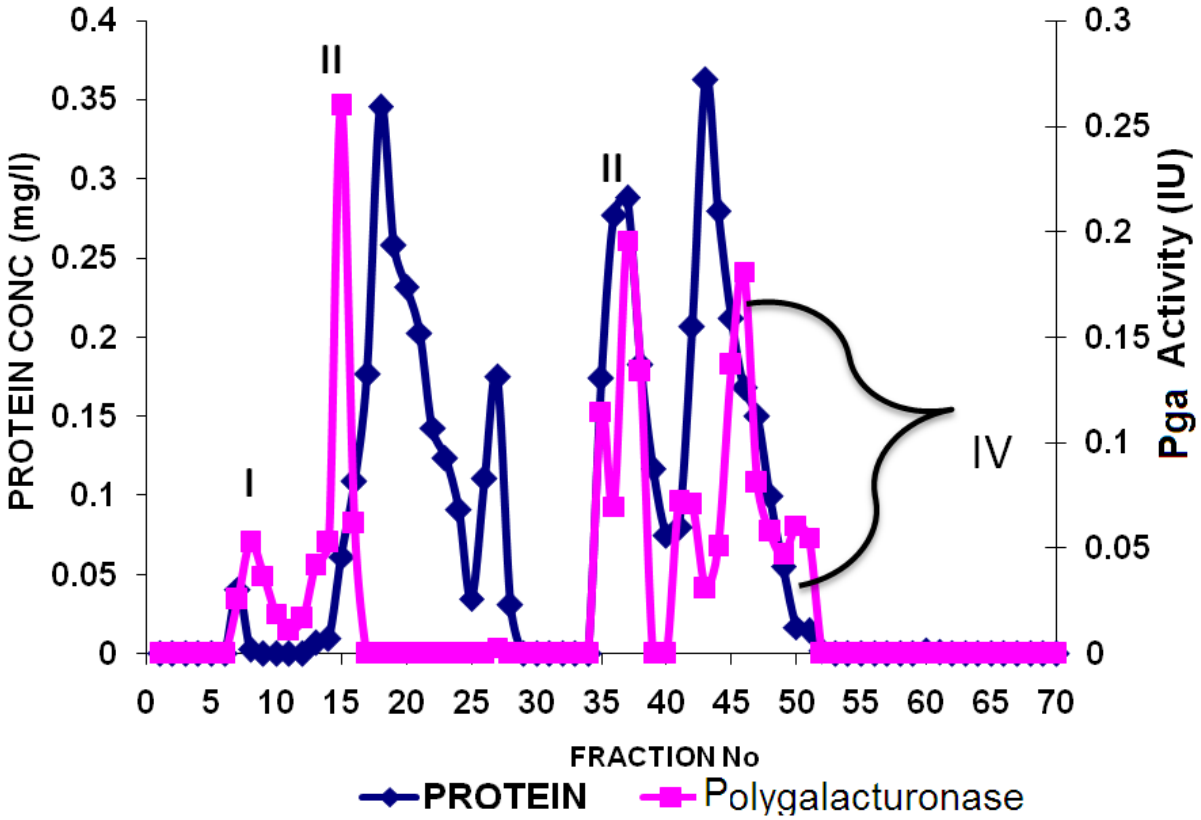


Figure 4.28: Chromatograph of partially purified polygalacturonase produced by *A.niger* using DEAE-Sephadex A25-120.

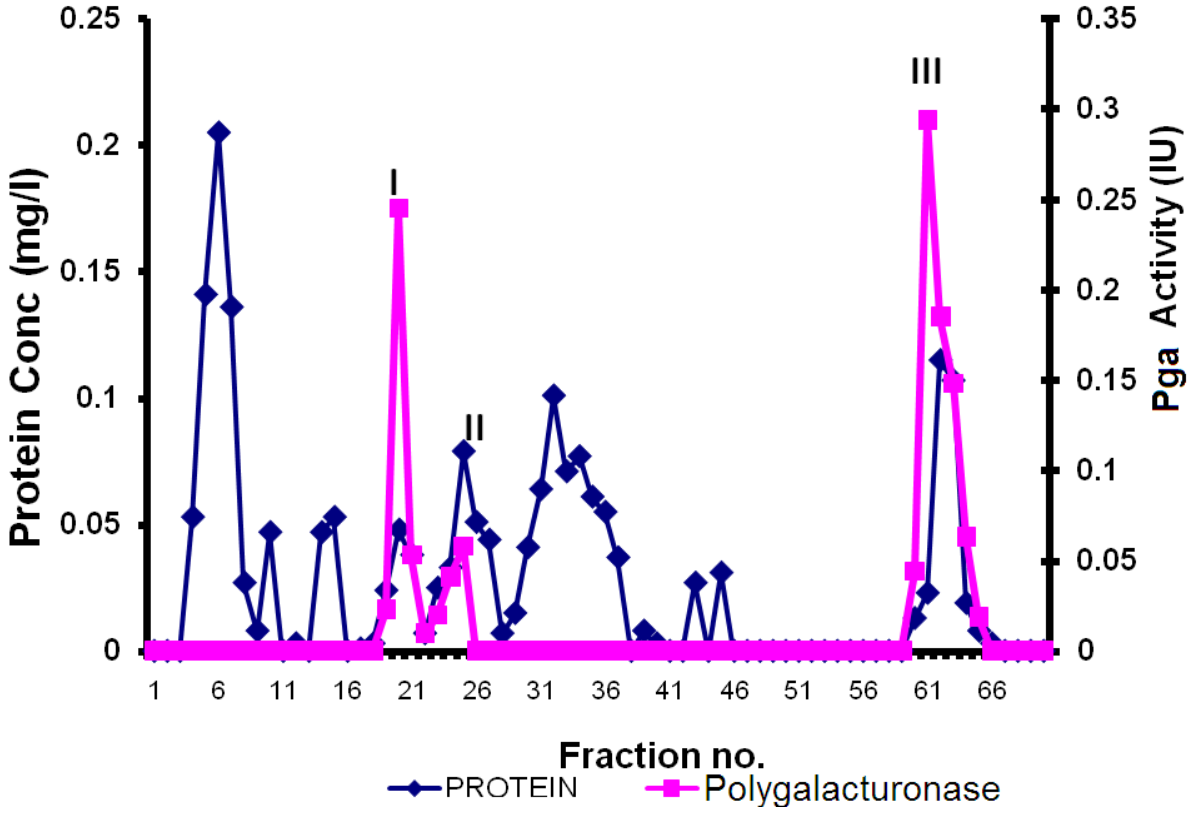


Figure 4.29: Chromatograph of polygalacturonase produced by *P.chrysogenum* using DEAE-Sephadex A25-120.

