SCREENING OF SOME MOULDS FOR PECTINASE PRODUCTION AND OPTIMIZATION OFFERMENTATION PARAMETERSUSING ORANGE PEELS AS SUBSTRATE

BY

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DECEMBER, 2021

DECLARATION

I, EleojoIKANI, hereby declare that the work in this Dissertation entitled “SCREENING OF SOME MOULDS FOR PECTINASE PRODUCTION AND OPTIMIZATION OF FERMENTATION PARAMETERS USING ORANGE PEELS AS SUBTRATE” has been performed by me in the department of Microbiology.The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

EleojoIKANI——————————————————
P17LSMC8036 Signature Date

CERTIFICATION

This Dissertationentitled “SCREENING OF SOME MOULDS FOR PECTINASE PRODUCTION AND OPTIMIZATION OF FERMENTATION PARAMETERS USING ORANGE PEELS AS SUBTRATE”by EleojoIKANI meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God and to my dearly beloved parents Mr J.H. Ikani and Mrs J.O. Ikani.

ABSTRACT

Enzymes are increasingly being used in different industrial processes globally as a result of their extreme efficiency and highly specific bio-catalytic activities. Pectinases are among the most important industrial enzymes and their demand is increasing day by day, hence, this study was undertaken with the aim of isolating and screening mould species from local soil for pectinase production and optimizing the production parameters for optimal enzyme yield using orange peel wastes via solid state fermentation. The proximate composition of the orange peel substrate was determined following standard procedures. Isolation of pectinolytic moulds was carried out using the spread plate and streaking method to obtain pure cultures. Screening of the isolates for their pectinolytic activity was done by culturing on Pectinase Screening Agar Medium (PSAM) and flooding with iodine-potassium iodide solution. The effects of incubation period, initial pH, initial temperature and nitrogen supplementation on the production of pectinase were investigated. Pectinase was produced by *Aspergillus niger*and *Sclerotium rolfsii* isolates using orange peel as substrate by solid state fermentation under predetermined optimum conditions. The pectinase activity was assayed with 3, 5-dinitrosalysalic acid method. The pectinase produced was purified by Ammonium Sulphate Precipitation, Gel filtration chromatography and Ion exchange chromatography. Proximate analysis of the orange peel revealed moisture content of 26%, Ash 3.86%, Lipid 3.40%, Protein 13.20% and Carbohydrate 53.45%. Seven (7) mould species; *Aspergillus niger, Monilia sitophila, Sclerotium rolfsii, Penicillium, Fusarium, Cladiosporium* and *Curvularia* species were isolated from soil obtained from the Botanical garden of Ahmadu Bello University, Zaria. Two (2) isolates; *Sclerotium rolfsii* and *Aspergillus niger*had the highest zone of hydrolysis (35 mm and 31 mm respectively) upon screening, thus they were selected for production studies. Maximum pectinase activity was achieved at pH 5.0, and a temperature of 30°C with peptone supplementation on day 5 of incubation for *Sclerotium rofsii* andday 6 for *Aspergillus niger*. The maximum enzyme yield for *Sclerotium rofsii was* 108.54 ± 2.15 U/mL while *Aspergillus niger*gave an overall enzyme yield of104.12 ± 0.53 U/mL. Results of the partial purification of extracellular pectinase produced revealed a decrease in the total enzyme activity with a corresponding increase in the specific activity of the enzyme. For *Sclerotiumrofsii*, total pectinase activity of the crude enzyme decreased from108,540 U/mL to a final value of 45,075 U/mL after the three stages of purification, while the specific activity increased from 10.30- 45.58 U/mg. The total protein content of the enzyme decreased from 10,540 - 989 mg, while the protein concentration increased from 10.54-19.78 mg/mL. For *Aspergillus niger*; total pectinase activity of the crude enzyme dropped from 104,120 U/mL to a final value of 41,701 U/mL after ion exchange chromatography, while the specific activity increased from 5.12 -32.58 U/mg. The total protein content of the enzyme decreased from 20,320 - 1,280 mg, while the protein concentration increased from 20.32 - 25.60 mg/mL. The high yield of pectinase obtained in this study revealed that orange peel substrate contains sufficient organic nutrients for growth and pectinase production by *Sclerotium rofsii* and*Aspergillus niger*. The study also revealed that *Sclerotium rolfsii* and*Aspergillus niger*isolated from botanical garden in Ahmadu Bello University, Zaria, Nigeria have potential for pectinase production.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Enzymes obtained from microorganisms are known to be of superior quality and they catalyse reactions in bio-processes in an economical and environmentally friendly way as opposed to the chemical catalysts hence in recent years there has been an increasing interest in the development and use of enzymes derived from microorganisms; more so as microbial enzymes are generally considered safer than the chemical catalysts (Torimiro *et al*., 2018). In a recent business intelligence report on 'Global Enzymes Market', the enzymes market size was worth USD 9.9 billion in 2019 and is anticipated to exhibit an annual growth rate of 7.1% over 2020-2027, with a net value of USD 17.17 billion by the end of 2027 (Sudeep *et al*., 2020).Factors such as the multifunctional benefits of industrial enzymes across various applications and the technological innovations to reduce the consumption of chemicals contribute to the growth of the industrial enzymes market (Sudeep *et al*., 2020). Pectinases are one of the upcoming industrial enzymes with commercial importance accounting for 25% of the global food and industrial enzyme sales (Oliyad and Abate, 2017).

Primarily, these enzymes are responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells; they are now an integral part of fruit juice and textile industries as well as having diverse and innumerable biotechnological applications since they can be applied in various industrial sectors wherever the degradation of pectin is required for a particular process (Oliyad, 2017). The structural constituents of a plant cell wall are cellulose, hemicellulose and pectic substances. While the cellulose micro fibrils provide strength to the cell wall, the hemicelluloses and pectic substances (which are colloidal polysaccharides, with galacturonic acid backbone linked by α (1-4) linkage) act as the cementing substance for the cellulose network. Pectins (or pectic substances) contribute to complex physiological

processes like cell growth and cell differentiation and so determine the integrity and rigidity of plant tissue (Tapre and Jain, 2014; Oliyad, 2017). Pectinase is an enzyme that hydrolyses the pectin substances present in plants and its use in diverse applications indicates its importance for green process (Mohandas *et al*., 2018).

Recently, pectinases have become the industrial enzymes of choice in food processing industries for wine and fruit juice extraction and clarification, tea and coffee processing. Other processes requiring pectinases include textile manufacturing, paper and pulp industries, oil extraction industries, industrial waste-water treatment plants, manufacture of pectin free starch; refinement of vegetable fibres; degumming of natural fibres (Samreen *et al*., 2019). Furthermore, enzyme cocktails having pectinases are used for the preparation of animal feeds(Kashyap *et al*., 2001), Microbial sources have occupied an important place in the production of pectinases, among microorganisms; fungi (especially moulds) as enzyme producers have many advantages since they are normally GRAS (generally regarded as safe) strains and the produced enzymes are extracellular which makes recuperation of products from fermentation medium quite easy (Torimiro *et al*., 2018).

Microorganisms are currently the primary source of pectinase with 50% originating from fungi; 35% from bacteria, while the remaining 15% are of plant origin, priority is however given to the fungi (especially moulds) because of their ability to colonize the substrate by apical growth and penetration which gives them a considerable ecological advantage over non-motile bacteria and yeast, which are less able to multiply and colonize on low moisture substrate (Abdullah *et al*., 2018). Microbial production of pectinase has been widely studied and*Aspergillus niger*is the most commonly used fungal species for industrial production of pectinolytic enzymes (Murad and Azzaz, 2011).

Two fermentation techniques can be used for pectinase production, these techniques are; Solid State Fermentation (SSF) and Sub-merged Fermentation (SmF).Solid state fermentation is

defined as the cultivation of microorganisms on moist solid supports that can be used both as carbon and energy source (Pandey, 1992). The low moisture content indicates that fermentation can only be carried out by a limited number of microorganisms, mainly fungi. This process occurs in the absence or near absence of free flowing water (Pandey *et al*., 2000a). In contrast to solid state fermentation, in submerged fermentation (SmF) the nutrients and microorganisms are both suspended in water (Pandey *et al*., 2000b). Solid state fermentation offers distinct advantages over submerged fermentation including economy of space needed for fermentation; simplicity of the fermentation media; higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water insoluble substrates, lower demand for sterility due to the low water activity, greater product yield, lower capital and low recurring expenditures in industrial operation, absence of foam build up; and easier control of contaminants due to the low moisture level in the system (Kapilan, 2015).

The most promising residues for solid state fermentation include agricultural and forestry residues, which are very abundant and usually underutilized especially in developing countries. The most widely used substrate for solid state fermentation for pectinase production are materials of mainly plant origin comprising of agricultural and food processing wastes such as fruit peels and pulps, wheat bran, cassava peels, sugar beet pulp, corn cob, sugarcane bagasse, cassava bagasse, cereal bran such as wheat bran, rice bran, oat bran and soybean bran, coffee pulp and husks, corn cobs, straws and husks of different origins (Soccol *et al*., 2017). Orange peels constitute 40-45% of the total bulk of fruit mass and form a major part of by-products along with waste pulp from fruit processing industries causing potential environmental problems, particularly water pollution (Farinas, 2015).

Sweet orange (*Citrus sinensis*) is the commonest and the most widely cultivated and consumed fruit in Nigeria with the following states being the major producers; Cross River,

Imo, Anambra, Osun, Ondo, Lagos, Ogun, Oyo, Kwara, Benue, Abia, Plateau, Kogi, Kaduna, Enugu and Bauchi states (Inienger and Udoh, 2020). Orange which belongs to the family Rutaceae appears as a well-known promising source of an efficient, inexpensive, and environment friendly platform for the production of pectinase; these fruit residues which are generally discarded as waste in the environment could serve as low-cost raw materials for enzyme production (Rafiq *et al*., 2016). Among the various substrates that have been reported in literatures, orange peel was found to be primarily suitable containing about 20-30% pectic substances which is used as a good inducer of pectinase (Steudler *et al.*, 2019). Microorganisms that are particularly suitable for solid state fermentation are the filamentous fungi, since the technique simulates their natural habitat, in this condition; they are able to synthesize considerable amounts of enzymes and other metabolites (Farinas, 2015; Soccol *et al*., 2017).

1.2 Statement of Research Problem

Pectinase is in high demand owing to the discovery of its existing and emerging significant biotechnological uses.

Commercial production of the enzyme is very expensive because it is produced from refined substrates and usually patented organisms; another problem facing the commercialization of new sources of enzymes is high cost of production.

Pectinase is not produced in Nigeria; hence, the high cost of importation has led to the high cost of finished industrial products. Nigeria spends millions of dollars on importation and this depletes the nation's foreign reserves. Some other negative effects of importation include dependence on other economies, exposure to unpredictable price changes and low chances of achieving sustainable development.

Enormous amount of waste materials are produced by agricultural and fruit processing industries, the frequent use of fruits such as oranges for production of juices, nectars,

concentrates, jams and jelly powders generate lots of wastes which pose serious waste disposal problems and ultimately leads to pollution.

The consumption of sweet Orange (one of the most common fruits in Nigeria) is accompanied by the production of wastes, these fruit wastes cause waste disposal problems since they are being thrown around indiscriminately and they are in the form of peel, pulp and seeds and citrus peel is rich in pectin.

1.3 Justification for the Study

There has been a great increase in the industrial applications of pectinase, hence, the use of high yielding strains, optimal fermentation conditions and cheap raw materials as carbon and nitrogen sources will reduce the cost of the production and make the enzyme cheaply available for wider applications in industrial processes.

It is imperative that cheaper substrates from local sources be investigated and fungi with good enzyme-producing capacity be isolated locally and harnessed for large scale production to reduce cost of commercialization.

Since the enzyme (pectinase) is not produced in Nigeria, carrying out researches to locally produce this enzyme would mitigate the problems associated with importation, also, local production would help to boost job opportunities and bring about increase in productivity and buoyancy of industrial economy as well as general economic growth. Microbial pectinases are known to be of superior quality as they catalyse reactions in bioprocesses in an economical and environmentally friendly way and most of all the commercial preparations of pectinases are produced from mould sources (Gummadi and Panda, 2003; Babu and Bayer, 2014; Torimiro *et al*., 2018).

The use of waste products (transformation of orange peel wastes to a source of industrial wealth) is economically viable as it provides a safer and cheaper means of waste management which is important for developing Nigerian economy.

1.4 Aim of the Study

The aim of the study was to screen moulds for pectinase production and optimize the fermentation parameters using orange peels as substrate.

1.5 Objectives of the Study

The specific objectives of the study were to:

i. determine the proximate composition of sweet orange peels;
ii. isolate, identify and screen moulds from soil for pectinolytic activity;
iii. produce pectinase by the selected isolate using orange peel-based medium and optimize the solid state fermentation process parameters (temperature, pH and nitrogen sources);
iv. partially purify the pectinase produced.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Enzymes

Enzymes are important biocatalysts used for various industrial and biotechnological purposes; they are produced by microorganisms, animals and plants and can work in many adverse conditions compared to chemical catalysts (Barman *et al*., 2015). Due to increasing demand for enzymes in the global market; the discoveries of microorganisms that have ability to produce enzymes with desirable biochemical and physico-chemical characteristics at low production cost have become a focus of research (Oliyad, 2017). Microorganisms are considered the best source for enzyme production because of their short life cycle, high yielding capacity and cost effectiveness; microorganisms are microscopic creatures that can produce tremendous amounts of various metabolites and enzymes in very short span of time (Bibi *et al*., 2018). Approximately 50% of currently used enzymes are produced from fungi, 35% are produced from bacteria, while the remaining 15% are from plants and animals (Barman *et al*., 2015).

Great biodiversity of pectinolytic microorganisms are found in nature; their common sources are soil, rinds of fruits (peels), rotten vegetables and fruits and also their dump yards (Tariq and Latif, 2012). Enzymes particularly, due to their specific nature of activity, are widely utilized in various processes in different sectors of industrial, environmental and food biotechnology and may be obtained by screening microorganisms sampled from new localities; one such enzyme is pectinase - used in different industries and produced from different microorganisms such as bacteria and fungi (especially moulds) (Javed *et al*., 2018). Pectinase constitute about 10% of overall enzymes used in various industrial processes due to its applicability in various fields of biotechnology hence the demand for commercial pectinase is continually on the increase and though large number of pectinase producing

microorganisms have already been explored and characterized, the ever increasing demand for pectinase has made further exploration of new sources of this enzyme the need of the day (Bibi *et al*., 2018). The compounds which are catalysed by pectinolytic enzymes have a generic name known as pectic substances or pectin. Fruits are the major sources of pectin and the high viscosity of fruit juices is due to mechanical crushing of fruits which are rich in pectin (Tapre and Jain, 2014).

2.2 Pectin

Pectin was discovered by Vauquelin in 1790 and was later crudely characterized by Braconnot in 1825 (Caffall and Mohnen, 2009).According to Oliyad (2017), Pectic substances (Pectin) are polysaccharides of high molecular weight, with a negative charge, appearing mostly in the middle lamella and the primary cell wall of higher plants; they are formed by a central chain containing a variable amount although in high proportion of galacturonic acid residues linked through α-(1-4) glycosidic bonds partially esterified with methyl groups. Namasivayam *et al*., (2011) reported that about 30% pectic substances are found in peels of oranges and various fruits. The plant cell wall is a complex macromolecular structure that envelops and protects the cell; it is a distinguishing characteristic of plants and is essential to their survival. The structure formed by the polysaccharides, proteins, aromatic, and aliphatic compounds of the cell wall enables plants to flourish in diverse environmental niches. The plant cell develops the middle lamella and the primary wall during initial growth and expansion of the cell. The primary wall is thought to contribute to wall structural integrity, cell adhesion, and signal transduction (Oliyad and Abate, 2017).

2.2.1 Classification of pectic substances

Based on the type of modifications of the backbone chain, the American Chemical Society classified pectic substances into four main types as protopectin, pectic acid, pectinic acid and pectin (Abalos *et al*., 2002).

2.2.1.1 Protopectin

This is a parent pectic substance and upon restricted hydrolysis yields pectin or pectinic acid. Protopectin is occasionally a term used to describe the water-insoluble pectic substances found in plant tissues and from which soluble pectic substances are produced.

2.2.1.2 Pectic Acids

These are the galacturonans that contain negligible amounts of methoxyl groups (they are often known as demethylated pectin or polygalacturonic acid. Normal or acid salts of pectic acid are called pectates.

2.2.1.3 Pectinic Acids

These are the galacturonans with various amounts of methoxyl groups. Pectinic acid alone has the unique property of forming a gel with sugar. Normal or acid salts of pectinic acids are either normal or acid pectinates respectively. Under suitable conditions, pectinic acids are capable of forming gels with sugars and acids.

2.2.1.4 Pectin (polymethylgalacturonate)

It is a polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall. Pectins are the soluble polymeric materials that contain pectinic acids as the major component.They can form insoluble protopectins with other structural polysaccharides and proteins located in the cell wall. Demethylated pectin is known as pectic acid or polygalacturonic acid.