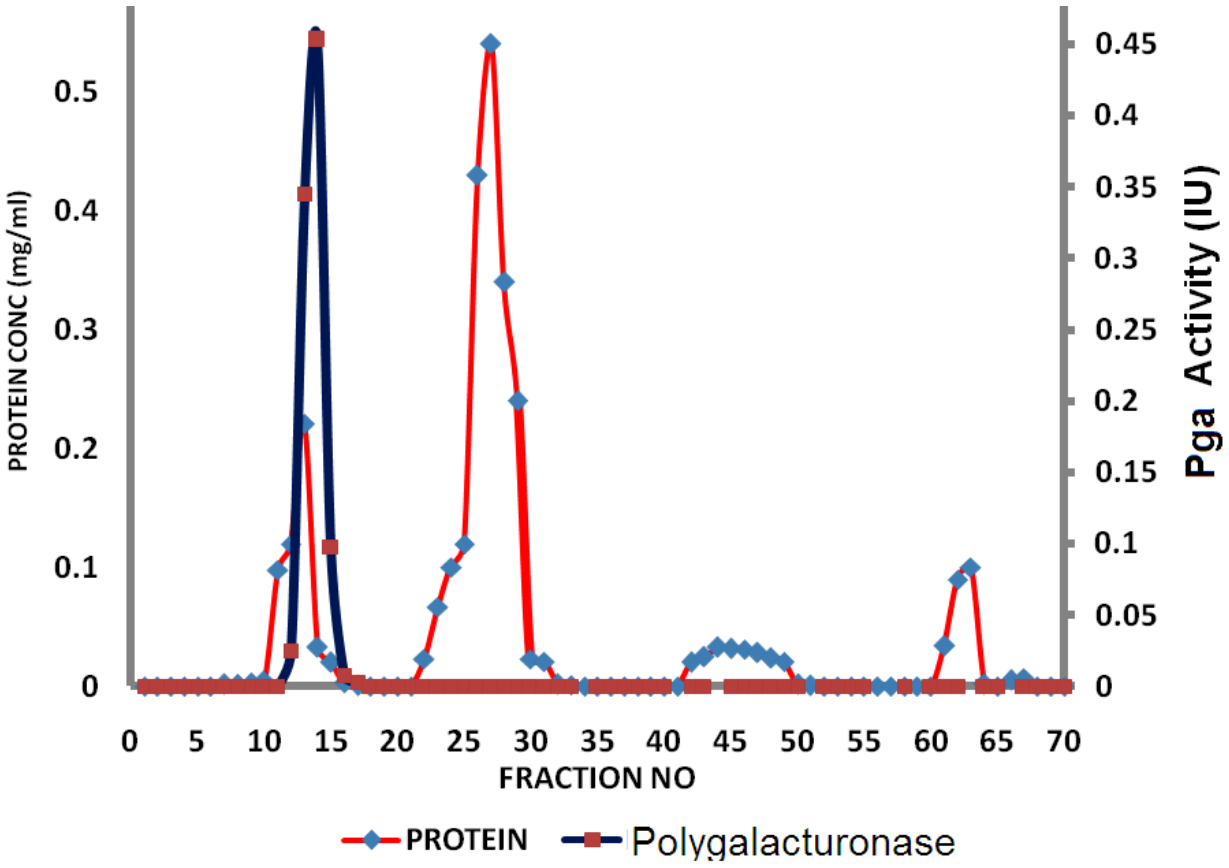


Figure 4. 30: Chromatograph profile of polygalacturonase produced by *A.niger* Sephadex G120.

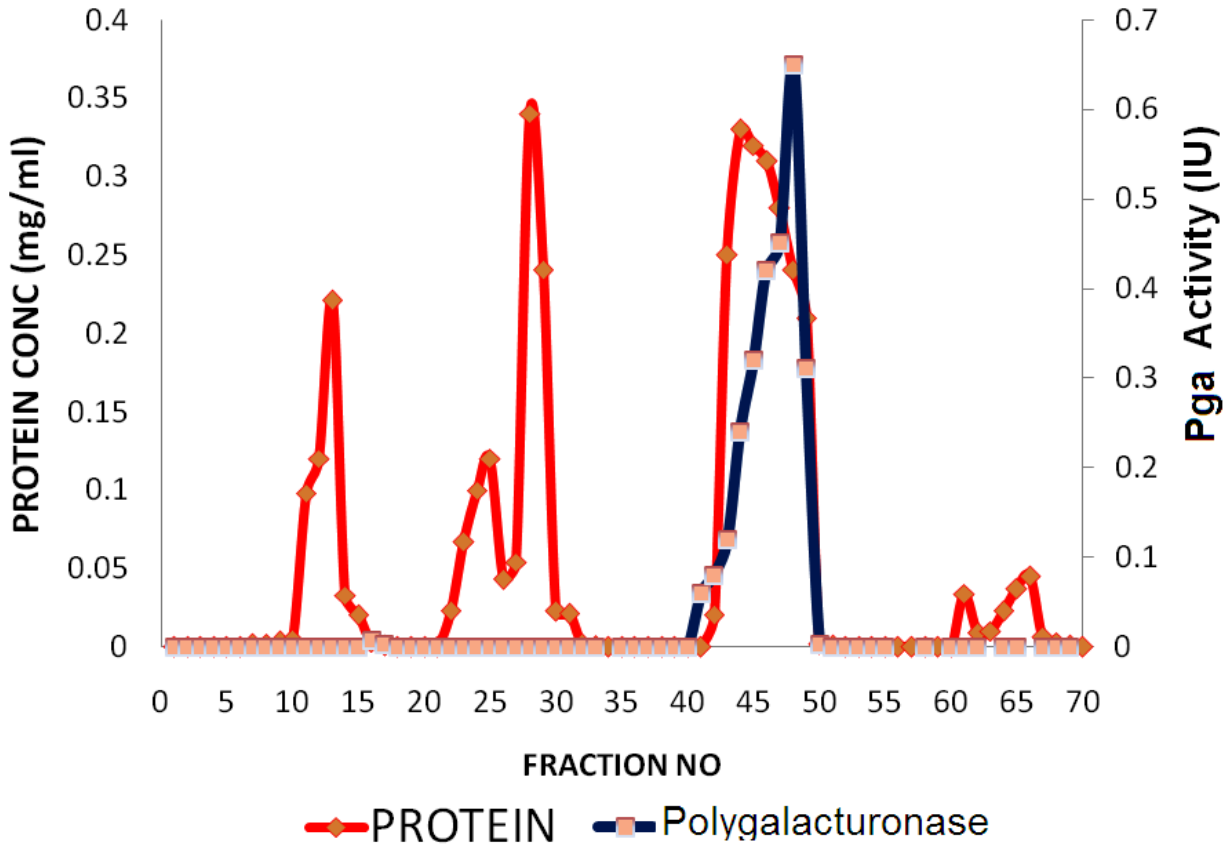


Figure 4.31: Chromatograph profile of polygalacturonase produced by *P.chrysogenum* Sephadex G120.

4.10.1. Molecular Weight of the polygalacturonase Fractions

Figure 4.32 shows the Sodium dodecyl suphate-polyacrilamide gel electrophoresis (SDS-PAGE) of pooled fractions of active polygalacturonase from Sephadex G120 chromatography of *A.niger* and *P.chrysogenum.* The molecular weight of these fractions weres calculated using the ratio of relative mobility (R_f) to log of molecular weight of standard proteins electrophoresed under the same conditions. The molecular weight of the active fraction of *A.niger* and *P.chrysogenum* were 105 and 95Kda respectively.