2.2.2 Structure of pectic substances

Pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1→4) linkage, the side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose. The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by Sodium,

Potassium or Ammonium ions (Kuhad *et al.,* 2004). Pectin is one of the most complex bio-macromolecules in nature and it can be composed of as many as seventeen (17) different monosaccharides with at least seven (7) different polysaccharides. The structural classes of the pectic polysaccharides include homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II), and rhamnogalacturonan I (RG -I) (Jayani, *et al*., 2005).

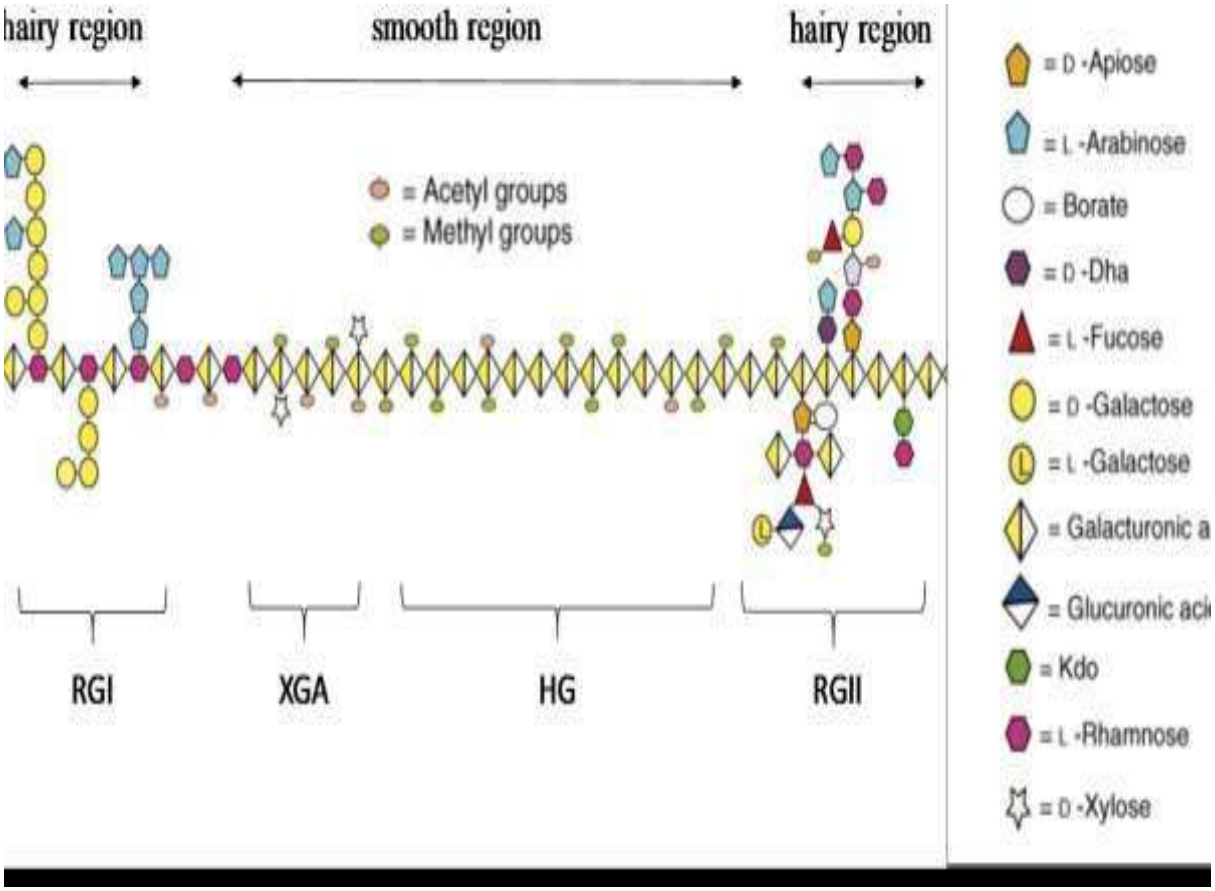


Figure 2. 1: Schematic Pectin Structure Adapted from Mellinas et al., (2020)

2.3 Pectinase

Pectinase is a collective name for a heterogeneous group of enzymes that catalyse the breakdown of pectin, it constitutes approximately 10% of the total enzyme production in the world market and 25% of global sale in the food industry; According to the Allied Market Research repports, the global enzyme market was valued at $7,082 million in 2017 and is

projected to reach up to $ 10,519 million by 2024, at a Compound Annual Growth Rate (CAGR) of 5.7% from 2018 to 2024 (El Enshasy *et al*., 2018). Pectinase is widely used in the fruit juice and textile industry, the wine production industry, vegetable waste treatment, pulp and papermaking processes, in the degumming of plant fibres, the oil extraction processes, and for the chocolate, tea, and coffee fermentation processes (Oliyad and Dawit, 2017). The crucial roles of pectinases in food industries especially and the biotechnological sector at large cannot be overemphasized; in the fruit juice industry, they are used for production and clarification of vegetable nectars and fruit juices by reduction in viscosity, increasing of juice yield by enzymatic liquefaction and maceration of pulps, enhancing pigmentation by extracting more anthocyanin and for haze removal from wines (Samreen *et al*., 2019). Pectinase splits polygalacturonic acid into monoglacturonic acid by opening the glycosidic linkages and breaking ester bonds between carboxyl and methyl groups (El Enshasy *et al*., 2018).

The enzymes of the pectinase complex include: polygalacturonase and Polymethyl galacturonases (EC 3.2.1.15) which catalyse the hydrolytic cleavage of α-1,4-glycosidic linkage in pectic acid and pectin respectively; pectin lyases (Polygalacturonate Lyase and Polymethyl galacturonate Lyase) (E.C. 4. 2. 2. 10) which catalyse the cleavage of α-1,4glycosidic linkage in pectic acid and pectin respectively by trans-elimination reaction forming unsaturated galacturonates and methyl galacturonates respectively and lastly pectin esterase (EC. 3.1.1.11) which catalyses the de-esterification of the methoxy or methylated carboxylic ester groups present in pectin into pectic acid and methanol; these enzymes are inducible, produced only when needed and they contribute to the natural carbon cycle (Hoondal *et al*., 2002). In plants, pectic enzymes are very important since they play a major role in elongation and cellular growth as well as in fruit ripening, also, the pectolytic activity of microorganisms which in turn brings about these enzymes plays many significant roles,

especially in the pathogenesis of plants since these enzymes are the first to attack the tissue (Jansirani *et al*., 2014).

2.3.1 Classification of pectinases

According to the cleavage site, pectinases are divided into three groups: (1) hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); (2) lyases/transeliminases comprising pectin lyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); (3) esterases comprising pectin esterase, PE (EC 3.1.1.11) (Bijay *et al.*, 2016).

2.3.1.1 Polygalacturonase (EC 3.2.1.15)

Polygalacturonases (PGases) are the pectinolytic enzymes that catalyse the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge (Kashyap *et al*., 2001).

2.3.1.2 Pectin lyase (EC 4.2.2.10)

Pectin lyase catalyzes the random cleavage of pectin, preferentially high esterified pectin, producing unsaturatedmethyloligogalacturonates through transelimination of glycosidic linkages. Pectin lyases do not have an absolute requirement of Ca2+ but they are stimulated by this and other cations (Jayani *et al*., 2005).

2.3.1.3 Pectate lyase (EC 4.2.2.2)

Pectatelyase (PGL) cleaves glycosidic linkages preferentially on polygalacturonic acid forming unsaturated product through transelimination reaction. PGL has an absolute requirement of Ca2+ ions. Hence it is strongly inhibited by chelating agents such as EDTA (Jayani *et al*., 2005).

2.3.1.4 Pectinesterase (PE)

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 (Kashyap *et al.,* 2001).

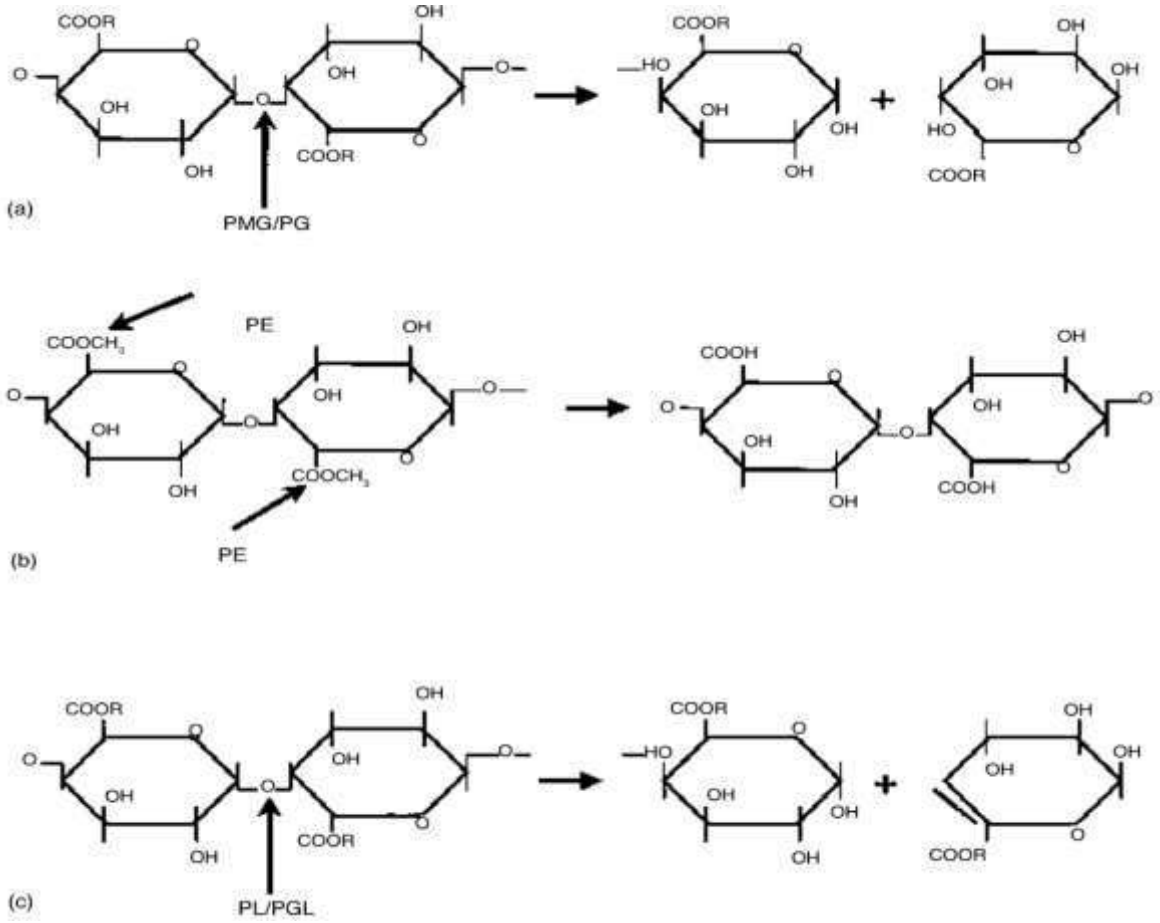


Figure 2. 2: Mode of action of pectinases: (a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH3 for PL. the arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases; PE, pectinesterase; PL, pectin lyase (Jayani et al., 2005).

2.4 Production of Microbial Pectinase

Microbial pectinases are important in the phytopathologic process, in plant-microbe symbiosis and in the decomposition of dead plant material, contributing to the natural carbon cycle. (Pedrolli *et al.,* 2009). The microbial world has shown to be very heterogeneous in its ability to synthesize different types of pectin degrading enzymes with different mechanisms of action and biochemical properties; they are currently the primary source of industrial enzymes with 50% originating from fungi and yeast; 35% from bacteria and the remaining 15% from plant

origin. It has been reported that microbial pectinases account for 25% of the global food enzymes sales (Jayani *et al*., 2005). There are many advantages in the use of microorganisms for the production of pectinase including the application of selection methods, increase of biosynthesis via the conditions of cultivation, an in-depth interaction on various substrates, a wide spectrum of enzyme complex and the possibility of genetic modification of the organisms being used (Torimiro *et al.,* 2018). The most important factor to be considered during the microbial production of pectinase is the choice of microorganisms; microorganisms that are particularly suitable for pectinase production through fermentation are the filamentous fungi since the technique simulates their natural habitat and they are able to synthesize considerable amounts of enzymes and other metabolites (Samreen *et al.,* 2019).

2.4.1 Biotechnological applications of microbial pectinases

Application of enzymes in biotechnological processes has expanded considerably in recent years, for example in food and related industries, major importance is being attached to the use of enzymes in upgrading quality of products, increasing yields of extractive processes, stabilizing products, improving flavours of foods and food products and in the profitable utilization of by-products. On the bases of their applications, pectinases are mainly of two types: acidic pectinases and alkaline pectinases (Murad and Azzaz, 2011).

2.4.1.1 Acidic Pectinases

Acidic pectolytic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger*(Kashyap *et al*., 2001). Potential applications of acidic pectinases are briefly described below:

2.4.1.2 Fruit juice extraction and clarification

To extract juice by mechanical method is difficult; hence pectinase along with some other enzymes replaces the mechanical extraction process and clarify the juice obtained from fruits, the crushing of pectin-rich fruits results in high viscosity juice which stays linked to the fruit

pulp in a gelatinous structure, hindering the juice extraction process by pressing (Javed *et al*., 2018). Pre-treatment of juices with pectinases or the addition of it in the extraction process improves the fruit juice yield through an easier process by lowering the amount of pectin present and decreasing the juice viscosity and degrading the gel structure, thus improving the juice concentration capacity which in turn accelerates the subsequent filtration process and alsohelps to increase the clarity of the juice. In the case of fruit juice, extraction by enzymatic maceration can increase yields by more than 90% compared to conventional mechanical juicing, besides improving the organoleptic (colour, flavour) and nutritional (vitamins) properties and technological efficiency (Pedrolli *et al.*,2009).

2.4.1.3 Wine processing

The wine processing industry also recognizes the importance of acidic pectinases. The addition of pectinases during crushing of the fruits increases the juice yield and also accelerates the release of anthocyanins into the juice. Pectinase treatment at the prefermentation or fermentation stage, settles out suspended particles while after fermentation the enzyme is added to the wine to increase its clarity and filtration rate (Kashyap *et al*., 2001).

2.4.2. Alkaline pectinases

Alkaline pectinases are mainly used in the degumming and retting of fibre crops and pretreatment of pectic waste water from fruit juice industries (Rathinam and Sani, 2017). In the industrial sector, alkaline pectinases are applied for the following purposes.

2.4.2.1 Paper and pulp industry

During papermaking, pectinase can depolymerise pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Reid and Ricard, 2000).

2.4.2.2 Retting and degumming of plant bast fibres

Bast fibres are the soft fibres formed in groups outside the xylem, phloem or pericycle,. The fibres contain gum, which must be removed before its use for textile making. The chemical degumming treatment is polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanase presents an eco-friendly and economic alternative to the above (Sani and Krishnaraj, 2017). Pectinases have been used in retting of flax to separate the fibres and eliminate pectin (Hoondal *et al*., 2002).

2.4.2.3 Textile processing and bio-scouring of cotton fibres

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and eco-friendly manner, replacing toxic caustic soda earlier used for the purpose. Bio-scouring is a process for removal of noncellulosic impurities from the fibre with specific enzymes and pectinases have been used for this purpose without any negative side effect on cellulose (Hoondal *et al*., 2002).

2.4.2.4 Pectic waste water treatment

The waste water from various processing industries contains pectinaceous materials that are barely decomposed by microbes during the activated-sludge treatment, pre-treatment of these waste waters with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Sani and Krishnaraj, 2017).

2.4.2.5 Animal feed

Pectinases are part of the enzyme cocktail used for the production of animal feeds. This cocktail reduces the feed viscosity which increases absorption of nutrients; this is achieved either by hydrolysing non-biodegradable fibres or by liberating nutrients blocked by these fibres (Jayani *et al.*, 2005).

2.4.2.6 Oil extraction

Vegetable oils, citrus, sunflower seed, olives and kernel oils which are traditionally produced by extraction with organic solvents, such as the potentially carcinogenic hexane can be extracted with pectinases. By degrading cell wall components like pectin pectinase promotes the oil liberation, the enzyme destroys the emulsifying properties of pectin, which interferes with the collection of oils (Pedrolli *et al.*, 2009).

2.4.2.7 Purification of plant viruses

Pectinases have also been reported to work on purification of viruses. But they are yet to be commercialized. When virus particle is restricted to phloem, to release the virus from the tissues, alkaline pectinases and cellulases are used. This gives very pure preparations of the virus (Reid and Ricard, 2000).

2.4.2.8 Coffee and tea fermentation

Fermentation of coffee using pectinolytic microorganisms is done to remove the mucilage coat from the coffee beans and to enhance the tea fermentation and foam forming property of tea. Pectinases are also used in the manufacture of tea. Enzyme treatment accelerates tea fermentation, although the enzyme dose must be adjusted carefully to avoid damage to the tea leaf (Pandey *et al*., 2000b).

2.5 Microorganisms Commonly Used for Pectinase Production

Microbial production of pectinases using various genera of microorganisms and the potential applications of the produced enzymes has been studied during recent years (Moubasher *et al*., 2016). Pectinase production has been reported from bacteria including actinomycetes (Beg *et al*., 2000), yeast (Reid and Ricard 2000) and fungi (Murad and Azzaz, 2011). Moulds especially *Aspergillus niger* are most widely used in submerged and solid-state fermentation for industrial production of pectinase, this is largely as a result of the ability of such microbes to colonize the substrate by apical growth and penetration which gives them a considerable

ecological advantage over non-motile bacteria and yeasts which are less able to multiply and colonize on low moisture substrate (Gummadi and Panda, 2003).

2.5.1 Moulds

For thousands of years, humanity has practiced rudimental forms of biotechnology: by fermenting starch and sugars present in grains and fruits ancient civilizations were able to produce bread, beer, wine, and other alcoholic beverages. Prior to the late nineteenth and early twentieth century, these processes were carried out without knowledge of the underlying biological events. Now, brewery and wine making is a well-understood, controlled industrial process. Similarly, in just a century, industrial biotechnology has changed remarkably and flourished, from initial proof-of-principle experimentation in Erlenmeyer flasks, to a multibillion dollar industry producing megatons of useful molecules. Fungal biotechnology is undoubtedly a major contributor and driver of this success, as an example, the estimated market volume for plant-degrading enzymes from filamentous fungi in 2016 was €4.7 billion, which was expected to reach up to €10 billion within the next decade (Cairns *et al.,* 2018). Pectic enzymes are widely distributed in nature and are produced by bacteria, yeast, fungi and plants, fungi produce pectinase and cellulose enzymes that break down cellulose and pectin into simpler forms (Babu and Bayer, 2014).

The ability of filamentous fungi to secrete pectinase and other hydrolyzing enzymes into their culture media has led to the harvesting of these useful enzymes (Adeleke *et al*., 2012). Moulds are widely distributed in nature having the capability to exploit pectin as their sole source of carbon and energy (Pedrolli *et al.*, 2009). The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. The success of this particular group is explained by the facts that; they are not very selective with respect to their abiotic growth conditions, they can degrade a wide variety of organic molecules and that they produce high numbers of asexual and sexual spores that are dispersed over short and long

distances, hence, fungi generally and the *Aspergillus* genus in particular has drawn a great deal of attention from various researchers worldwide (Priya and Sashi, 2014a).

2.5.1.1 Aspergillus niger

Aspergillus is an anamorph genus, which comprises between 260 (Samson & Varga, 2009) and 837 species (Hawksworth, 2011). These species are classified into about ten (10) different teleomorph genera, for example, *A. nidulans* belongs to the teleomorph genus *Emericella*, while *A.fumigatus* and *A. flavus* belong to the genera *Neosartorya* and *Petromyces*, respectively, and this indicates that *Aspergillus* is a diverse group of fungi (Geiser, 2009). *Aspergillus* species are among the most abundant fungi worldwide and they are not very selective with respect to abiotic growth conditions for instance, they can grow at relatively low humidity, furthermore; *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue but, they are predominantly found on complex plant polymer (Krijgsheld *et al*., 2013). The success of *Aspergillus* is also explained by their effective dispersal; spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances (Bennett, 2010). *Aspergillus* speciessecrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For example, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material (Meyer *et al.,* 2011).

In nature, species of *Aspergillus* grow within and on solid substrates. A colony can result from a single sexual or asexual spore but it may also arise after conidia and/or germlings that are in close vicinity to each other fuse together (Sethi *et al*., 2015). In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, Aspergilli form radial symmetrical macro-colonies. The mycelium of *A. niger* extend their diameter with

approximately 0.25 mm per h in excess of nutrients and at a temperature of 30 °C (Krijgsheld *et al*., 2013).

About one-third of the described species of *Aspergillus* have a known sexual stage (Geiser, 2009). However, analysis of genome sequences suggests that most, if not all, *Aspergillus* species should be able to reproduce sexually (Dyer & Paoletti, 2005). *Aspergillus* species with a sexual state can be either homothallic or heterothallic. Homothallic species undergo sexual reproduction without the need to cross with a compatible partner while the opposite is the case for heterothallic species. The genomic sequences of *A. niger* predicts that the fungus is either heterothallic or truly asexual (Pál *et al.*, 2008).

Three stages can be distinguished during germination of conidia. In the first phase of germination, dormancy is broken by environmental signals such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars (Osherov & May, 2001). Spores grow isotropically in the second phase of germination. In the third phase of germination, a germ tube is formed by polarised growth, to this end; the morphogenetic machinery is redirected to the site of polarisation (Harris, 2006).

*Aspergillus niger*is a member of a group of species named *Aspergillus* section Nigri, formerly known as *A. niger* group. The productive metabolism of *A. niger* acquired a great economic importance when James Currie in 1917 published a study describing the ability of the fungus to biosynthesize high amounts of citric acid by culturing it in sugar solutions at low pH. This remarkable discovery showed the direct influence of the ambient and nutritional factors in the yield of the citric acid production and was the basis for the birth of the biotechnology industry in 1919 by Pfizer (Max *et al*., 2010). Species from *Aspergillus* genus section Nigri present a thin stalk with a round black conidial head made up of spores of a characteristic shape, which bud from the organism's body as part of asexual reproduction. Its name is derived from this appearance since it resembles the holy water sprinkler called aspergillum, used by

priestsduring the Asperges ceremony.*A. niger* is considered a cosmopolitan asexual saprophyte, occurring in almost all aerobic environments. The optimal pH for this fungus growing is 6, although it tolerates wide pH range (from 1.5 to 9.8). In addition, the most favourable water activity and relative humidity to observe the growth of this species is 0.97 and 96-98%, respectively. The black spores of *A. niger* apparently provide protection from sunlight and UV irradiation, leading to a competitive advantage over other microorganisms in their habitats. These abilities besides the profuse production of conidiospores spread through air ensure its more frequent occurrence in warm and humid habitats. Endophyte strains of *A. niger* were the most predominant sources used in chemical studies, which was closely followed by strains associated to marine habitats and others. This latter comprises those strains coming from either fungi collections or without information of their source habitat. Additionally, it was possible to note a significant contribution of chemical investigations of strains derived from genetic mutation. It is important to mention that some compounds were produced by strains from different sources/habitats, suggesting that environmental conditions had no influence on this fungus metabolism (Lima *et al*., 2019). A large number of unique proteins involved in certain mechanisms do not occur in other filamentous fungi, proving that this species is quite versatile at the level of cellular production. These discoveries significantly contributed to the fundamental understanding of enzyme function and to the production of numerous extracellular enzymes, such as pectinase, amylase, cellulase, among others. Studies have shown the expression of twenty-six (26) pectinolytic genes from *Aspergillus niger*under sixteen (16) different growth conditions to obtain an expression profile for each gene. The results of these findings provide strong indication for a general activating system for pectinolytic gene expression in *A. niger* responding to the presence of D-galacturonic acid or a metabolite derived from it. This system regulates both genes encoding enzymes acting on the main chain of pectin as well as genes encoding accessory enzymes. Regulation of the

pectinolytic genes appears to be complex; individual genes are expressed at different times and in response to different D-galacturonic acid containing carbon sources (Pedrolli *et al*., 2008).

2.5.1.2 Sclerotium rolfsii

Almost all fungi produce some type of durable, quiescent microscopic structure such as a spore that is important for dispersal and/or survival under adverse conditions; however, some fungi also produce dense aggregations of fungal tissue called sclerotia. These persistent structures help fungi to survive challenging conditions such as freezing temperatures, desiccation, microbial attack, or the long-term absence of a host (Parvin*et al.*,2016).

Sclerotium rolfsii is a common soil-borne fungal plant pathogen of tropical, sub-tropical and warm temperate regions, *S. rolfsii* typically forms abundant white mycelium and small, brown, round sclerotia on the mycelium. *Sclerotium rolfsii* is a soil borne plant pathogen causing diseases on a wide range of agricultural and horticultural crops (Songvillay *et al*., 2012). It has wide geographic diversity and commonly found in the tropics, subtropics and other warm temperate regions especially in the Southern United States, Central and South America, Southern European countries bordering the Mediterranean, Africa (Smith *et al*., 2014).*Sclerotium rolfsii* represents both unity and diversty in fungi. It may be a plant pathogen, a parasite, a symmbiont, or a saprophyte. It occurs throughout the world and can damage any part or all of a plant. *Sclerotium rolfsii* produces pectinases and cellulases which function in degradation and penetration (Ordoñez *et al.*,2014).

2.6 Fermentation

For enzyme production, both submerged fermentation (SMF) and solid-state fermentation (SSF) are usually used, however, the production of enzyme is found better in SSF (Priya and Sashi, 2014b).

2.6.1 Solid state fermentation (SSF)

The term Solid State Fermentation (SSF) has been defined in many ways. Many researchers in the field have come up with diverse ways to define the technique. For example, in 1997, Viniegra-Gonzàlez defined SSF as a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients. Subsequently, in the years 2000 and 2006 respectively; Pandey *et al* defined SSF as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can also be used as carbon and energy source while Rahardjo *et al* in their review article, came up with a definition that SSF is the growth of microorganisms on moistened solid substrate, in which enough moisture is present to maintain microbial growth and metabolism, but where there is no free-moving water and air is the continuous phase. Manan and Webb, (2017) defined solid state fermentation as a system in which the growth of selected microorganism(s) occurs on solid materials with low moisture contents. Whatever the definition, we can understand that SSF is referring to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted.

The technique incorporates microbial growth and product formation on or within particles of a solid substrate under aerobic conditions (Ghosh, 2016). Solid state fermentation has been identified as a potentially important methodology and technique in biotechnology, today, it is considered one of the most economically viable and practically acceptable technologies for large-scale bioconversion and biodegradation processes. Development of sustainable SSF and bioprocess technology is an emerging, multidisciplinary field with possible application to the production of enzymes, chemicals, biofuel and pharmaceuticals. Solid state fermentation offers many advantages over conventional submerged fermentation (SMF) such as; simple technique, simple and inexpensive substrates, elimination of the need for rigorous control of

many parameters during fermentation, lower capital investment, lower levels of catabolite repression and end product inhibition, higher product yields, lower energy requirements, lower waste water formation, no foam generation and relatively easy and better recovery of end products. SSF provides flexibility in terms of the raw materials to be used and their capability to produce various value-added products (Lemo *et al*., 2019).

SSF has emerged as a viable technology for bioremediation and the biodegradation of hazardous compounds as well as the recycling of food and agro-industry wastes and biomass conservation; it is a promising technology and is reliable for the development of various biotechnological products with various advantages that have been identified through literature (Manan and Webb, 2017). They include;

2.6.1.1 Biological advantages

- Products produced in high volume
- Higher productivity level of the products
- Higher stability of products
- Absence of catabolic repression
- Tolerance to high substrate concentration
- Absence of rigorous control of fermentation process
- Easier aeration
- Low water demand helps to minimize contamination
- Absence of foam production

2.6.1.2Processing advantages

- Bioreactors are usually in small volumes and are compact
- The growth substrates are from natural sources
- Pre-treatment and treatment of the natural resources can be very simple
- The process does not involve anti-foam chemicals

2.6.1.3Environmental advantages

- Produce less liquid waste
- Solving waste problem as biological detoxification
- Substrates are usually raw materials – natural carbon sources which are extremely cheap variable and abundant agro-industrial and food wastes.

2.6.1.4 Economic advantages

Substrates are usually natural materials- natural carbon sources which are extremely cheap, variable and abundant agro-industry and food wastes.Bioreactors used are simple, cheap and user friendly Low recovery cost in downstream processing.

2.6.2 Factors that influence Solid State Fermentation

The advantage of SSF comes from its simplicity and its closeness to the natural habitat of many microorganisms. Through modern biotechnology, there are new initiatives to improve and enhance the productivity of SSF. Each microorganisms, solid substrate, and bioreactor system plays a major role in the success of SSF. The performance of the SSF process can be influenced by various factors. Broadly, the factors that influence the performance of SSF can be divided into three major categories, namely; i. Biological factors ii. Physico-chemical factors and iii. Mechanical factors;

2.6.2.1Biological factors

Type of microorganism: The most important criterion in SSF is the selection of a suitable microorganism, which has the ability to degrade the solid substrate. SSF processes are due mainly to the fermentation activity of fungi alone, bacteria alone, a mixture of fungi and yeasts or fungi followed by a mixture of bacteria and yeast. The selection of microorganism is usually dependent on the type of solid substrate, growth requirements and targeted final product; these general criteria will affect the fermentation design and downstream processing (Krishna, 2005). Filamentous fungi continue to dominate as an important microorganism in

SSF due to their mycelia mode of growth as well as their neutral physiological capabilities (Mitchell *et al*., 2011). The use of a single microorganism, especially in industrial SSF processes, has the advantage of improved rate of substrate utilization and controlled product formation (Nigam and Pandey, 2009).

Inoculum: Inoculum can be described as a preparation containing high numbers of viable microorganisms added to a substrate to bring about desirable changes in the solid substrate (Visintin *et al*., 2017). The age of the inoculum, the medium used for its cultivation, and therefore its physiological state are of the utmost importance in many fermentation processes (Asmahan and Muna, 2009).

Substrates: Carbon sources supplied in the medium are of great importance to fungi since they provide the carbon source needed for the biosynthesis of cellular constituents; this includes carbohydrates, proteins, lipids, nucleic acids, and their oxidation provides energy for the cell (Zepf and Jin, 2013). The solid substrate is a major element in SSF; in addition to providing nutrients such as carbon and nitrogen, the solid substrate also performs the role of the physical structure that supports the growth of microorganisms (Rodríguez Couto, 2008). Another important factor in the selection of substrate is the water holding capacity that maintains moisture content of the fermented substrate (Nigam and Pandey, 2009). Generally, most of the solid substrates used in SSF are based on food and agro-industry crops and residues (Rodríguez Couto and Ma Ángeles, 2006).

2.6.2.2Physico-chemical factors

pH: In solid state fermentation, pH is quite difficult to measure and control because fo the nature of solid substrate, low water content (lack of free water) and heterogeneity in the conditions of the systems (Behera and Ray, 2016). Individual groups of microorganisms react in different ways to the pH value of the fermentation environment, fungi generally prefer slightly acidic pH values (Durand, 2003).

Temperature: In solid state fermentation, the problem regarding temperature arises as a result of the heat generated from microbial activity and accumulated in the system (Nignam and Pandey, 2009). The heat needs to be removed from the system to avoid overheating and thereby disturbing the growth of microorganisms and the formation of products this is the reason why in SSF, most studies on solid state bioreactor designs are focused on maximising heat removal (Finkler *et al*., 2017). The problem becomes crucial in large-scale systems where heat evolution leads to huge moisture loss which in turn disturbs fungal growth (Khanahmadi *et al*., 2004). Another problem is that heat creates condensation such that a large amount of water is returned back to the fermented solid. This will create heterogeneity in the solid substrate. Because of this, it is difficult to maintain the temperature at an ideal range. To overcome this, air is usually blown into the system to force out the heat generated via a gas outlet however, the flow rate of the air blown into the system needs to be monitored to avoid the loss of moisture content from the fermented substrate to overcome this problem, a cooling system can also be installed into the system (Castro *et al*., 2015).

Gaseous environment: The gases of interest are oxygen and carbon dioxide; adequate supply of oxygen (must diffuse from the inter-particle space to the biomass) is required to maintain aerobic conditions while carbon dioxide on the other hand must diffuse from the biomass to the inter-particle space to be removed from the system, this can be achieved by aeration or mixing of the fermenting solids (Nava *et al*., 2011). Oxygen limitation may occur at deep

areas of the substrate; this situation can be overcome by turning the fermenting substrate through mixing processes (Nava *et al*., 2011).

Aeration: Microorganisms normally vary in their oxygen requirements; aeration plays two important roles in SSF (Manan and Webb, 2017):

i. Meeting the oxygen demand in aerobic fermentation and

ii. Heat and mass transport in a heterogeneous system

Aeration provides and maintains high oxygen levels and low carbon dioxide levels in the inter-particle solid substrates. The points to consider as regards aeration are the flow rate and air quality. Dry air at high flow rate will have an effect on the moisture of fermented substrate even though it has an advantage in terms of heat removal. Aeration rate was shown to have a positive effect on microbial growth and product formation (Assamoi *et al*., 2008). Alternatively, using saturated air is a common strategy to avoid substrate drying by maintaining moisture levels. In addition, the rate of aeration by saturated air controls the temperature and the moisture gradients of the solid medium (Umsza-Guez *et al*., 2011).

Particle size: The particle size properties of solid substrates will determine the shape, accessible area, surface area and porosity of the solid substrates (Richard *et al.*, 2004). Processes like chopping, grinding and cutting create a condition for microorganisms to be active at the initial stages of growth and increase the degradation and hydrolysis rate since the solid substrate is insoluble (Ramana *et al*., 1993). The most important physical factor is the particle size that affects the surface area to volume ratio of the solid substrate (Krishna, 2005). Smaller particle size would provide a larger surface area per volume and allow full contact of microorganisms with the nutrients but the diffusion of oxygen would be affected (Nignam and Pandey, 2009) Larger particle size provides small area per volume ratio and gives excellent diffusion of oxygen but contact with nutrients is affected (Karimi *et al*., 2014). A suitable

particle size should satisfy both mycelial growth and the demand for oxygen and nutrients (Schmidt and Furlong, 2012).