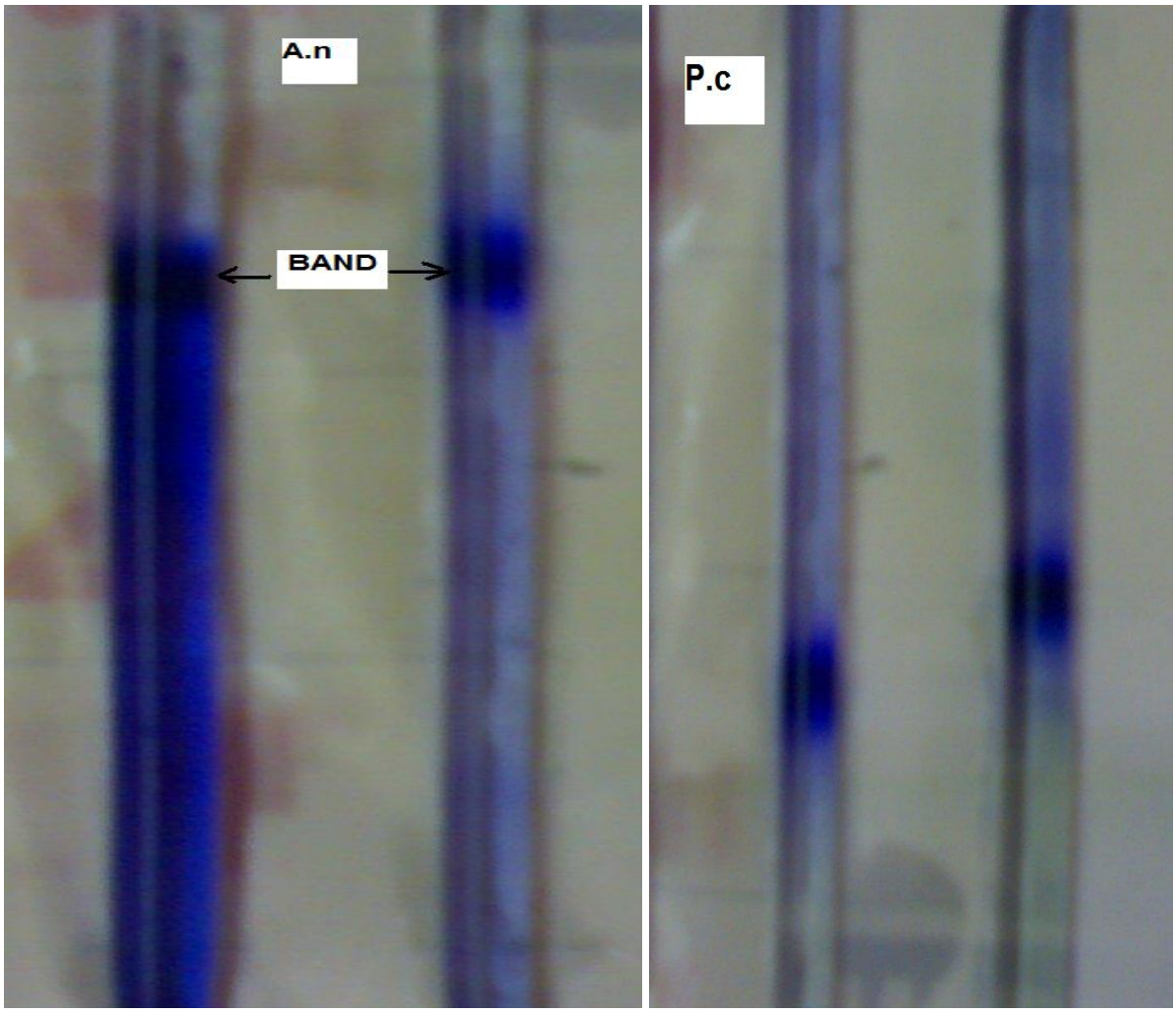


Figure 4.32: Digital images of SDS-PAGE showing the bands of partially purified polygalacturonase

4.11. Properties of Polygalacturonase (Pga) (EC: 3.2.1.15):

4.11.1. Effect of Temperature:

Figures 4.33 and 4.34, illustrate the effect of temperature on Pga activity of 2 DG UV M2 *(A.niger)* and 2 DG UV W1 (*P.chrysogenum).* An optimal temperature of 40^{o}C was found to be suitable for Pga of *A.niger* and *P.chrysogenum.*

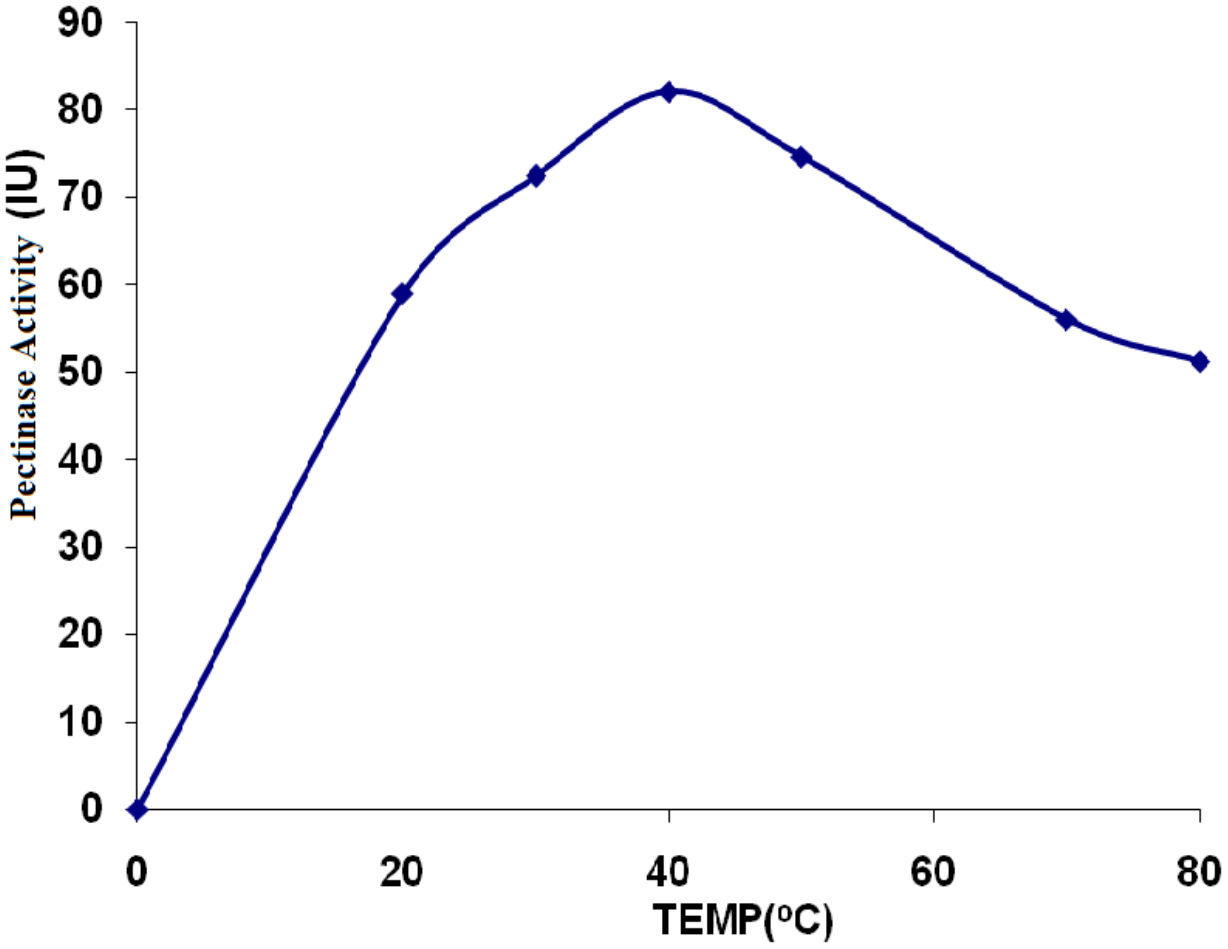


Figure 4.33: Effect of temperature (20-80^{o}C) on polygalacturonase of 2 DG UV M2 *A. niger*.