2.6.2.3Mechanical factors

Agitation/Mixing: Agitation or mixing plays the same role as aeration. In addition, agitation is a possible alternative to solve heterogeneity problems in SSF and might improve homogeneity and disrupt gradients (Assamoi *et al*., 2008). Another benefit of agitation is that airflow is more evenly distributed which improves the conditions for microbial growth within the entire fermented bed (Mitchell *et al*, 2011). However, agitation affects mycelium formation as shear forces due to agitation can destroy the mycelium, it may also create problems related to cell damage especially when filamentous fungi are used a slower agitation speed might be necessary and some bioreactors perform this by using intermittent agitation to avoid serious damage to the mycelium (Mitchell *et al*, 2011)..

2.6.3Bioreactors forsolid state fermentation

Solid State Fermentation (SSF) bioreactor design greatly depends on the solid substrate. The bioreactor is the core of biological process. There are four major roles of the bioreactor they are: (Bastos *et al*., 2016)

i. To contain the substrate
ii. To contain the process microorganism
iii. To protect the process microorganism against contamination and
iv. To control environmental conditions to optimize growth and product formation

2.6.4 Submerged fermentation (SmF)

The process which uses broth media to culture organisms is called submerged fermentation; it is the cultivation of microorganisms in broth where the nutrients and microorganisms are both submerged in water with continuous agitation (Thangaratham and Manimegalai, 2014). A colossal amount of effluents is released in this process.Submerged fermentation techniques for

enzyme production on an industrial scale are generally conducted in stirred tank reactors under aerobic conditions using batch or fed batch systems, high capital investment, energy costs and infrastructural requirements for large-scale production make the application of submerged fermentation techniques in enzyme production not practical in a majority of developing countries (Javed *et al*., 2018)**.**

2.6.5 Substrate for fermentation

The most promising residues for pectinase production include agricultural residues and fruit peels: usually, these agro residues are not only a solid support for nutrients absorption and biomass growth, they are also a source of carbon and nutrients (Samreen *et al*., 2019).

2.6.5.1 Orange Peels: Citrus is one of the most important fruit crops known to humans since antiquity and is a good source of vitamin "C" with high antioxidant potential (Gorinstein *etal*., 2001). Citrus originated from south-eastern Asia, China and the east of Indian Archipelago from at least 2000 BC (Gmitter and Hu, 1990). The fruit is widely grown in Nigeria and many other tropical and subtropical regions.Sweet orange (*Citrus sinensis* L. Osbeck) commonly called orange is a member of this Citrus family, it is one of the most popular fruit crops in the world and it contains active phytochemicals that can protect health; in addition to this, it provides an abundant supply of vitamin C, folic acid, calcium, potassium, thiamine, niacin and magnesium potassium and pectin, furthermore, the contribution of citrus species in deterrence of life threatening diseases have been studied and it has been reported that citrus fruits, citrus fruit extracts and citrus flavonoids exhibit a wide range of promising biological properties due to their phenolic profile and antioxidant properties (Rafiq *et al*., 2016). Citrus fruits are highly consumed worldwide as fresh produce, juice and most often the peel is discarded as waste which contains a wide variety of secondary components with substantial antioxidant activity in comparison with other parts of the fruit. Global production of citrus fruit has significantly increased during the past few years and has reached 82 million tons in

the years 2009–2010, of which oranges – commercially the most important citrus fruit accounts for 80% of the world's citrus fruit productionwith the peels constituting 40-45% of the total fruit mass (Sidana *et al*., 2013; Farinas2015).

Therefore, a large amount of peel is produced every year. Citrus peel, the primary waste, is a good source of pectin. A number of studies have recognized the presence of polyphenols, vitamins, minerals, dietary fibres, essential oils and carotenoids content which makes citrus a health-benefit promoting fruit. To this regard several examples about the use of citrus fruits as therapeutic remedies can be cited: oranges to cure scurvy (Magiorkinis *et al*., 2011) orange, lime, and lemon juices as remedies for the prevention of kidney stones formation, grapefruits as agents able to lower blood pressure and to interfere with calcium channel blockers (Sica, 2006), citrus flavonoids as effective in vivo agents able to modulate hepatic lipid metabolism, orange juice to prevent and modulate inflammatory processes (Assis *et al*., 2013). Rarely occurring valuable biologically-active components such as oxyprenylated natural products for example, 3,3-dimethylallyloxy-(C5), geranyloxy- (C10), and the farnesyloxy-(C15) related compounds have been recognized in the last 12 years in citrus varieties (Munakata *et al*., 2012). There is an increasing acceptance that phenols, amino acids, essential oils, pectin, carotenoids, flavonoids, and vitamin C present in citrus fruits exert beneficial effects in the prevention of degenerative diseases (Wang *et al*., 2014). Antioxidants are currently employed to inhibit the formation of compounds that result in decrease in sensory and nutritional quality such as butylatedhydroxyanisole, butylated hydroxytoluene (BHA, BHT). Citrus species of various origins have been evaluated for their phytochemical composition and its contribution in health promotion and it has been recognized that citrus species exhibit promising biological properties including antiatherogenic, antiinflammatory, antitumor activity, anticlotting and strong antioxidant activity (Guimara˜es *et al*., 2009).

Botany: Sweet orange (*Citrus sinensis* L. Osbeck) (to distinguish it from closely related species like sour orange, *C. aurantium C. reticulata* and mandarin orange), is a small evergreen tree 7.5 m high and in some cases up to 15 m. It originated from southern China where it has been cultivated for many years, but is today grown commercially worldwide in tropical, semitropical and some warm temperate regions to become the most widely planted fruit tree in the world (Ehler, 2011). Orange produces leathery and evergreen leaves of different shapes,. It bears fragrant white flowers either singly or in whorls of 6, about 5 cm wide, with 5 petals and 20-25 yellow stamens. The small, white or purple scented hermaphroditic flowers produce nectar for pollination by insects. The fruit, which may be globose to oval is 6.5 to 9.5 cm wide, and ripens to orange, yellow or even green colours. Anatomically, the fruit consists of two distinct regions: the pericarp also called the peel, skin or rind, and the endocarp, or pulp and juice sacs. The skin consists of an epidermis of epi-cuticular wax with numerous small aromatic oil glands that gives it its particular smell. The quantity of wax is dependent on the variety, climatic conditions and growth rate. An abundance of microflora consisting mainly of fungus and bacteria are present on the skin and these can be more in damp climates; this justifies the need for appropriate washing of the fruit before eating or proceeding to extract juice and essential oils. The pericarp consists of the outer flavedo, or epicarp largely made of parenchymatous cells and cuticle. Beneath the epidermis is the flavedo, with its characteristic yellow, green or orange colour as indicated in figure 2.3. The flavedo is very fine and fragile containing oliferous vesicles on the inside which can be collected by scraping on the flavedo layer. The flavedo is a generally colorless, spongy inner layer of mesophyll that changes character and thickness throughout fruit development, properties that determine ease of peeling. The albedo, or mesocarp lying beneath the flavedo consists of tubular-like cells joined together to constitute the tissue mass

compressed into the intercellular area. The albedo is rich in flavonoids, which if transferred to the juice imparts a bitter taste (Etebu and Nwauzoma, 2014).

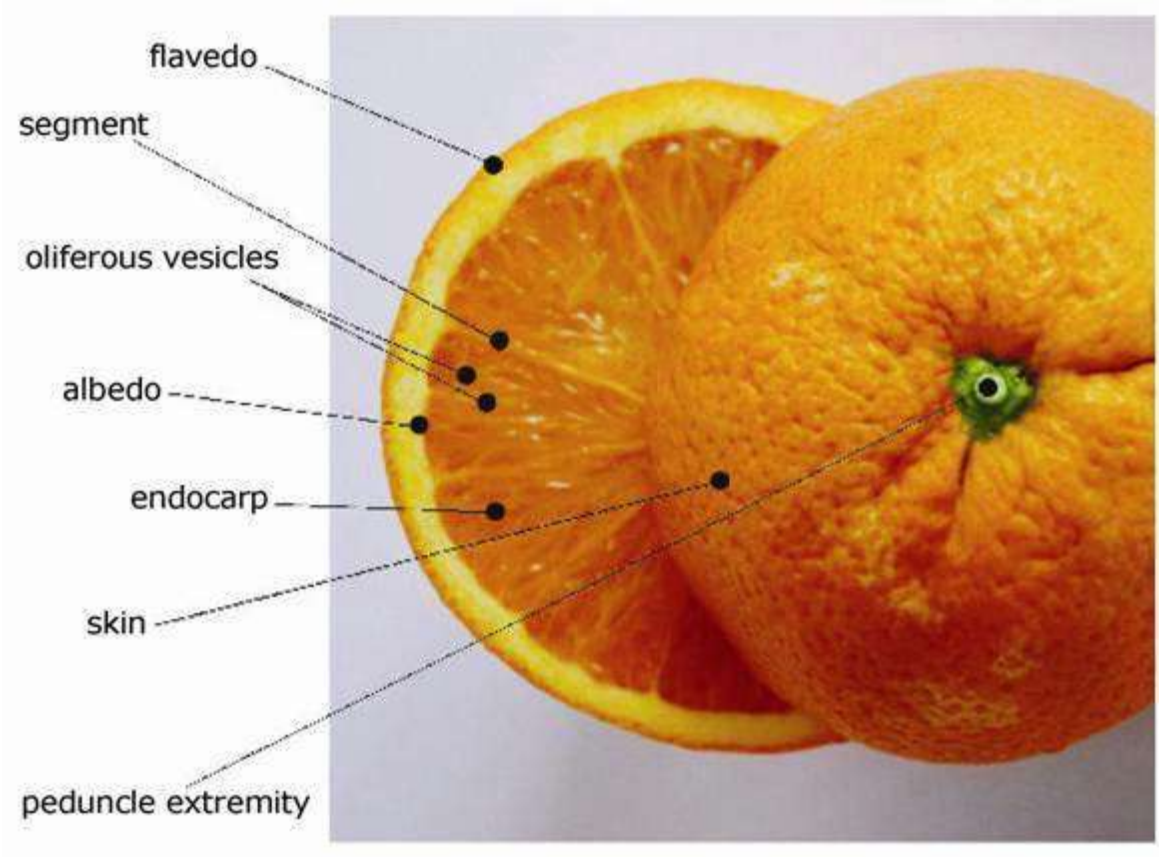


Figure2. 3: Structure of Citrus fruit. (Etebu and Nwauzoma, 2014).

2.7 Optimization

Some previous studies have investigated the influence of operational parameters on the production of enzymes following SSF. The enzymes considered include pectinase, amylase, xylanase, lipase and manganese peroxidase (Pirota *et al*., 2013).

2.7.1 Process optimization

Optimization of the various parameters is of utmost importance for the production of pectinase in large quantities. Various physical and chemical factors have been known to affect the production of pectinase such as pH, temperature, Incubation period, and carbon and nitrogen sources (Das *et al*., 2013; Lemo *et al*., 2019) and they are explained below;

2.7.1.1Temperature

The influence of temperature on pectinase production is related to the growth of the organism meaning that the optimum temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi most pectinase production studies have been done with mesophilic fungi within the temperature range of 25-30^{o}C.

2.7.1.2pH

pH is one of the most important factors that determine the growth and development of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium (Singh and Mandal, 2012). Several studies have revealed that fungi require slightly acidic pH for optimum growth for instance studies have revealed that fungi of *Aspergillus* sp. such as *A. niger* has been found to give significant yields of pectinase at pH of 5.0 to 6.0 in SSF.

2.7.1.3Carbon sources

Agricultural wastes are being used for fermentation in enzyme production to reduce the cost of fermentation media. The waste consists of carbon and nitrogen sources necessary for the

growth and metabolism of organisms. These nutrients sources include orange wastes, corn, tapioca, wheat and a host of others.

2.7.1.4Nitrogen sources

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce biomolecules such as amino acids, nucleic acids, proteins, and cell wall components, and also for metabolite secretion. Different sources of organic and inorganic nitrogen include; yeast extract, peptone, tryptone, glycine, urea, ammonium chloride, ammonium nitrate, ammonium sulphate e.t.c.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Media and Reagent Preparations

3.1.1 Potato Dextrose Agar (PDA)

Potato Dextrose Agar (PDA) with 1% citrus pectin was used for the isolation of pectinolytic moulds. It was prepared following the manufacturer's instruction.

3.1.2 Pectinase Screening Agar Medium (PSAM)

Pectinase Screening Agar Medium (PSAM) was used for the screening of pectinolytic moulds. It was prepared as per the following composition; 0.3g/100mL (NH_4) H_2PO_4 (Ammonium di hydrogen phosphate), 0.3g/100mL KH_2PO_4 (Potassium di hydrogen phosphate), 0.3g/100mL K_2HPO_4 (di potassium hydrogen phosphate), 0.01g/100mL $MgSO_4$ (Magnesium sulphate), 2.5g/100mL–Agar and 1g/100mL–*Citrus*Pectin. The pH of the medium was set at 6.0.

3.1.3 Tween 80 solution

To prepare the Tween 80 solution which was used to prepare the fungal spore suspension, 2.5mL of the concentrated Tween 80 was measured and transferred into a conical flask containing 97.5mL of distilled water, it was swirled and shaken thoroughly to achieve a homogeneous mixture and then autoclaved to obtain a sterile solution.

3.1.4 Iodine-Potassium Iodide solution

One gram **(**1g) of Iodine and 5g of potassium-Iodide was dissolved in 330 mL distilled water. The solution was thoroughly shaken to obtain a homogenous mixture.

3.1.5 3, 5-Dinitrosalicylic Acid (DNSA)

One gram (1g) of DNSA was dissolved by warming in boiling 20mL of 2N NaOH. Then 6.85g of Sodium Potassium tartarate was dissolved by warming in 30ml of distilled water in a separate conical flask, this was then added to the DNSA-NaOH solution to form a homogeneous mixture and made up to 100 mL with distilled water.

3.1.6 Biuret' reagent forprotein estimation

Three grams(3g) of copper sulphate pentahydrate, 9g of Sodium potassium citrate and 5g of Potassium iodide were dissolved into 1000mL of 0.2N NaOH and then used as a colour binding solution. The standard protein used was Bovine Serum Albumin.

3.1.7 Standard galacturonic acid concentration

One gram (1g) of galacturonic acid powder was dissolved in 100ml of distilled water to obtain a stock solution. From the stock solution, serial dilution was prepared to obtain different concentrations (1 - 10mg/ml). The different concentrations of galacturonic acid were used to prepare galacturronic acid standard curve for the determination of pectinase activity.

3.2 Collection of Samples

3.2.1 Orange peels

Disposed sweet orange peel wastes were collected from local fruit selling points within Samaru in Zaria metropolis in clean polythene bags. The bagscontaining the sample were sealed and transported to the Food/Industrial Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

3.2.2 Soil

Soil sample was collected from the botanical garden of Ahmadu Bello University Zaria. The samples were collected in clean polythene bags.

3.3 Sample Preparation

3.3.1 Preparation of Orange peel substrate

The peels were oven-dried at 65°C and pulverized into fine powder using the laboratory milling machine. The processed peels were placed in a clean polythene bag and kept in the refrigerator (4^{o}C) until required for further analysis.

3.4 Determination of Proximate Analysis of Orange Peel Samples

This was carried out based on the procedure of the Association of Official Analytical Chemists (AOAC, 2000) as cited by Egbuonu *et al*., (2016). It was carried out at the Institute for Agricultural Research (IAR), Zaria, in order to determine the moisture, ash, fat, protein, crude fibre and carbohydrate contents.

3.4.1 Moisture content

The moisture content of the orange peel was determined by taking 2g of the sample (orange peels) and placing on a previously weighed moisture can after which it was dried in an oven set at 105°C for three hours. The moisture loss was determined and expressed as a percentage of the sample weight (Egbuonu *et al*., 2016).

The percentage moisture content was calculated using the formula:

$$\text{Moisture content}(\%) = \frac{w_2 - w_3}{w_2 - w_1} \times 100$$

W_1 = Weight of empty can

W_2 = Weight of can and 2g of sample

W_3 = Weight after oven drying

3.4.2 Ash content

Two grams (2g) of the pulverized sweet orange peels was placed on a previously weighed porcelain crucible. The sample was charred with Bunsen burner before it was transferred to a muffle furnace set at 550°C. The sample was burnt to grey. Thereafter, the crucible was removed, cooled and re-weighed. The ash content was then calculated as a percentage of the sample weight (Egbuonu *et al*., 2016).

$$\text{Ash content}(\%) = \frac{w_3 - w_1}{w_2 - w_1} \times 100$$

W_1 = Weight of empty crucible

W_2 = Weight of crucible and 2g of sample

W_3 = Weight after removing from the furnance

3.4.3 Crude Protein content

Digestion:2g of the sample was put in a Kjeldahl flask with copper (catalyst) and 15mL of concentrated H_2SO_4 was added. The mixture was heated in a fume cupboard for digestion to occur. After heating, the mixture was allowed to cool after which the digest was transferred to a 100mL conical flask and distilled water was added to make it up to that mark.

Distillation:a 100mL conical flask containing 10mL of Boric indicator was placed under the condenser of the distillation apparatus. 10mL of the distilled digest was then pipetted into the apparatus via the small funnel aperture then it was washed down with distilled water followed by 10mL of 40% NaOH solution. Steam was passed through the apparatus for seven (7) minutes and then Ammonium Sulphate was collected.

Titration: A 50 mL portion of the distillate was titrated against hydrochloric acid solutions to observe a colour change from green to a deep red end point and the nitrogen content was calculated. A reagent blank was also digested, distilled and titrated in a similar way as the sample.

$$\% \text{ Protein} = \frac{\text{Final reading} - \text{Initial reading} - \text{blank (0.2)} \times \text{standard number of nitrogen (1.4)}}{\text{Initial weight (2)} \times \text{standard number of Protein (6.25)}}$$

3.4.4 Fat/ lipid content

The continuous solvent extraction method was used to determine the fat content using a Soxhlet extractor. A 2g portion of the sample was wrapped with a pre-weighed filter paper (Whatman, No. 40) and placed in Soxhlet column flask. This flask was mounted onto a weighed oil extraction flask containing about 300 mL of petroleum ether at 40-60°C boiling points and left for 8 h. After that the sample was removed and transferred into the oven to dry and finally to a dessicator where it was allowed to cool. The fat content was determined by

expressing the difference in weight as a percentage of the original sample (Egbuonu *et al.*, 2016).

$$\text{Fatcontent}(\%) = \frac{w_2 - w_3}{w_2 - w_1} \times 100$$

W_1 = Weight of empty filter paper

W_2 = Weight of filter paper and 2g of sample

W_3 = Weight after removing from the dessicator.

3.4.5 Crude fibre

Two grams (2g) of the powdered sweet orange peels was defatted and boiled in a 200ml solution containing 1.25% H_2SO_4 under reflux for 30 minutes. Thereafter, the sample was washed with hot water and a two-fold muslin cloth was used to trap the particles. The washed sample was transferred to a weighed porcelain crucible and placed in an oven (set at 105°C) to dry. The crucible was transferred to a dessicator to dry before it will be re-weighed. The crude fibre content was calculated as the percentage weight loss.

$$\text{Crude fibre content}(\%) = \frac{C_2 - C_3}{W} \times 100$$

C_2 = weight after oven drying

C_3 = weight after incineration in a furnace

w= weight of sample (2g).

3.4.6 Carbohydrate (CHO) content

The carbohydrate content is also known as the nitrogen free extract (NFE). This was estimated as carbohydrate by difference when the values of the other components of the sweet orange peels had been determined; these values (excluding the % fibre) were added together and then subtracted from 100. It was calculated using the formula:

Nitrogen free extract (% CHO) =100 - % (a + b + c + d)

Where: a = moisture, b= ash, c= protein, d = fat

3.5 Preparation of Soil Sample and Serial Dilution

Twenty gram (20g) of soil sample was placed in 180mls of sterile distilled water to make the stock solution from which serial dilutions of up to 1×10^{-5} were prepared; from the stock solution, 1ml was taken and transferred into a bottle containing 9ml of sterile distilled water to make dilution of 1×10^{-1}, from this same bottle another 1ml was taken and transferred into a second bottle also containing 9ml of sterile distilled to make dilution of 1×10^{-2}, this process was repeated until a dilution of 1×10^{-5} was obtained.

3.6 Isolation of Pectinolytic Moulds

For the isolation, 0.1ml aliquot of samples from appropriate dilutions of soil sample was aseptically inoculated using the spread plate method onto sterilized and solidified Potato Dextrose Agar (PDA) enriched with 1% citrus pectin and incubated at room temperature for seven (7) days after which the plates were observed for fungal growth. All morphologically contrasting colonies were purified by repeated streaking and sub-culturing on separate PDA plates; this process was continued untill pure mould cultures were obtained (Geetha *et al*., 2012).

3.7 Identification of Pectinolytic Mould Isolates

Pure mould cultures were identified by their morphology, hyphal characteristics, presence or absence of asexual spores, arrangement of conidia and reproductive structures, to achieve this, a sterile straight wire was used to tease out a small portion of the pure culture from the PDA plate unto a sterile glass slide, this was then stained using Lactophenol Cotton Blue dye, the slide was covered with a sterile cover slip and viewed under the x40 magnification of the light microscope. Identification was carried out by relating the microscopic features and the micrographs in the “Illustrated genera of Imperfect Fungi” by Barnett and Hunter (1972).

3.8 Screening and Selection of Pectinolytic Mould Species

The pectinase positive isolates were screened by sub-culturing them unto pectinase screening agar medium (PSAM) and then incubated at ambient temperature for three days. After the third day, the plates were flooded with concentrated Potassium Iodide solution and after decanting the excess dye solution they were left to stand for one hour. Pectinolytic moulds produced clear zones after staining.The diameter of the clear zone observed during that span of time was measured in order to select the isolate with highest pectinase activity as the highest value was assumed to contain the highest activity; the isolates with highest diameter values were then selected for the enzyme production.

3.9 Production of Pectinase by Selected Mould Strain using Solid State Fermentation Technique

Solid state fermentation was carried out as follows; 5gof solid substrate (Orange peel powder) was moistened with 10ml of nutrient solution (composed of 0.1% NH_4NO_3; 0.1% $NH_4H_2PO_4$; 0.1% $MgSO_4.7H_2O$ in 250mL conical flask (Dange and Harke, 2018). The inoculum was prepared by adding 10mL of sterile Tween 80 solution into a seven day-old fungal slant culture and mixing thoroughly (the spores were washed with sterilised Tween 80 solution by shaking vigorously for 1 minute. Spores were counted with a haemocytometer to obtain approximately 2 $x10^7$ spores/mL). 5mL of inoculum was added into the flask and incubated at 30°C for 96 hours. After the incubation period, the enzyme was harvested by adding 50mL of autoclaved distilled water into the fermentation medium and placed in the shaker for one hour after which the fermented slurry was filtered through the muslin cloth and centrifuged. The supernatant served as crude enzyme solution and was used to assay for pectinase activity (Garlapati *et al*., 2015; Abdullah *et al*., 2018).

3.10 Assay for Pectinase Enzyme Activity

Pectinase activity was determined by measuring the release of reducing sugars from the citrus pectin using the 3, 5- dinitrosalicyclic acid reagent assay as described by Miller, (1959); a sample of 0.5mL of the crude enzyme filtrate was placed in a test tube and incubated with 0.5ml of 1% pectin in 0.1M Sodium acetate buffer of pH 6.0 at 40ºC for 10 min. Then, 1mL of DNSA reagent (3, 5-dinitrosalicylic acid) was added to the reaction mixture and this mixture was heated by placing in boiling water bath for 15 minutes after which 1mL of distilled water was added. The mixture was allowed to cool and then the absorbance was spectrophotometrically measured at 595nm. A standard graph was generated using standard galacturonic acid solution curve. One unit of Pectinase activity is defined as the amount of enzyme which liberates 1µ mole of galacturonic acid per minute under standard assay condition.

3.11 Optimization of Fermentation Parameters for Optimal Enzyme Production

Various fermentation parameters such as temperature, pH and nitrogen sources were studied to determine the optimum conditions for pectinase production.

3.11.1 Effect of initial pH

Pectinase production was carried out at various pH values (4.0, 5.0, 6.0, 7.0 and 8.0,) and then the enzyme activity was assayed.

3.11.2 Effect of temperature

Pectinase production was carried out at different temperatures of 30ºC, 35ºC, 40ºC, 45ºC and 50ºC and the enzyme activity was assayed after production.

3.11.3 Effect of nitrogen sources

Different nitrogen sources (peptone, yeast extract, potassium nitrate, ammonium chloride and ammonium sulphate) were supplemented into the production medium at a concentration of 0.5% w/v and then the pectinase activity was assayed.

3.12 Protein Determination

Protein concentration of the enzyme was determined usingthe Biuret's method. Test tubes were arranged in duplicates containing 0.0- 1.0mL of 10mg/mL of protein stock solution (10mg/ml BU reagent) and brought up to 1mL with distilled water. For the test mixture, 0.5mL of Biuret reagent was added to 2mL of the crude enzyme and absorbance was read at 540nm.

3.13 Extraction and Purification of Pectinase Produced

3.13.1 Extraction

The enzyme produced was extracted using sodium acetate buffer at pH 6.0 and assayed by measuring the D-galacturonic acid released from polygalacturonic acid as described by Miller's method (1959). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µ mole of galacturonic acid per minute.

3.13.2 Purification: ammonium sulphate precipitation

Pectinase in the crude extract was purified using 40-70% of ammonium sulfate precipitation. 10 mL each of crude enzyme solution was placed in separate centrifuge tubes, then 40%, 50%, 60% and70% of ammonium sulphate were added to the enzyme solution in appropriately labelled test tubes and stored at 4ºC overnight after which centrifugation was done at 12000 rpm for five minutesminutes. The pellets were dissolved using sodium acetate buffer and activity was determined as previously described to ascertain the percentage saturation of ammonium sulphate salts that gave the highest activity.

3.13.3 Gel filtration chromatography

Ten millilitres (10mL) of the enzyme stock collected after ammonium sulphate precipitation was applied to sephadex G-150 column (2.5 x 30cm^2) equilibrated with phosphate buffer at pH 6.0. Stepwise elution was carried out with phosphate buffer (pH 6.0). A total of ten (12) fractions (4mls each) of the enzyme were collected in tubes and tested for pectinolytic activity

and protein assay was also carried out. Fractions exhibiting the highest activity were pooled and subjected to ion exchange chromatography (Maria *et al*., 2006).

3.13.4 Ion exchange chromatography

Ion exchange chromatography was carried out using Diethylaminoethyl as ion exchange resin. The enzyme sample was applied to a DEAE Cellulose column in phosphate buffer at pH 6.0. The protein was eluted using a linear gradient of 0.1M NaCl using the same buffer. Twelve (12) fractions were collected in series of tubes and tested for Pectinolytic activity as well as protein concentration (Somyok and Phimchanok, 2009).

3.14 Data / Statistical Analysis

Data obtained from the study were subjected to statistical analysis using the SAS JMP Pro version 14 statistical packages. One way Analysis of Variance (one way ANOVA) was used to compare the effect of fermentation parameters on pectinase production by the isolates. Results were then presented in tables, charts and figures were applicable.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition of Orange Peels

Table 4.1 shows the result of the proximate analysis carried out on the Orange peel sample for percentage moisture, ash, fat, protein, fibre, and total carbohydrate content. The average moisture content was 26.08%, ash 3.86%, fat 3.40%, protein 13.20% and total carbohydrate content was estimated to be 53.45%.

4.2 Isolation and Identification of Pectinolytic Moulds

A total of seven (7) isolates were identified from soil samples obtained from the botanical garden of Ahmadu Bello University, Zaria. Based on cultural morphology on the selective growth media and microscopic characterization; the isolates were all found to be filamentous fungi. Genus identification was done by examining both macroscopic and microscopic features of a seven day old pure culture. Colour, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation and spore shapes were examined. Based on these characteristics *Aspergillus niger, Monilia, Fusarium, Sclerotium rolfsii, Penicillium, Cladiosporium* and *Curvularia* species were confirmed. Details of the cultural characteristics are given in Table 4.2 and Plates I and II also show the cultural and microscopic features respectively.

Table 4. 1: Proximate Composition of Orange Peels Sample

Parameters	Percentage Composition (%)
Moisture content	26.08
Ash	3.86
Crude fibre*	16.88
Crude protein	13.20
Fat	3.40
Total Carbohydrate	53.45

Key *(Crude fibre) is not included in the calculation of total carbohydrate

Table 4. 2: Cultural Characteristics of Mould Isolates

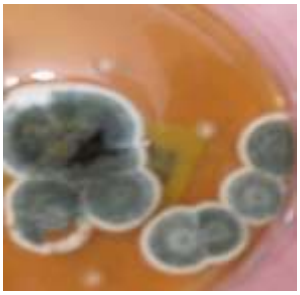
Isolate	Colour	Surface features	Edge	Reverse colour	Presumptive Identity
1	Whitish grey	Thin fluff	White, circular	Grey	*Monilia sitophila*
2	White	Cottony	White circular	Bluish brown	*Fusarium* spp
3	Black	Granular	Black, irregular	Cream	*Aspergillus niger*
4	White	Granular	White, irregular	Peach	*Sclerotiumrolfsii*
5	Bluish green	Powdery	White irregular	Brownish red	*Penicillium* spp
6	Green	Cottony	Irregular	Black	*Cladosporium* spp
7	Grey	Woolly	Circular	Black	*Curvularia* spp

1(*Monilia sitophila*)

2 (*Fusarium* spp)

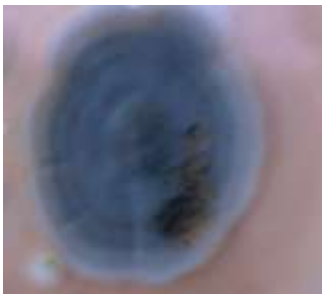
3 (*Aspergillus niger*)

4 (*Sclerotiumrolfsii*)



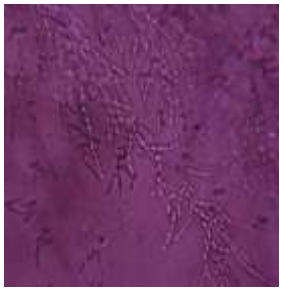
5 (*Penicillium* spp)

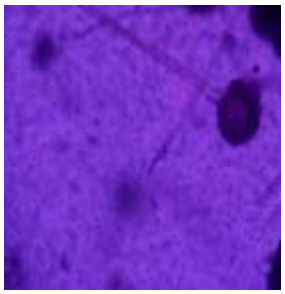
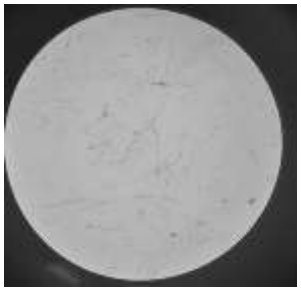
6 (*Cladosporium* spp)



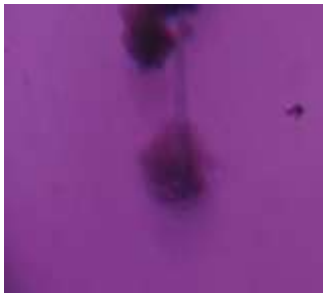
7 (*Curvularia* spp)

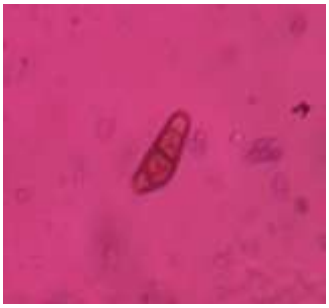
Plate I: Cultural appearance of mould isolates



1 (*Monilia sitophila*) 2 (*Fusarium* spp) 3 (*Aspergillus niger*) 4 (*Sclerotium rolfsii*)





5 (*Penicillium* spp) 6 (*Cladosporium* spp) 7 (*Curvularia* spp)

Plate II: Microscopic characteristics of mould isolates (x40 magnifications)

4.3 Screening of Mould Isolates for PectinaseActivity

The seven isolates were screened and found to be capable of producing pectinase which was identified by zone of clearance signifying hydrolysis of pectin on agar plate. *Sclerotium rolfsii* had the highest (35mm) zone of hydrolysis followed by *Aspergillus niger* (31mm) while *Fusarium* spp had the least (9mm) zone of hydrolysis.

Table 4. 3: Screening for Pectinase Activity by Mould Isolates

Mould species	Diameter of zone of Pectin hydrolysis (mm)
Monilia sitophila	11.00
Fusarium spp	9 .00
Aspergillus niger	31 .00
Sclerotiumrolfsii	35 .00
Penicillium spp	16 .00
Cladosporium spp	15 .00
Curvularia spp	12 .00

4.4 Optimum Conditions for Pectinase Production by *Sclerotium rolfsii*

The result of Pectinase activity (U/mL) recorded at 24h interval over a seven (7) day fermentation period is shown in Figure 4.1**.** The highest activity observed was 103.01 U/ml at day 5 (120h) of the fermentation period and the lowest activity 66.53 U/ml was at day 1 (24h) of incubation. Pectinase production was also observed to decline after day 5.Figure 4.2 shows the results of pectinase activity (U/mL) recorded at different initial pH values of the fermentation medium.The highest activity of 116.65 U/mL was observed at initial pH of 5 of incubation and the lowest activity was 36.05 U/mL at initial pH of 8.Results of pectinase activity (U/mL) obtained when production was carried out at varying temperature values is shown in Figure 4.3.The highest activity observed was 100.4 U/mL during fermentation at room temperature 30^{o}C and no activity was recorded at an elevated temperature of 50^{o}C of incubation.Figure 4.4 shows the results of pectinase activity (U/mL) recorded when production was carried out with addition of various nitrogen sources.The highest activity observed (114.12 U/mL) was at 0.1% supplementation with peptone, while a low pectinase activity of 34.10 U/mL was recorded when fermentation medium was supplemented with ammonium chloride.