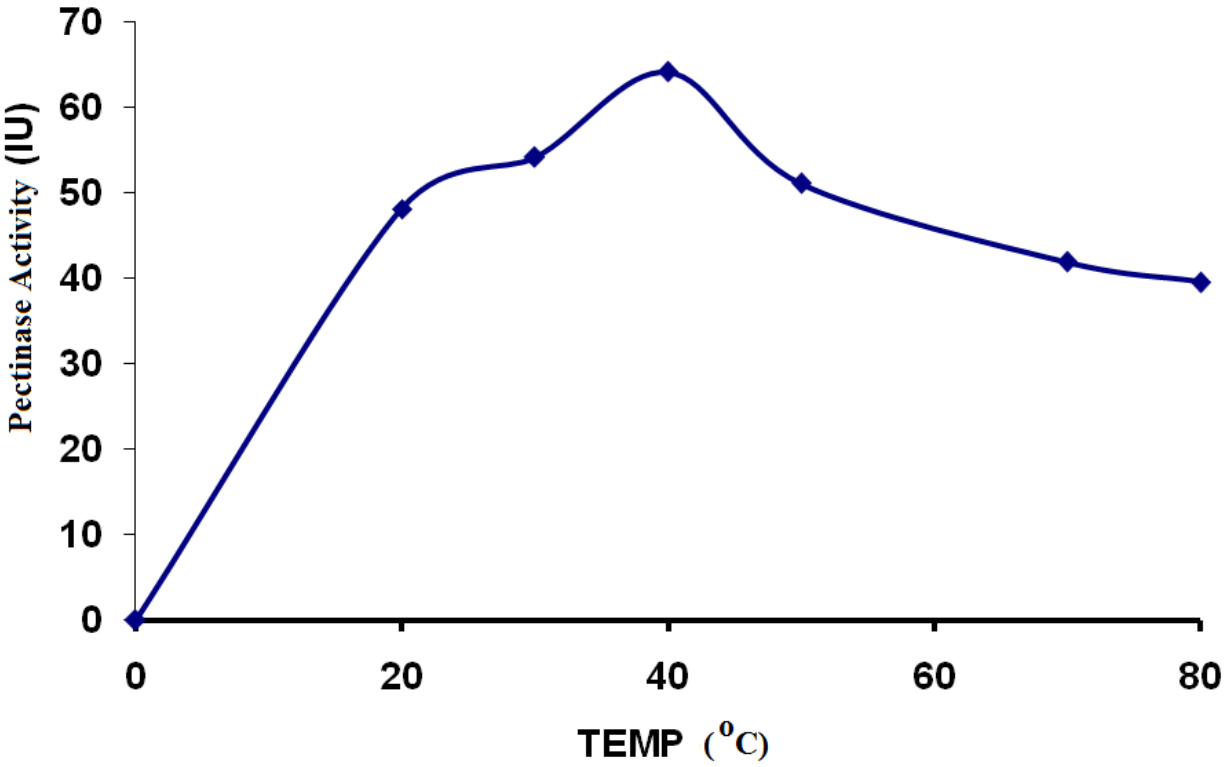


Figure 4.34: Effect of temperature (20-80°C) on polygalacturonase of 2 DG UV W1 *P. chrysogenum*.

4.11.2. Effect of pH

The results of the varying pH of the reaction mixture on the polygalacturonase activities of *A.niger* and *P.chrysogenum* showed a broad pH spectrum for both organism. An optimal pH of 4.5 and 5.0 were obtained for *A.niger* and *P.chrysogenum* respectively (Figures 4.35 and 4.36).

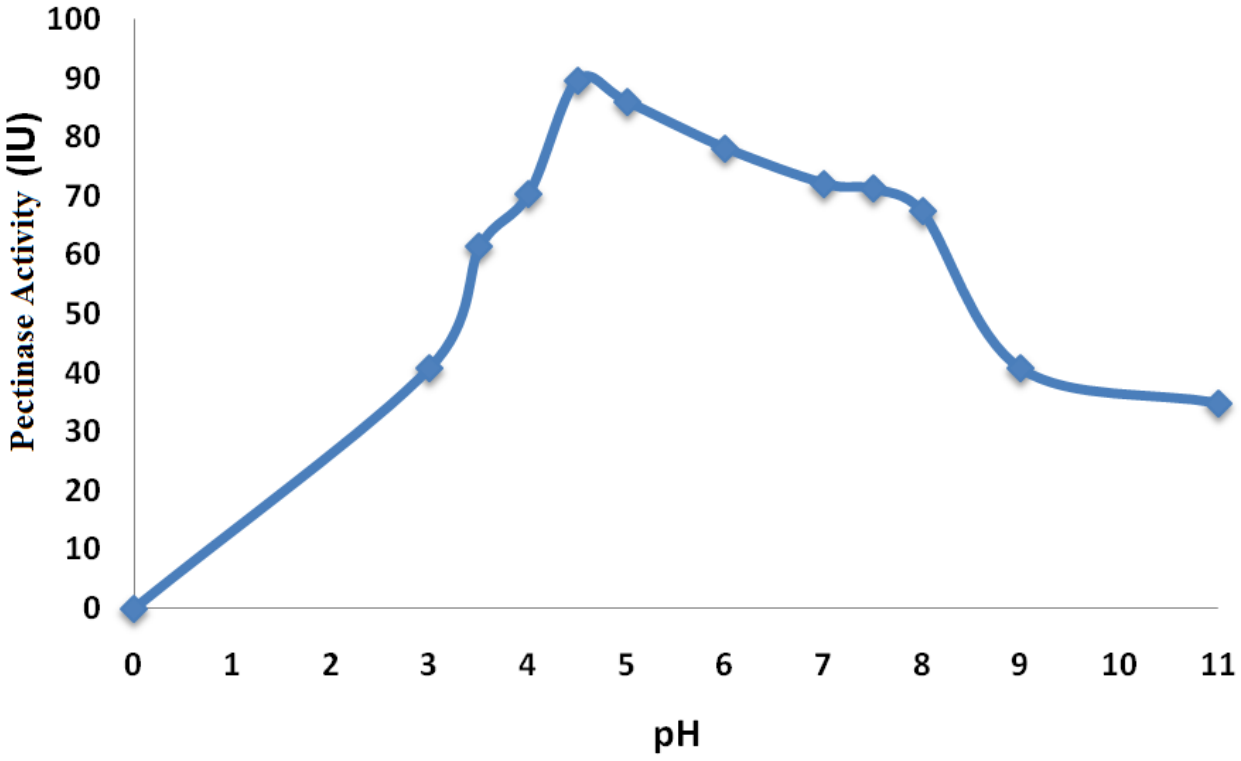


Figure 4.35: Effect of pH (3-11) on polygalacturonase of 2 DG UV M2 *A. niger.*

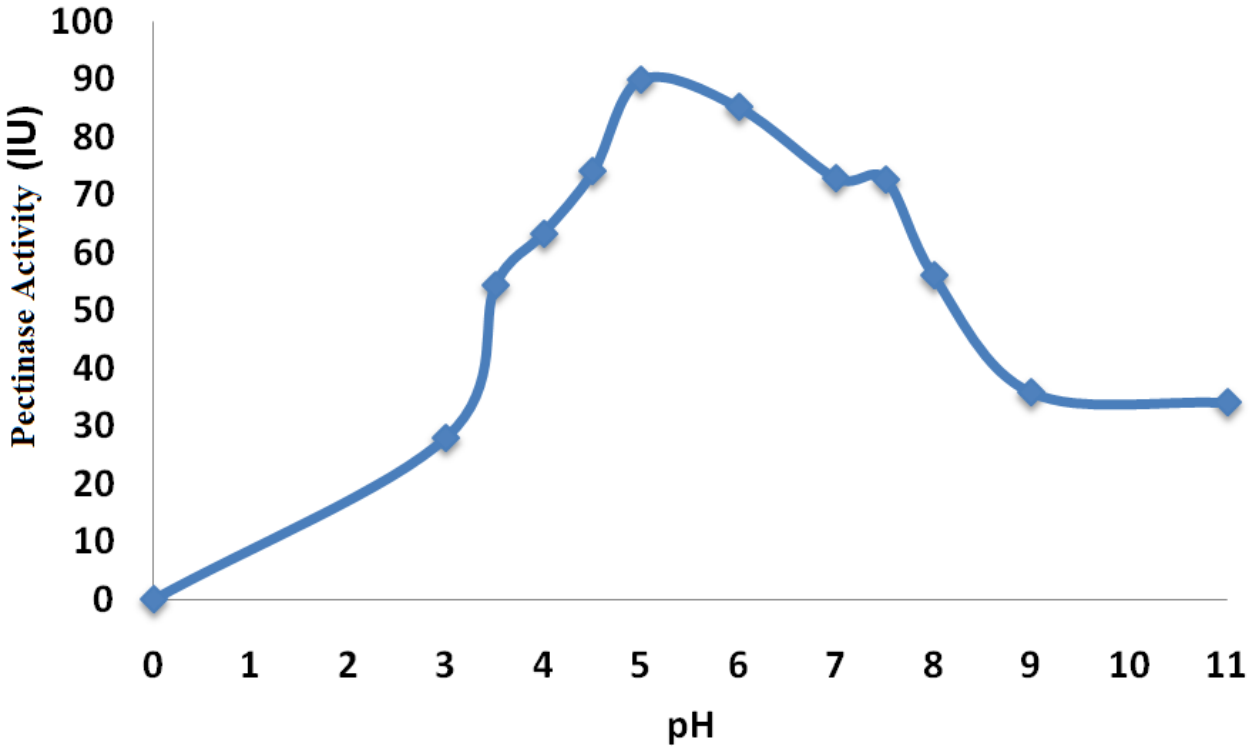


Figure 4.36: Effect of pH (3-11) on polygalacturonase of2 DG UV W1 *P. chrysogenum*.