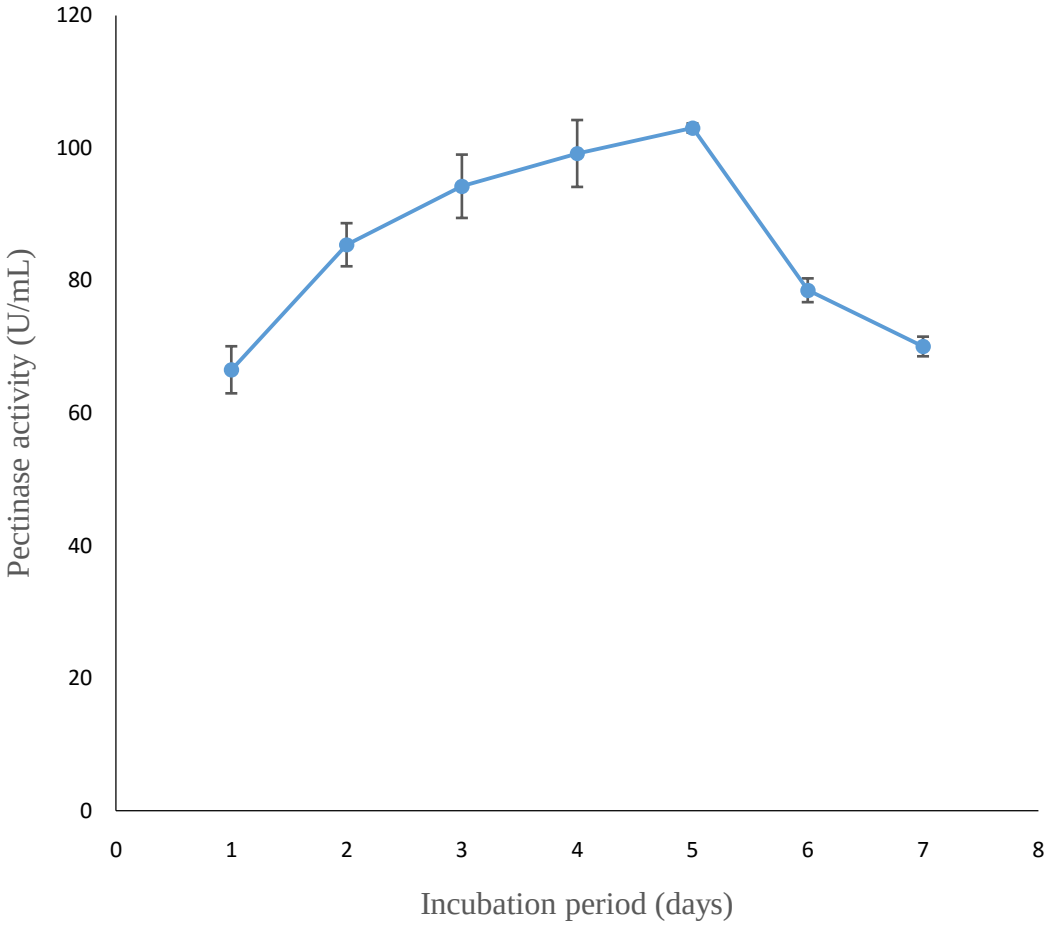


Fig. 4. 1: Effect of incubation period on pectinase production by *Sclerotium rolfsii*

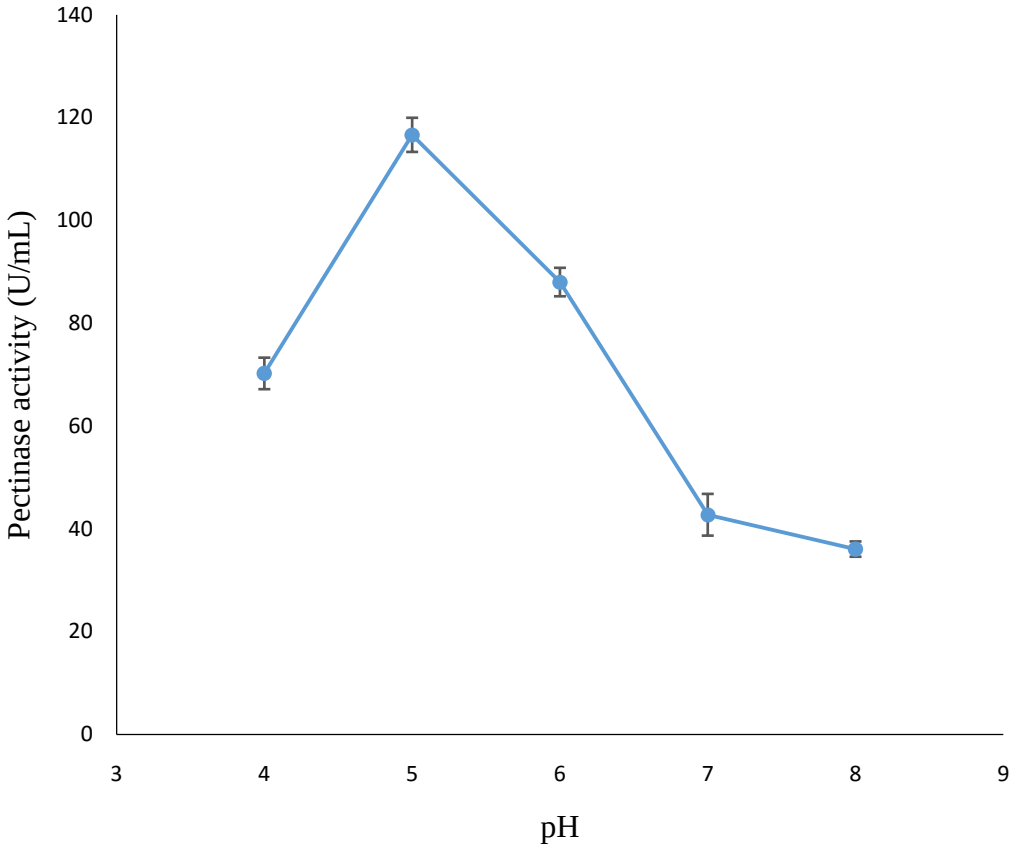


Fig. 4. 2: Effect of pH on pectinase production by *Sclerotium rolfsii*

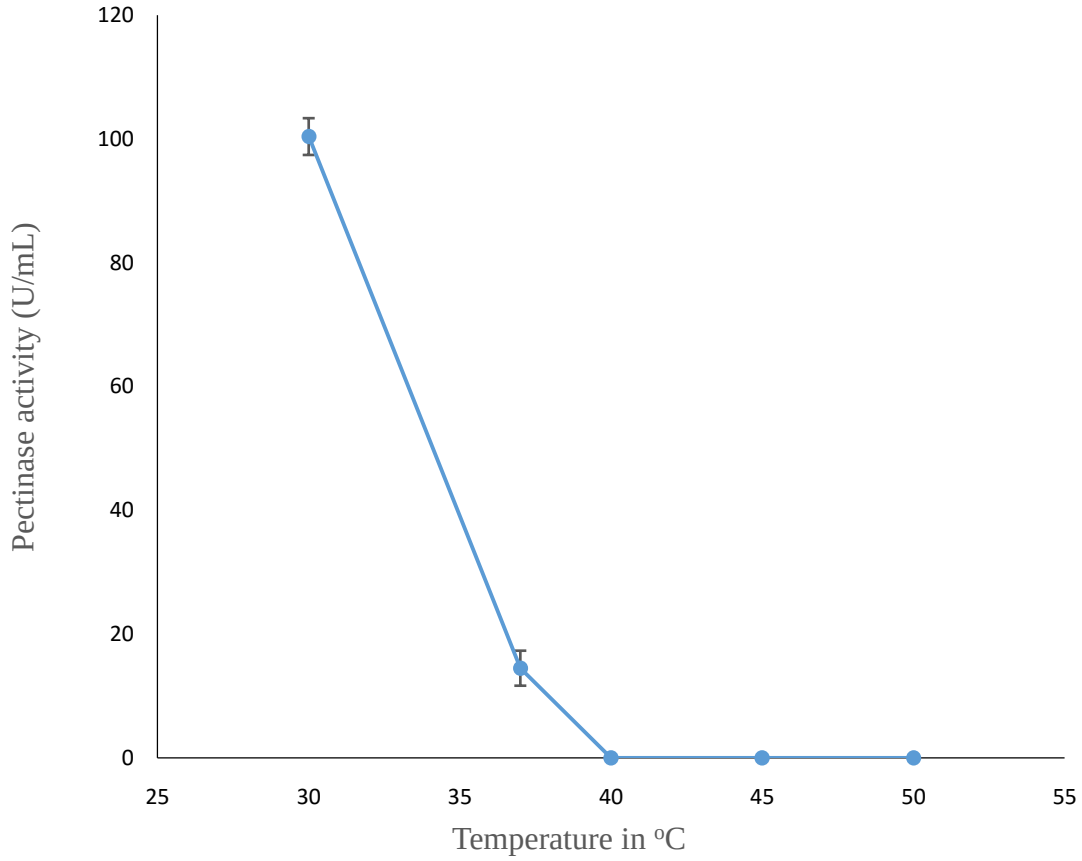


Fig. 4. 3: Effect of temperature on pectinase production by *Sclerotium rolfsii*

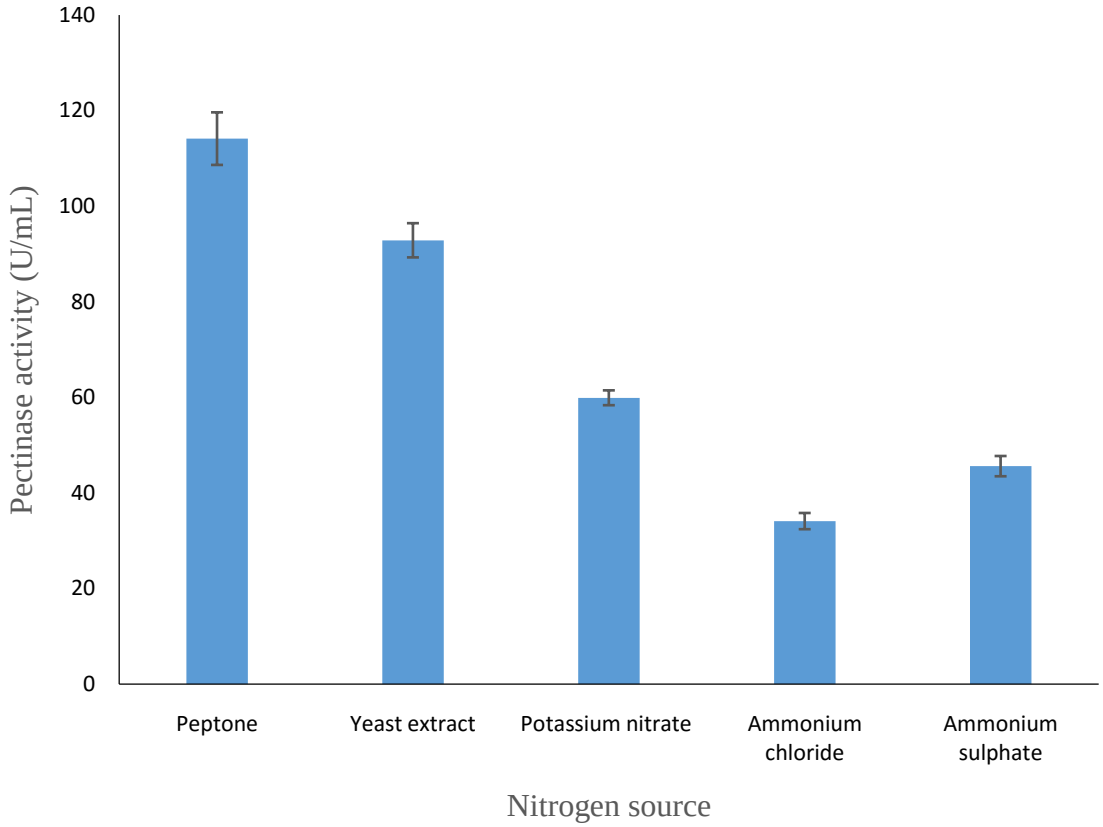


Fig. 4. 4Effect of nitrogen source on pectinase production by *Sclerotium rolfsii*

4.5 Optimum Conditions for Pectinase Production by *Aspergillus niger*

Results of pectinase production by *Aspergillus niger* recorded at 24h interval over a seven (7) day fermentation period is shown in Figure 4.5**.** The highest activity observed was 106.96 U/mL on the sixth day (144h) of the fermentation period and the lowest activity (49.90 U/mL) was at day 1 (24h) of incubation.Figure 4.6 shows the results of pectinase activity (U/mL) recorded at different initial pH values of the fermentation medium.The highest activity observed was 95.6 U/mL at initial pH of 5 and the lowest activity (23.67 U/mL) was obtained at initial pH of 8.Figure 4.7 shows the results of pectinase activity (U/mL) recorded when production was carried out at varying temperature values.The highest activity observed was 93.7 U/mL during fermentation at ambient temperature (30^{o}C) and no activity was record at elevated temperature of 50^{o}C of incubation.Results of enzyme activity (U/mL) obtained when the fermentation medium was supplemented with additional nitrogen sources are shown in Figure 4.8. The highest activity observed (102.33 U/mL) was at 0.1% supplementation with peptone, while a low pectinase activity (35.6 U/mL) was recorded when ammonium chloride was added to the fermentation medium.

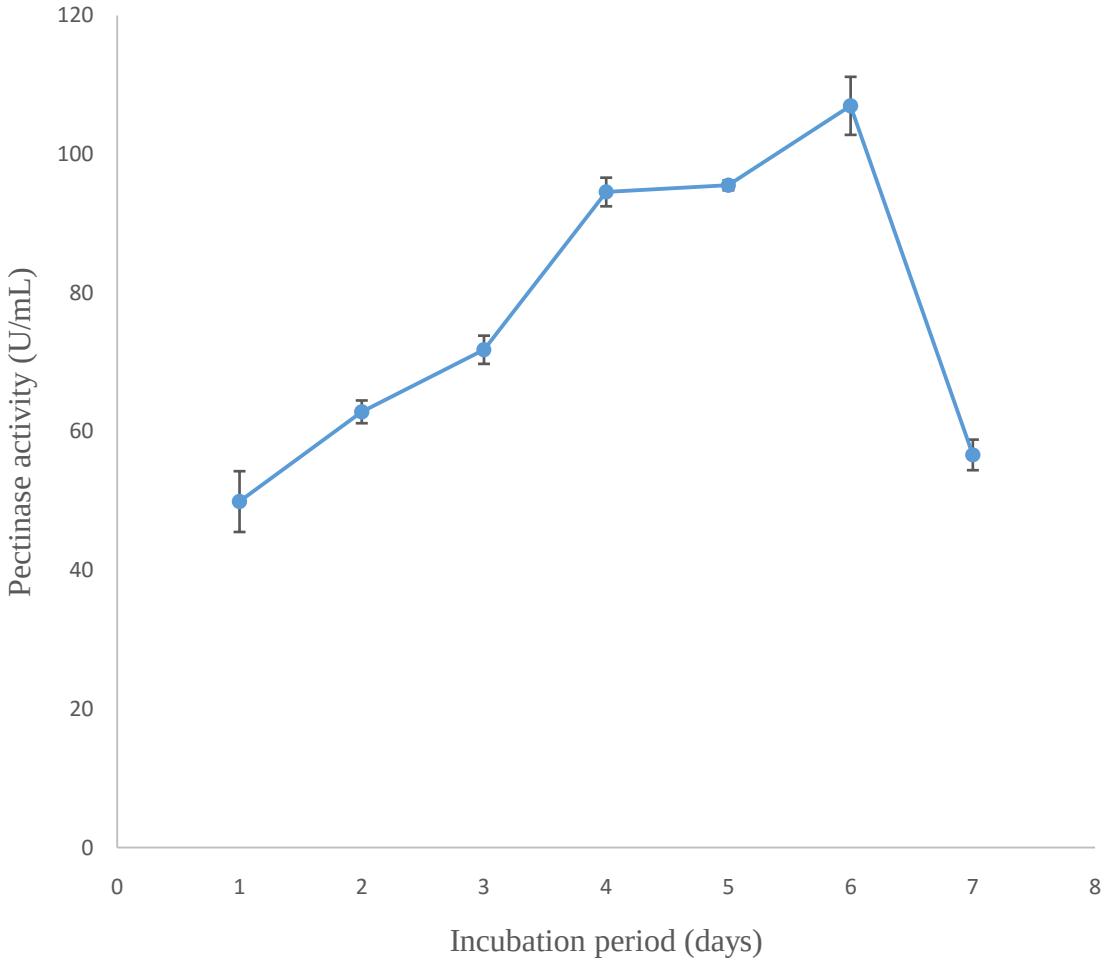


Fig. 4. 5: Effect of incubation period on pectinase production by *Aspergillus niger*

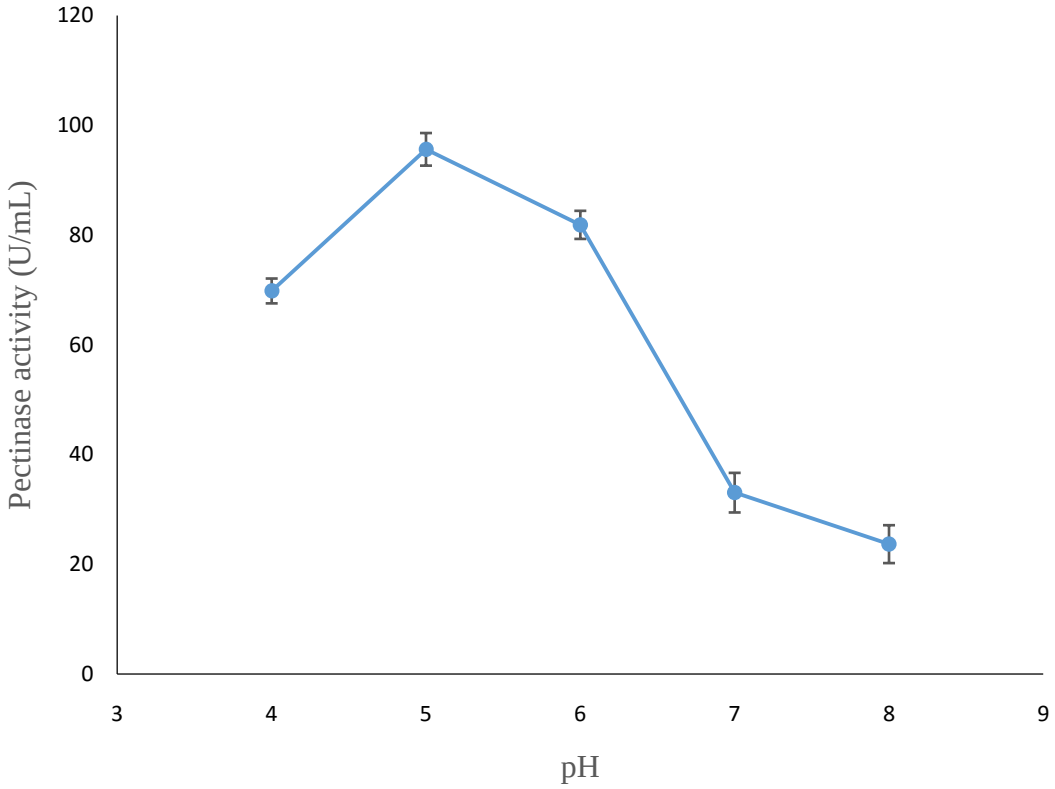


Fig. 4. 6: Effect of pH on pectinase production by *Aspergillus niger*

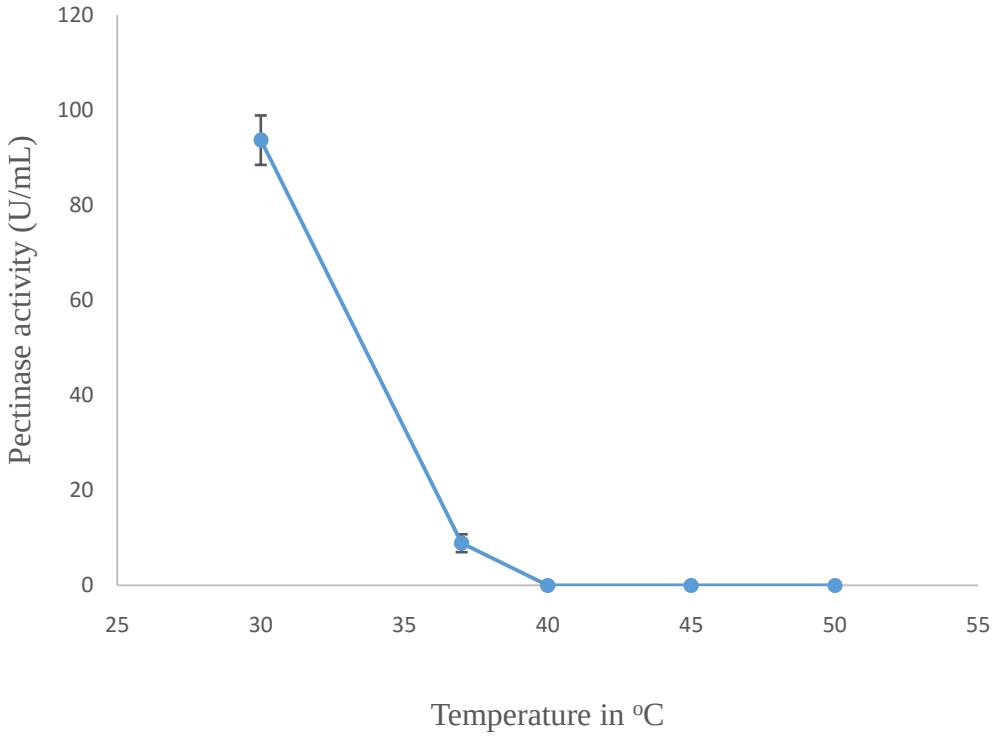


Fig. 4. 7: Effect of temperature on pectinase production by *Aspergillus niger*

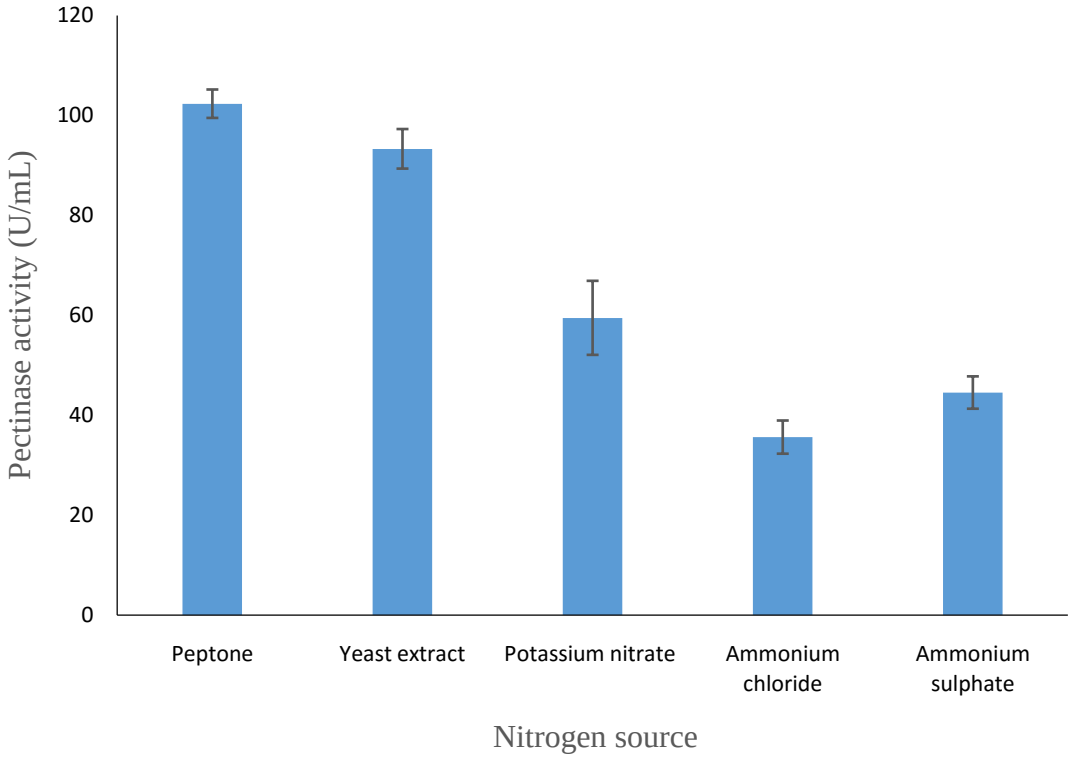


Fig. 4. 8: Effect of nitrogen source on pectinase production by *Aspergillus niger*

4.6 Overall Optimum Yield of Pectinase Produced by *Sclerotium rolfsii* and *Aspergillus niger* Using OrangePeel under Optimum Fermentation Conditions

Optimum pectinase yield of 108.54 ± 2.15 U/mL and 104.96 ± 0.53 U/mL as presented in (Table 4.4) was obtained from the orange peel substrate with the *Sclerotium rolfsii*and *Aspergillus niger*isolates under predetermined optimum fermentation conditions of incubation temperature (30^{o}C), initial pH (5.0), incubation period of days 5 and 6 (for *Sclerotium rolfsii* and *Aspergillus niger*respectively) and nitrogen supplementation with peptone.

4.7 Partial Purification of Extracellular Pectinase Produced by *Sclerotiumrofsii*

The result of the three stage purification revealed a decrease in the total enzyme activity with a corresponding increase in the specific activity of the enzyme. The total pectinase activity of the crude enzyme was 108,540 U/mL, but this consistently decreased after each purification step to a final value of 45,075 U/mL after the ion exchange chromatography stage, while the specific activity increased from 10.30 - 45.58 U/mg. The total protein content of the enzyme decreased from 10,540 - 989 mg, while the protein concentration increased from 10.54 - 19.78 mg/mL. Details of the purification parameters and enzyme activity values obtained from the study are summarized in Table 4.6.

Table 4. 4: Yield of Pectinase Produced by Sclerotium rofsii and *Aspergillus niger*using Orange Peel under Optimum Fermentation Conditions (Mean ± SE)

Isolate	Yield of Pectinase (U/mL)
Sclerotiumrofsii	108.54 ± 2.15
Aspergillus niger	104.12 ± 0.53

Table 4. 5: Purification of Pectinase Produced by *Sclerotium rolfsii*

Purification step`	Volume (mL)	Protein Conc. (mg/mL)	Activity (U/mL)	Total activity (U/mL)	Total protein Conc. (mg/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude Pectinase	1000	10.54	108.54	108540	10540	10.30	1.00	100
70% Ammonium sulphate precipitation	500	16.04	177.16	88580	8020	11.04	1.10	81.61
Gel filteration chromatography	70	17.14	707.35	49514.5	1200	41.26	4.01	45.62
Ion exchange Chromatography	50	19.78	901.50	45075	989	45.58	4.43	41.53

4.8 Partial Purification of Extracellular Pectinase Produced by *Aspergillus niger*

Table 4.6 indicates the result obtained from the three stages of purification of the enzyme produced by *Aspergillus niger*; a decrease was observed in the total activity with a corresponding increase in the specific activity of the enzyme. The total pectinase activity of the crude enzyme dropped from 104,120 U/mL to a final value of 41,701 U/mL after ion exchange chromatography, while the specific activity increased from 5.12 - 32.58 U/mg. The total protein content of the enzyme decreased from 20,320 - 1,280 mg, while the protein concentration increased from 20.32 - 25.60 mg/mL Details of the purification parameters and enzyme activity values obtained from the study are summarized in Table 4.7.

Table 4. 6: Purification of pectinase produced by *Aspergillus niger*

Purification step`	Volume (mL)	Protein Conc. (mg/mL)	Activity (U/mL)	Total activity (U/mL)	Total protein Conc. (mg/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude Pectinase	1000	20.32	104.12	104120	20320	5.12	1.00	100
70% Ammonium sulphate precipitation	500	26.37	188.21	94104.18	13185	7.14	1.39	90.38
Gel filtration chromatography	70	23.03	695.79	48705	1612	30.21	5.90	46.78
Ion exchange Chromatography	50	25.60	834.02	41701	1280	32.58	6.36	40.05

CHAPTER FIVE

5.0 DISCUSSION

Pectinase enzyme has been the focus of research for many years due to its potential and wide applications in various industrial processes. An increasing demand for this enzyme necessitates the need to explore various microbial strains capable of producing novel pectinases with improved activities (Kahtri *et al*., 2015). In this study, an attempt has been made to isolate the mould strains that produce pectinase and to optimize the production parameters of this important enzyme.

Proximate composition of the orange peel substrate revealed percentages of moisture contents 26.08%, ash 3.86%, crude fibre 16.88%, crude protein 13.20%, fat 3.40% and carbohydrates 53.37%. The presence of crude protein reveals the potential of the orange peel as a nitrogen source required for growth and efficient enzyme expression, while the high carbohydrate content indicates the availability of sufficient amount of fermentable sugars required for growth and enzyme production by the organism. The ash content indicates the richness of the substrate in minerals required for growth of the organism (Enyisi *et al*., 2014).

Moulds are well-known for having the ability to produce many varieties of extracellular enzymes. In nature, extracellular enzymes primarily serve to procure nutrients for the survival and growth of fungi (Steudler *et al.*, 2019). Pectinase (especially from fungal sources) is notably one of the most important industrial enzymes that have a wide range of applications and accounts for about 25% of the world's food enzyme production (Bajpai, 2018).

Isolation is the first step in the screening of microorganisms with potential industrial applications; beginning first with the identification of the environmental source, which ideally should be richer in the desired types of microorganisms. The isolation method was

designed to favour the growth of a specific organism possessing the characteristics of interest. In the present study, seven (7) (*Aspergillus niger, Monilia, Fusarium, Sclerotium rolfsii, Penicillium, Cladiosporium* and *Curvularia* species) mould isolates were selectively isolated by enrichment method using a medium containing pectin as the sole carbon source. Similar techniques for isolation of pectinase producing microorganism using pectin as sole carbon source were previously used by Ketipally and Ram (2018). The occurrence of pectinolytic organisms in soil agrees with the earlier report of Sangilimuthu *et al.* (2018), where soil was described as a repository for pectinase producing organisms.

Isolates were subjected to plate agar screening method using citrus pectin as the sole carbon source and zones ofhydrolysis were used to select isolates with highest pectinase producing potential. Majority of the strains were positive for pectinase activity in a plate agar screening method.

Out of seven (7) mould isolates identifiedand screened for pectinase production ability, *Sclerotium rolfsii* had the highest zone of pectin hydrolysis followed by *Aspergillus niger* while the lowest zone of pectin hydrolysis was observed with *Fusarium* spp. This variation might be due to differences in the metabolic capabilities of the different isolates. This result is similar to the work of Sudeep *et al*. (2020) who reported that an isolate (*Aspergillus* sppGm) showed the maximum zone of pectin hydrolysis around the colony.

Other Researchers such as Munir *et al*. (2019) and Ketipally and Ram (2018) also used the same plate agar screening method with evidence of hydrolysis around the colony indicated by zone of clearance, however, Souza *et al*. (2003) applied the cup-plate assay method for isolation and screening of pectinolytic fungi and reported that all isolates showed pectinase production potentials.

Isolates (*Aspergillus niger*and *Sclerotium rolfsii*) that showed relatively higher clear zone to colony diameter ratio were employed in solid state fermentation for optimization of

fermentation conditions for enzyme production. The capacity of microorganisms to produce extracellular enzymes is influenced by environmental conditions such as temperature, pH, aeration, inoculum and the presence of inducer or repressor substrates (Oliyad and Abate, 2018). In this study, various parameters that affect the pectinase enzyme production have been screened.

Growth of fungi in any medium varies according to physical and chemical factors governing that environment. Basically, enzymes are produced during the exponential phase; hence, the level of enzyme production varies with the time duration in the fermentation processes (Oliyad and Abate, 2018). In this study, the effect of incubation period on pectinase production by *Sclerotium rolfsii* and*Aspergillus niger* under solid state fermentation (SSF) revealed that 120 h and 144 h respectively were the optimum (100.40 ± 0.13 U/mL and 93.70 ± 0.25 U/mL respectively) among the tested incubation periods. The pectinase production from the selected isolates increased continuously until 120 h (in the case of *Sclerotiumrolfsii*) and 144 h (with *Aspergillus niger)* of incubation; after these respective hours of incubation, the pectinase activity decreased. Maximum enzyme production by *Aspergillus niger* was reported on the sixth day of incubation which contrasts the observation made by Adeleke *et al*. (2012) where maximum production of pectinase by the same organism was reported on the fifth day, the different optimum incubation time might be due to differences in the species and strains of organisms used. The reduction in pectinase production after the 120 and 144 hours of incubation by the respective organisms investigated in this study might be due to change in pH during fermentation, denaturation or decomposition of enzyme depletion of nutrients and accumulation of toxic wastes in the medium(Oliyad and Abate, 2018). Producer strains are required to have shorter fermentation cycles for the purpose of their cost effectiveness (Oliyad and Abate, 2018) and from the results of this study, 120 and 144 hours

of incubation for high pectinase production is a relatively short incubation time, thus, it has good prospect for industrial application.