4.11.3. Effect of substrate concentration

The *Km* for the polygalacturonase of 2 DG UV M2 of *A.niger* and 2 DG UV W1 of *P.chrysogenum* obtained from the plot *of* double reciprocal plot method (Lineweaver-Burk plots) were 1.27 g/l and 2.6 g/l respectively and the Vmax for the enzyme were the same (156.25 IU) for 2 DG UV M2 of *A.niger* and 2 DG UV W1 of *P.chrysogenum* (Figures 4.37 and 4.38).

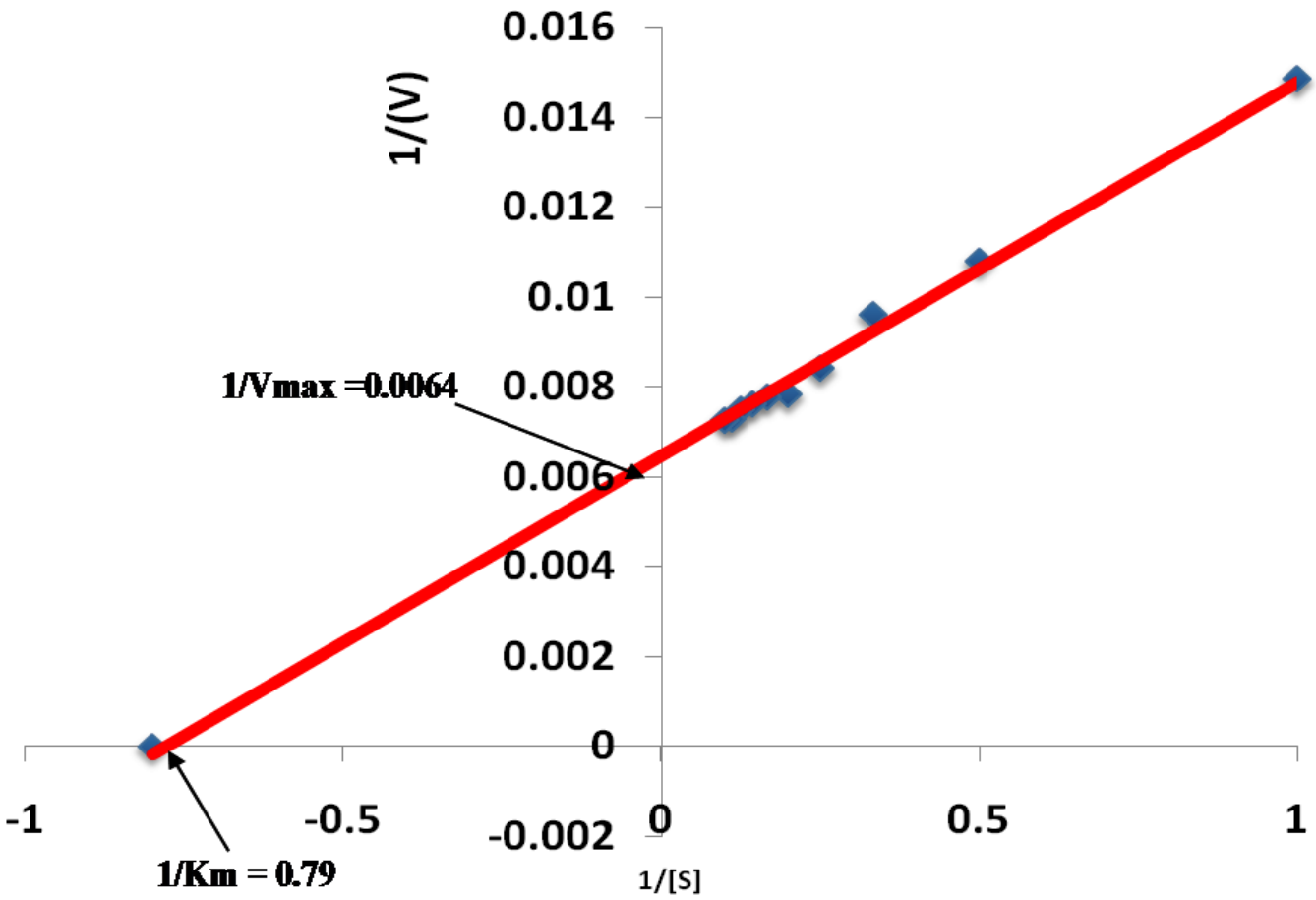


Figure 4.37: Lineweaver -Burk plot of pectinase activity produced by *A. niger*

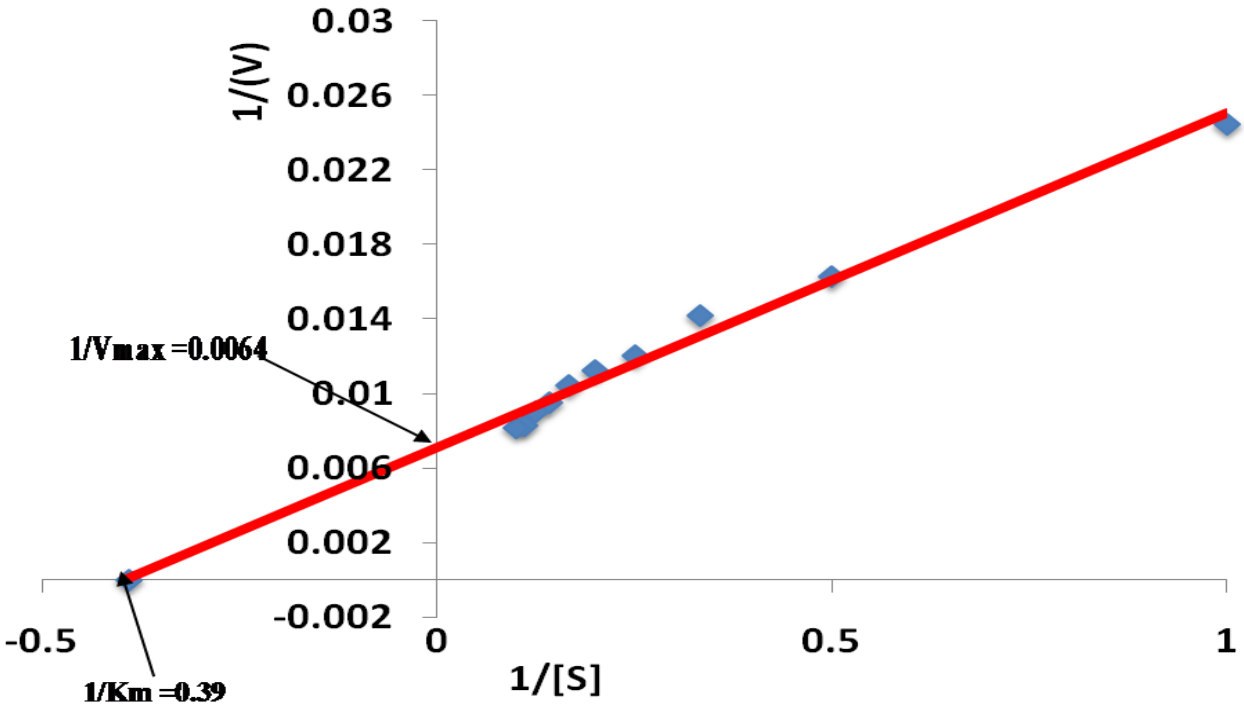


Figure 4.38: Lineweaver -Burk plot of pectinase activity produced by *P. chysogenum*

4.12. Effect of Metal Ions and EDTA on Polygalacturonase Activity

Tables 4.9A and B show the effect of EDTA and various metal ions on polygalacturonase activity of 2 DG UV M2 (*A.niger*) and 2 DG UV W1 (*P.chrysogenum*). There was stimulation of polygalacturonase activity of 2 DG UV M2 of *A. niger* by Mg^{2+} with percentage of 52%. Zn^{2+} ion inhibited the enzyme by 16%. However, polygalacturonase of *P.chrysogenum* 2 DG UV W1, was highly stimulated by Mg^{2+} with a percentage stimulation of 82%, while Hg^{2+} inhibited the activity of the polygalacturonase produced by *P.chrysogenum* by 41%.

Table 4.9 A: Effect of various metal ion and EDTA on pectinase activity of 2 DG UV M2 of *A.niger*

SALTS	ION	% ACTIVITY	% INHIBITION	% STIMULATION
CONTROL	CONTROL	100.0	0.0	0.0
$MgSO_4.7H_2O$	Mg^{2+}	152.0	0.0	52.0
$MnSO_4.5H_2O$	Mn^{2+}	128.0	0.0	28.0
$FeSO_4.7H_2O$	Fe^{3+}	135.0	0.0	35.0
$CaCl_2$	Ca^{2+}	96.0	4.0	0.0
$CuSO_4$	Cu^{2+}	106.0	0.0	6.0
$ZnSO_4$	Zn^{2+}	84.0	16.0	0.0
$HgCl_2$	Hg^{2+}	149.0	0.0	49.0
EDTA	N/A	78.0	22.0	0.0

Table 4.9 B: Effect of various metal ion and EDTA on pectinase activity of 2 DG UV W1 of P.chrysogenum

SALTS	ION	% ACTIVITY	% INHIBITION	% STIMULATION
CONTROL	CONTROL	100.0	0.0	0.0
$MgSO_4.7H_2O$	Mg^{2+}	182.0	0.0	82.0
$MnSO_4.5H_2O$	Mn^{2+}	142.0	0.0	42.0
$FeSO_4.7H_2O$	Fe^{3+}	115.0	0.0	15.0
$CaCl_2$	Ca^{2+}	84.0	16.0	0.0
$CuSO_4$	Cu^{2+}	86.0	14.0	0.0
$ZnSO_4$	Zn^{2+}	64.0	36.0	0.0
$HgCl_2$	Hg^{2+}	59.0	41.0	0.0
EDTA	N/A	74.0	26.0	0.0

CHAPTER FIVE:

DISCUSSION

Isolation, screening and growth of pectinolytic fungal strains

In this study, five fungal species were isolated from decomposing fruit wastes they include– *Aspergillus clavatus, Aspergillus niger, Fusarium sp., Trichoderma harzianum.*and *Penicillium chrysogenum.* *Aspergillus niger* and *Penicillium chrysogenum* were selected as the best producers of pectinase using agro wastes as inducers in a plate assay method. The plate assay approach was previously employed by Makeshkumar and Mahalingam (2011) for isolation and characterization of rapid cellulose degrading fungal pathogens from compost of agro wastes. Also Phutela *et al.,* (2005), used a semi-quantitative plate assay approach to screen for pectinolytic fungi. They isolated a thermophilic fungal strain TF3 (*Aspergillus fumigatus* MTCC 4163) which was selected as the best producer of pectinase and polygalacturonase. Various fungi species are known to inhabit agro wastes, they survive by production of various enzymes and secondary metabolites that destroy or inhibit the growth of other microorganisms (Said *et al.,* 2001).

The growth of *A. niger* and *P.chrysogenum* monitored in various mineral salt medium containing agro wastes as the sole carbon source revealed a possible means of cultivating these organisms on a cheap and readily available medium where byproducts (residual matter) can be used for other cost effective bioprocess (like animal feed, biocomposites and in mushroom production). Nwodo *et al.,* (2007a) illustrated the possible used of agro wastes based media in cultivation of fungal strains, and their growth rate was monitored compared to commercially expensive media.

The result of this study showed that the simple sugars (glucose and sucrose) and the agro wastes were capable of supporting the growth of selected fungi. However, the growth in glucose

containing media was highest while wheat bran supported the growth of these fungal strains best as compared to other agro wastes tested. Akinyele and Adetuyi (2005) studied the effect of various agro wastes on the growth of *Volvariella volvacea* (an edible mushroom). They found that the growth of this strain of mushroom was best supported by rice husk. In another study, Asper*gillus niger* ANL 301 strain using different carbon sources, good growth were observed in all the agro wastes tested (Chinedu *et al.,* 2010). Thus there is greater tendency for organisms to grow very rapidly in media containing the cheaper source of carbon like agro wastes.

The pectinase production profile and the effect of physicochemical factors associated with enzyme production were studied. The fermentation period to achieve maximum peak for pectinase production was 48h with *P.chrysogenum* producing higher pectinase than *A.niger* in a wheat bran containing medium (MS-WB). A 2–day incubation period to achieve peak pectinase activity was also recorded by the isolate *A. fumigatus* TF3 (Phutela *et al.,* 2005), which is suitable from the commercial viewpoint. An earlier study by Said *et al.,* (2001) reported maximum pectinase activity in *Penicillium frequentans* after culturing for 48 h, whereas maximum polygalacturonase activity was observed on the third day of incubation as reported by Maller *et al.,* (2011) in *Aspergillus niveus*.

The pectinase production by *A.niger* and *P.chrysogenum* isolated in this study were ably supported by wheat bran, which is a cheap and readily available carbon source; similar findings were reported by Minussi *et al.,* (2007). In recent years, there has been an increasing trend towards efficient utilization and value-addition of agro wastes (Hossain *et al.,* 2011; Masutti *et al.,* 2012).

A fermentation system is generally regulated by mainly physicochemical and nutritional factors. The nutritional parameters could be effectively monitored in the process for the maximum

production of the end product, keeping physicochemical parameters as constant (Lenihan *et al.,* 2010).

It was observed that after 72h post fermentation, urea best supported the production of pectinase, while polygalacturonase production was optimized when yeast extract was considered as the nitrogen source in *A.niger*. However, when *P.chrysogenum* was investigated, urea and peptone best supported the production of pectinase and polygalacturonase. Ganbarov *et al.,* (2001) reported that pectinase production by *Bjerkandera adusta* 40 was stimulated (127 %) by the addition of $NaNO_3$ as the nitrogen source, whilst that of *Coriolus versicolor* 24 was stimulated (154 %) by the addition of $(NH_4)_2SO_4$. Phutela *et al.,* (2005) observed that the supplementation media with yeast extract + $(NH_4)_2SO_4$ greatly enhanced the pectinase and polygalacturonase production and promoted more than twice the enzyme activity as compared to urea+ $NaNO_3$.

Most studies on the effect of nitrogenous salts on the production of pectinases revealed divergent views on the most effective nitrogenous salts for this class of enzyme (Maller *et al.,* 2011: Azzaz *et al.,* 2012; Sampriya *et al.,* 2012). The type of nitrogenous salt with the most effective enzyme production could be linked to the source and strain of the microorganism in question. An acidic pH 3.5 and 5.5-6.5 were optimal for pectinase production by *A.niger* and *P.chrysogenum*, respectively. The pH range 3.5-8.0 supported pectinase yield. This might be as a result of other active enzymes produced by these strains since pectinase is a group of hydrolytic enzymes comprising mainly polygalacturonase, pectin lyase and pectin methylesterase. These enzymes function synergistically. The report of Phutela *et al.,* (2005) confirmed the acidic pH (pH 4.0-5.0) of pectinase and polygalaturonase production by *A.fumegatus.* Fungi and yeasts produce mainly acidic PGases, whilst alkaline pectinases are mainly produced by bacteria

(Fevela-Torres, 2006). The results show that such changes in pH during fermentation cannot bring about a great effect on the efficiency of these fungal strains during pectinase production.

Hyper production of pectinase using UV mutagenesis

The UV irradiation of spores suspension containing $1x10^6$ spores/ml show that over 80% lethality was reached after 25 min. De Nicolas-Santiago *et al.,* (2006) showed that UV irradiation of *A. niger* spores that caused 50% lethality was achieved after 30 min of irradiation. The results of various UV irradiation studies showed a lack of correlation of all data, similar observation has been previously reported (De Nicolas-Santiago, *et al.,* 2006; Haq, *et al.,* 2006; Kovacs, *et al.,* 2008; Irfan, *et al.,* 2011). This might be as a result of the UV target rate (dosage per unit time) of the spores.

In the 1930s, a theory to explain the target rate (survival curve) was established by J.A. Crowther, N.W Timofeeff-Ressovskky, and Max Delbruck and later developed by D. Lea, (Lea and Coulson, 1949). However one of the shortcomings of target theory is the probability and positioning of the target (in this case the fungal strains), which has led to various conclusions in elucidation of a fixed slope for a survival curve by most researchers.

Thus generation of mutants was conducted using a mutagenesis time of 25 min with two replicates, and the use of 2 deoxyglucose to inhibit the growth of these fungi. Three mutant strains for *A.niger* (2DG–UV M1, M2 and M3) and five strains for *P.chrysogenum* (2DG-UV W1, W2, W3, G1, and G2) suspected to be hyper-producing strains were isolated and screened for pectinase hyper activity as compared with wild strains. The analysis of the enzyme activity indices by the mutants show that the degree of enzyme activity indices differs among the mutant strains, but is higher than the wild strains.

2-Deoxy-D-glucose (2DG), a toxic glucose analogue, has frequently been employed to isolate glucose-deregulated mutants (Haq *et al.,* 2006). Haq et al. *(*2006) isolated a total of 110 UV mutants of *Aspergillus niger*. Out of all the mutants, only mutant GCBU 25 isolated after 25 mins of UV exposure increased the production of amyloglucosidase by 2 fold than the parent strain in submerged fermentation (SmF).

The relationship of growth (mycelia dry weight) of both the wild and the mutant strain of *Aspergillus niger* (2DG-UV M2) and *Penicillium chrysogenum* (2DG-UV W1) and their pectinase production under solid state fermentation using pectin, sugar cane pulps and wheat bran showed that the growth rate of the mutant strains of both *A.niger* and *P.chrysogenum* was reduced as compared to the wild (parent) strains. However, their ability to produce pectinase was better than the wild strains.

Most enzyme induction studies have been shown to be subject to catabolite repression (CR) when there is abundance of a fermentable substrates such as glucose, glycerol or other carbon sources (de Groot *et al.,* 2003; Favela-Torres, 2006). The effect of catabolite repression as discussed by Favela-Toress (2006) showcases the need for the mutant (hyper producer) to be resistant to easily metabolized carbon sources, which will increase biomass and enzymes production.

The catabolite repression experiments with both wild and mutant strains of *A. niger* and *P.chrysogenum* revealed that the pectinase induction was subject to catabolite repression in the wild strains of both isolates, but the 2 DG-UV M2 (mutant strain of *A. niger*) showed an increase in pectinase activities, above the basal pectin level with a slight depression when 10 g/l glucose concentration was used. However, when the catabolite was changed to sucrose, the level of repression was high in the wild of *A.niger*, while, the 2 DG UV M2 mutant strain of *A.niger* showed a resistant to catabolite repression with a slight depression in activity at 5 g/l of sucrose.

For *P. chrysogenum,* the results confirm that there was resistance to CR (glucose) effect on the 2 DG-UV W1 strain as the concentration of glucose increased compared to the wild strain of *P.chrysogenum.* The effect of CR (using sucrose) on both the wild and the 2 DG-UV W1 (*P.chrysogenum*) was relatively high compared to the percentage yield when pectin alone was used as the inducer. Therefore, *P.chrysogenum* is CR sensitive in the presence of sucrose. Catabolite repression plays an important role in the regulation and secretion of inducible enzymes.

Genomic Characterization of Wild and Mutant Strains using Random Amplified Polymorphic DNA (RAPD)-PCR Analysis

DNA polymorphism can be revealed by various molecular techniques, with some of them being even more sensitive (e.g. Sequencing). Nevertheless, differentiation of fungal strains and species by RAPD certainly is an easy tool to detect polymorphism in a large number of samples at relatively low cost (Motlagh and Anvari 2010).

The assessment of the strains using RAPD patterns generated by OPA 02, 04, OPC 02, and OPE 02 primers indicated genetic variability between the wild and mutants isolates. In spite of the small number of amplified polymorphic fragments between the species, these results indicate differences in the genome of these isolates. El. Sherbeny *et al.,* (2005) used RAPD-PCR to investigate the influence of ionizing radiation in inducing DNA-Polymorphisms. The number of amplified DNA fragments ranged between 81 and 78 in *R. rubra and H. anomala* respectively when 3 M RAD of gamma rays were used. El. Sherbeny *et al.,* (2005) suggested that fast neutrons were more efficient in inducing DNA polymorphism than gamma rays. *A. japonicus* and *A. aculeatus* strain were studied by Hamari *et al.,* (1997) using RAPD and isoenzyme analysis. Various research studies have applied the RAPD-PCR technique in the study of genetic

diversities in animals (Yaqoob *et al.,* 2007), plants (Singh *et al.*, 2009), fungi (Abadio, *et al.,* 2012) and microorganisms (Cardoso *et al.*, 2007).

The results of RAPD-PCR confirm the genetic variability between the different fungi, using RAPD-PCR.

Enzymes Purification Studies

The polygalacturonase of 2 DG UV M2 of *A.niger* was purified to 7.94 purification fold with a specific activity of 4.13U/mg protein while a purification fold of 4.7 with specific activity of 2.82 U mg protein^{-1} were obtained for 2DG UV W1 for polygalacturonase (EC:3.2.1.15) of *P.chrysogenum.*

The molecular weight of the polygalacturonase of *A.niger* 2DG-UV M2 and *P.chrysogenum* 2 DG UV W1 elucidated using SDS-PAGE was estimated to be 105 and 95 kDa respectively. De Vries and Visser (2001) reported a wide range of diversity in molecular weight of pectinase in various *Aspergillus species.* Within the *Aspergillus niger* strains reviewed a molecular weight as high as 80kDa and low as 35 kDa were reported. Hence, molecular weight of this enzyme-polygalacturnase might not be strain specific but a genotypic.

The polygalacturonase of these isolates exhibited the same optimum temperature of 40^{o}C, having about 50% of its optimum enzyme activity at 80^{o}C. The enzyme of both isolates had activity at pH range 3-11 with the highest enzyme activity at pH 4.5 and 5.0 for 2 DG UVM2 of *A.niger* and 2 DG UV W1 of *P.chrysogenum* respectively. Various fungi strains demostrated a wide range of optimal temperature, *A. alliaceus, A. carbonarius* and *A. niger* polyagalacturonase (EC:3.2.1.15) yielded an optimal temperature of 55, 50 and 50^{o}C respectively and the pH optimal of 5.0, 4.0 and 3.8 for *A. alliaceus, A. carbonarius* and *A. niger* polyagalacturonase (EC:3.2.1.15) (De Vries and Visser, 2001). The broad temperature and pH ranges of the isolates in this study is an important factor that could be harnessed in the industrial application of this

enzyme. The polygalacturonase of these isolate showed stability over a high range of temperature. The enzyme retained about 45% enzyme activity at 80^{o}C. This is equally a plus for the polygalacturonase of these isolates. Ortega *et al.,* (2004) reported that polygalacturonase retained around 40% of its activity at 65^{o}C and 75^{o}C. Maller *et al.,* (2011) investigated polygalacturonase of *A. niveus*, their result showed that the PG secreted was quite active and stable at 60°C. The optimum temperature of commercial enzyme Rapidase C80 was determined to be 55^{o}C. Similar optima temperature were detected for polygalacturonases in *S. thermophil* (Kaur *et al.,* 2004). Lower temperature optima were however reported for polygalacturonases from *T. reesei* (40 ^{o}C) (Mohamed *et al.,* 2006) and *A. niger* (40 ^{o}C) (Fahmy *et al.,* 2008). 45 to 50°C has been reported for pectinase production by *Thermomucor indicae-seudaticae* N31 (Martin *et al.,* 2010), and 50°C for thermostable exo-polygalactouronase from actinomycete (*Streptomyces erumpens* MTCC 7317 (Shaktimay and Ramesh, 2011).

The present study also showed that the V_{max} calculated from Lineweaver-Burk plots were 156.25IU/ mg protein for both 2 DG UV M2 of *A.niger* and 2DG UV W1 of *P.chrysogenum*. While the *Km* constant of 1.27 g/l for 2 DG UVM2 of *A.niger* and 2.6 g/l for 2DG UV W1 *P.chrysogenum*. Various researchers have attempted the purification of polyagalcturonase (Pga) from various sources. The kinetic studies of the enzyme show a wide difference in Vmax and *Km* values. These values range from 0.11 mgmL^{-1} to 4.47 mgmL^{-1} for *Km* and 1.68 μmol min^{-1} mg^{-1} to 1100 μmolmin^{-1} mg^{-1} for *V*max (Saeed *et al.,* 2007; Gomes *et al.,* 2009). Thus the results on kinetic parameters of polygalacturonase fall within this range with 2 DG UV M 2 of *A. niger* having a lower *Km* than 2 DG UV W1 of *P. chrysogenum.*

The effects of metal ions and EDTA on the polygalacturonase activities of the isolates showed that Mg^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+} acted as activators of Pga of both isolates. However Mg^{2+} and Hg^{2+} best supported the activity of Pga of the 2 DG UVM2 of *A.niger* isolate while

Zn^{2+} inhibited the enzyme and Mg^{2+} best supported polygalacturonase of 2 DG UV W1 *of P.chrysogenum* and Hg^{2+} inhibited the enzyme. Maller *et al.,* (2011) revealed that EDTA and Mn^{2+} increased the enzymatic activity of the Pga from *A. niveus*, and Hg^{2+}, Ba^{2+}, and Cu^{2+} inhibited actvity. Mohamed *et al.,* (2006) examined the effect of various metal cations on polygalacturonase activity; they reported a different and partial inhibitory effect on the activity of *T. harzianum* PGase except for Hg^{2+} which completely inhibited the enzyme activity. This similar effect was reported for polygalacturonase from Bacillus sp., while Cu^{2+}, Ni^{2+} and Zn^{2+} inhibited the enzyme activity (Kobayashi *et al.,* 2001). Thakur *et al.,* (2010) observed that addition of metal ions such as Mn^{+2},Co^{+2}, Mg^{+2}, Fe^{+3}, Al^{+3}, Hg^{+2} and Cu^{+2} had inhibitory effect on polygalacturonase production. The effects of metal ions and EDTA on Pga shows that there is no specificity in terms of enzyme activators or inhibitor, most research results revealed diverse opinion on Pga activators and inhibitors. The study of Sakamoto *et al.,* (2002) on the effects of metal ion on Pga, showed that HgCl increased the Pga 2 activity of *A. niger 3.4* times but did not affect Pga 1, this is in support of the above results reported of this study work.

The inhibitory effect of the divalent cations was probably a result of physical interactions with the pectin and not a direct effect on the enzyme (Saeed *et al.,* 2007). Thus, the contrast in results by many researchers could be associated with the type of microorganism used in the enzyme production. The cause of the inhibition with Ca^{2+} is that this ion enhances the gelling of the pectin molecules, thereby producing interlink and make the substrate inaccessible to the enzyme.

CONCLUSION

Current trend in the world today is to utilize and convert wastes into useful products and to recycle waste products as means of achieving sustainable development. This dissertation has investigated spectra of microbes inhabiting decaying agro wastes that are both beneficial and harmful to human. The organisms which were isolated from decomposing agro wastes and screened for pectinolytic activities; have medical, agricultural, industrial and economic importance. Pectinase production from various agro wastes is economically feasible within the fungi strains studied. The rich fungi flora of Lagos-Nigeria revealed high yield of pectinase production and capabilities of using various agro wastes as cheap carbon source.

The selected fungi used in this study were able to grow in various agro wastes which are cheap and readily available carbon source. Wheat bran best support pectinase production by both fungi. These fungi were UV mutated and the resultant mutants were less responsive to catabolite repression during pectinase production, catabolite repression is an important factor for production of inducible enzymes. Also the mutants were adaptable to both solid state and submerged fermentations.

RAPD-PCR was able to reveal the high genetic variability of the wild and mutant strains of these fungi. the observed genetic diversity can aid in the development of appropriate strategies to obtain better strains resistant to catabolite repression for industrial application.

The partially purified enzyme has unique industrial enzymes properties, stable at $80^{o}C$ and having enzyme activities over a wide range of pH. The results of this research work also revealed broad spectra of activators of the enzyme with Mg^{2+} ion activating the enzyme best.

The results presented in this thesis shows a promising biodegradation of agro waste into useful and valuable products, which can boast the economy of the nation and reduce unemployment.

Once agro wastes become a resource; the menace to the environment and health hazard contribute by the indiscriminate disposal of agro waste will become a thing of the past.

Contributions to Knowledge

1. The microfungi (*A.niger* and *P.chrysogenum)* were genetically improved (using UV mutation) to hyper produce pectinase.

2. A total of eight pectinolytic microfungal mutant strains were isolated using UV radiation- 2 DG UV M 1, 2 DG UV M2, 2 DG UV W 1, 2 DG UV W2, 2 DG UV W3, 2 DG UV G4, AND 2 DG UV G5.

3. The mutants 2-DG UV M1 and 2-DG UV W1 were less responsive to catabolite repression in both Submerged and Solid state fermentation thereby increasing their pectinase production potential using agro wastes.

4. The purified polygalacturonase has a good industrial potential based on its enzyme properties.

5. Wheat bran and sugar cane pulp were identified as best agro wastes capable of inducing pectinase production.

Recommendation for future work

This research has thrown up many questions in need of further investigation on pectinase production from other microorganisms locally sourced within major biomass generating cities in Nigeria, using the same experimental set up, this will generate more data/information on locally produced pectinases and would help to establish a greater degree of accuracy on microbial production of pectinase.

Although biological processes for the production of pectinase have been demonstrated in this study using various cultured microorganisms and biomass, these processes must still be integrated into a system capable of meeting basic requirements for overall efficiency of bioconversion of agro wastes into useful products, it is therefore recommended that more research work be done in view of industrial production efficiency of pectinase.

Furthermore, strain improvement of these microorganisms should be enhanced with a view of hyper-production of these enzymes. It is necessary to improve these strains in a bid to make them more adaptable to fermentation types, high yielding of enzymes and specificity.

There are a lot of industrial processes in Nigeria where pectinases can be applied to improve the quality and the yield of final products. In this way, it is important to investigate the production conditions and other physico-chemical characteristics involved in application of pectinase in final products quality and quantity.

Also, future efforts into pectinase research should be concentrated on elucidation of the regulatory mechanism of enzymes secretion at the molecular level and the mechanism of action of different pectinolytic enzymes towards pectic substrates. These studies can provide valuable tools to manipulate microorganisms making them able to produce efficient enzymes in high amounts.

As with any development, the sustainable re-use of agro/food waste resources would not be without difficulties, but it would open up the opportunity for biotechnological developments.

The same strategic imperatives, socio-economic growth and sustainable developmental issues, which drove Western countries' research into waste utilization since the 1970's are of even greater and progressing relevance to developing countries like Nigeria, there is, therefore, a definite need for agro wastes management. The general pathways of agro/industrial/food wastes generation should be reduced, recycled or reused and what is left must be treated and disposed of in an environmentally acceptable way.

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