The initial pH of the fermentation medium plays a vital role in determining the level of metabolite synthesis, the stability of the microbial metabolite is also dependent on the hydrogen ion concentration of the medium (Oliyad and Abate, 2018). In the present study, maximum production of pectinase by *Sclertium rolfsii* and*Aspergillus niger*occurred at pH of 5.0 (116.65 ± 0.92 U/mL and 95.60 ± 1.93U/mL respectively), thiscould be because Fungi are acidophilic making them to thrive and function well in acidic environments. The optimum pH for the two organisms was found to be the same; this may be due to the fact that the optimum pH for the production of pectinase is more related to the optimum conditions required for the growth of microorganism irrespective of the type of microorganisms. The enzyme yield noticeably increased from pH4 to pH 5 while a steady decrease in the enzyme yieldwas observed from pH 6 to 8. This might bebecause the metabolic activities ofmicroorganisms (and of Fungi especially) are very sensitive to changes in the pH of their environment. The results of this finding is in line with that of Adeleke *et al*.(2012) who observed a maximum pectinase production by *Penicillium atrovenetum* at initial pH of 5.0; however the result of this study differs from that of Ketipally and Ram (2018) who reported maximum production at pH 6.5 for the production of pectinase from *Aspergillus oryzae RR10,* this discrepancy may be due to the difference in species of *Aspergillus* used in the two investigations.

The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition and protein denaturation. The maximum pectinase production from the isolates used in this study was observed at a temperature of 30^{o}C (100.40 ± 0.13 U/mL and 93.70 ± 0.25 U/mL for *Sclerotium spp* and*Aspergillus niger* respectively). This result is similar to the findings of Sudeep *et al*. (2020) who also reported 30^{o}C as the temperature for

maximum pectinase production (96.7 U/mL) by *Aspergillus* spp strain Gm. At higher temperatures, the enzyme production declined and this observation agrees with that of Sudeep *et al*. (2020) who indicated a decline in pectinase activity at a higher temperature. However Khatri *et al.* (2015) reported that the maximum yield of pectinase enzyme from the members of genus *Aspergillus* spp. occurred at the temperature range of above 30^{o}C to 40^{o}C; this could be because the *Aspergillus* species used may have been the thermo-tolerant strains (Khatri *et al.*, 2015). Decrease in pectinase production as observed beyond the optimum temperature (30^{o}C) might be due to growth reduction or suppression of cell viability (and probably cell death) and enzyme inactivation. Nitrogenous compounds are used by the microbial cells for the synthesis of nucleotides, amino acids, proteins and other metabolites (Oliyad and Abate, 2018). The importance of nitrogen sources in fungal growth and consequently enzyme production cannot be over emphasized (Ketipally and Ram, 2018), therefore, supplementing nitrogen into the production medium accelerates biomass production and subsequently results in higher metabolite secretion (Das *et al*., 2013).

The effect of various organic and inorganic nitrogen supplements on pectinase production in this study showed that among the five nitrogen sources supplemented into the fermentation medium, peptone showed the highest activity of 114.12 ± 2.15 U/mL and 102.33 ± 0.53 U/mL by *Sclerotium rolfsii* and*Aspergillus niger* respectively. Similarly, Ketipally and Ram (2018) reported enhanced production of pectinase with the addition of peptone, the result also agrees with the work of Nazneen *et al*. (2011) in which peptone was found to support maximal production of pectinase (113.68 U/ml) as compared with the other sources of nitrogen (Yeast extract, ammonium sulphate). However, Adeleke *etal*. (2012) reported maximum pectinase production from fungi in the presence of $(NH_4)_2SO_4$, so also the findings of Kumar *et al.* (2011) where ammonium sulphate was found to be the best nitrogen source for pectinase production by*Aspergillus niger*MCIM 548 using SSF process.

The purification of pectinase can be done using a series of methods such as salt precipitation, gel filtration chromatography and ion-exchange chromatography; in the present study, Ammonium sulphate precipitation was performed as an initial step to purify extracellular pectinase produced by *Sclerotiumrofsii* and *Aspergillus niger.* Asaturation level of 70% was used and specific activities of 11.04 U/mg and 7.14 U/mg were recorded for *Sclerotiumrofsii* and *Aspergillus niger* respectively, this report is similar to that of Jalil and Ibrahim(2021) where specific activity of 11.41 U/mg was recorded for pectinase obtained from *A. niger* LFP-1 after ammonium sulphate at a concentration level of 80% was used for precipitation. A high concentration of ammonium sulphate is usually used since the salting-out process is dependent on the hydrophobicity of the protein and thus, high salt concentration promotes the aggregation of hydrophobic patches on the protein surface.

Also, the addition of salt in high concentration reduces the electrostatic repulsion between like-charged groups at the protein surface and disturbs the structure of water molecules around the protein, making the aqueous salt solution a poor solvent for proteins, which precipitate out (Farinas *et al.*,2011). Many other researchers also made use of the precipitation method as the first step in their purification process. Researchers like Batool *et al.* (2013) reported that the maximum enzyme activity (382.45 U/mL/) was achieved at 60% ammonium sulphate concentration. Also Ahmed *et al.* (2016) reported a specific activity of 23.6U/mg after purification by ammonium sulphate precipitation of pectinase from *A. niger* cultured on waste orange peels. Proteins can be further purified by the sequential application of chromatography. Gel filtration chromatography also referred to as size exclusion chromatography is a chromatographic technique that allows the separation of macromolecules based on their hydrodynamic size whereby the larger molecules can only penetrate larger pores (elute earlier) whereas the smaller molecules can access the higher number of pores and retain longer in the column (Raak *et al.*,2018). The present study

revealed that the pectinase produced was purified up to 4.01 and 5.90 folds with specific activities of 41.26 U/mg and 23.03 U/mg for *Sclerotiumrofsii* and *Aspergillus niger* respectively through gel filtration chromatography on Sephadex G-150 column. However, the purification folds and specific activities of enzymes purified by gel filtration chromatography may vary depending on the microbial strains and substrates used; for instance, Khatri *et al.* (2015) reported that the pectinase they obtained from *Aspergillus niger*strain MCAS2 was purified 8.5 folds with a specific activity of 60 U/mg by using Sephadex G-75 gel filtration chromatography; on the other hand, Ire and Vinking (2016) reported that pectinase from *Aspergillus niger*grown on banana peels was purified up to 42 folds with 166.67 U/mg of specific activity through gel filtration on Sephadex 100. Again in another study carried out by Jalil and Ibrahim(2021) the pectinase produced was purified up to 76 folds with a specific activity of 61.54 U/mg through gel filtration chromatography on Sephadex G-100 gel. The discrepancies in all these reports might not only be due to the different strains and substrates used but could also be due to the different gel sizes used in the various studies.

Ion-exchange chromatography is a common method used for protein purification and it relies on the charge-to-charge interactions between the ion-exchange resins and the proteins in the lysate, the method provides high resolution even though under mild conditions with high binding capacity (Saraswat *et al.*,2013). In the present study, the pectinase from *Sclerotiumrofsii* and *Aspergillus niger* respectivelywas purified up to 4.43 and 6.36 folds with specific activities of 45.58 U/mg and 32.58 U/mg through DEAE-cellulose ion-exchange chromatography; these values are slightly higher than the value obtained in the work of Okonji *et al.*(2019) who reported that pectinase produced by *A. fumigatus* was purified 1.74 folds with specific activity of 15.19 U/mg after purification process through CM Sephadex C-50 ion exchange chromatography; this difference could be as a result of the different strains of organisms used in

the studies. However, Jalil and Ibrahim(2021) reported that the pectinase from *A. niger* LFP-1 was purified up to 20.30 folds through DEAE-cellulose anion-exchange chromatography with specific activity of 16.50 U/mg. Similarly, Patidar *et al.* (2017) carried out the chromatography on DEAE-cellulose to purify pectinase from *niger* AN07 cultured on dried papaya peels and the enzyme was purified to 24.8 folds.

Overall, the purification steps showed a decrease in total activity and total protein - this phenomenon might be due to the removal of impurities from the crude which is responsible for high total enzyme activity and total protein content (Ire and Vinking, 2016). The three purification steps also demonstrated increase in specific activity (from 10.30 - 45.58U/mg and from 5.12 - 32.58U/mg for *Sclerotium rofsii* and *Aspergillus niger*respectively) which agrees with the observations of researchers such as Ubani *et al.* (2015) and Jalil and Ibrahim(2021) who revealed that the purification steps increased specific pectinase activity from crude (76.04 U/mg) to the purified enzyme (179.18 U/mg) and from (0.81 U/mg) to (61.54U/mg) respectively. Increase in specific enzyme activity after each purification step is in agreement with the report of Lukong *et al*. (2007) that for a purification procedure to be considered successful the specific activity of the desired enzyme must be greater than what it was before.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The findings of this study revealed that orange peel contains appreciable amounts of vital nutrients (protein 13.20%, carbohydrates 53.37%, fat 3.40%, ash 3.86% and moisture 26.08%) in quantities sufficient to support the growth of pectinolytic moulds.

Soil from the Botanical garden of Ahmadu Bello University, Zaria can serve as a good source of *Sclerotium rofsii* and *Aspergillus niger* for pectinase production studies and these two isolates possess the ability to produce extracellular pectinases when grown on pectin containing medium.

Sclerotium rolfsii had the highest zone of pectin hydrolysis (35mm) *followed* by *Aspergillus niger* (31mm) whereas, the lowest zone of pectin hydrolysis (9mm) was observed with *Fusarium* spp.

Pectinase production by *Sclerotium rofsii* and*Aspergillus niger*using orange peel as substrate under the solid state fermentation occurred on the fifth (5^{th}) and sixth (6^{th}) day respectively when the process was conducted at optimum fermentation conditions of 30°C, initial pH 6.0 and peptone supplementation giving overall enzyme yields of 108.54 ± 2.15 U/mL and 104.12 ± 0.53U/mL for*Sclerotium rofsii* and*Aspergillus niger* respectively. This shows that agricultural residues such as orange peel wastes could be a good substrate for the production of pectinase.

Purification of extracellular pectinases produced by *Sclerotium rofsii* and*Aspergillus niger* helps eliminate contaminating substances giving increased specific activity. Specific activity increased from 10.30 - 45.58 U/mg and from 5.12 - 32.58 U/mg for *Sclerotium rofsii* and *Aspergillus niger*respectively.

6.2Recommendations

i. The use of orange peel wastes should be encouraged for pectinase production because it is cheap, readily available and has the ability to yield considerably high amount of the enzyme.

ii. Further research and screening should be carried out on the use of other agro industrial wastes for their potentials to serve as substrates for solid state fermentation studies as regards to pectinase production.

iii. Further research and screening should be done to discover other pectinase producing strains and optimize other fermentation conditions to improve yield of the enzyme.

iv. The use of Orange peels and other agro wastes for enzyme production should be encouraged as it serves as a more economical and efficient form of waste management for environmental clean-up.

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APPENDICES

Appendix I: Contributions to Knowledge

1. This study revealed that orange peel contains appreciable amounts of vital nutrients required for pectinase production (protein 13.20%, carbohydrates 53.37%, fat 3.40%, ash 3.86% and moisture 26.08%).
2. This study revealed the presence of *Aspergillus niger, Monilia, Fusarium, Sclerotium rolfsii, Penicillium, Cladiosporium* and *Curvularia* species in the Botanical garden of Ahmadu Bello University, Zaria which are potential producers of pectinase.
3. *Sclerotium rofsii* and*Aspergillus niger* showed the highest (35mm and 31mm respectively) zones of pectin hydrolysis; which indicate the ability to produce extracellular pectinases when grown on pectin containing medium.
4. This study also revealed that the optimum fermentation conditions for pectinase production by *Sclerotium rofsii* in the solid state fermentation were 30°C, at initial pH of 6.0 and peptone supplementation on day 5 and for *Aspergillus niger,* it was 30°C, at initial pH 6.0 and peptone supplementation on day 6.
5. The study also revealed that *Sclerotium rofsii* and *Aspergillus niger* gave overall enzyme yields of 108.54 ± 2.15 U/mL and 104.12 ± 0.53U/mL respectively.
6. The study also revealed that purification of produced pectinase increases specific activity. Specific activity increased from 10.30 U/mg to 45.58 U/mg and from 5.12 U/mg to 32.58 U/mg for *Sclerotium rofsii* and *Aspergillus niger*respectively.

Appendix II: ANOVA tables for optimization

ANOVA for Incubation period on pectinase production by *Sclerotium rolfsii*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2432.601	6	405.4335	36.653	6.08E-05	3.865969
Within Groups	77.4298	7	11.0614			
Total	2510.031	13				

ANOVA for pH on pectinase production by *Sclerotium rolfsii*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8788.394	4	2197.099	234.6084	7.13E-06	5.1921678
Within Groups	46.8248	5	9.36496			
Total	8835.219	9				

ANOVA for Temperature on pectinase production by *Sclerotium rolfsii*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15300.02	4	3825.004	1137.04	1.4E-07	5.192168
Within Groups	16.82	5	3.364			
Total	15316.84	9				

ANOVA for Nutrients on pectinase production by *Sclerotium rolfsii*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8905.048	4	2226.262	210.5571	9.33E-06	5.192168
Within Groups	52.866	5	10.5732			
Total	8957.914	9				

ANOVA for Incubation period on pectinase production by *Aspergillus niger*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5848.798	6	974.7997	128.2342	8.49E-07	3.865969
Within Groups	53.212	7	7.601714			
Total	5902.01	13				

ANOVA for Incubation pH on pectinase production by *Aspergillus niger*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7766.841	4	1941.71	213.824	8.98E-06	5.192168
Within Groups	45.4044	5	9.08088			
Total	7812.246	9				

ANOVA for Temperature on pectinase production by *Aspergillus niger*

ANOVA

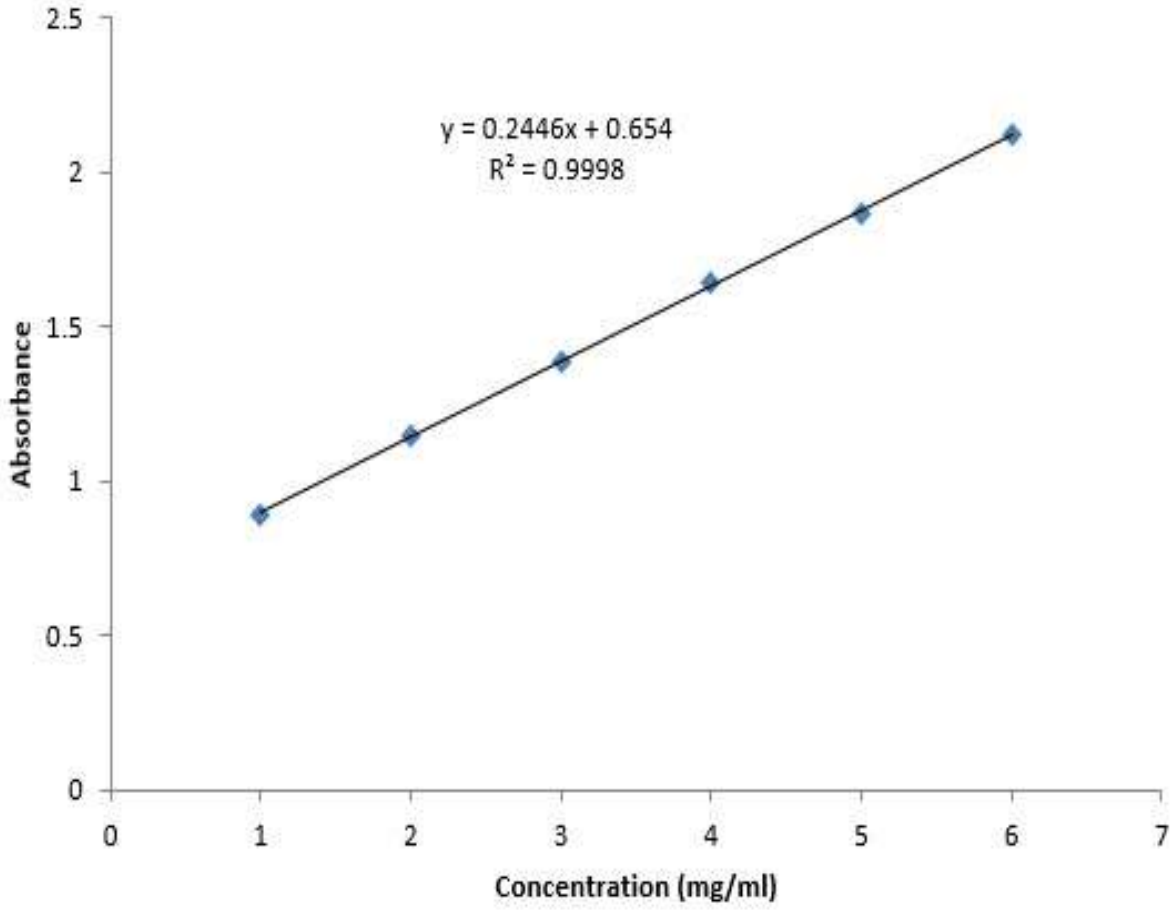
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13508.96	4	3377.239	551.4292	8.5E-07	5.192168
Within Groups	30.6226	5	6.12452			
Total	13539.58	9				

ANOVA for Nutrients on pectinase production by *Aspergillus niger*

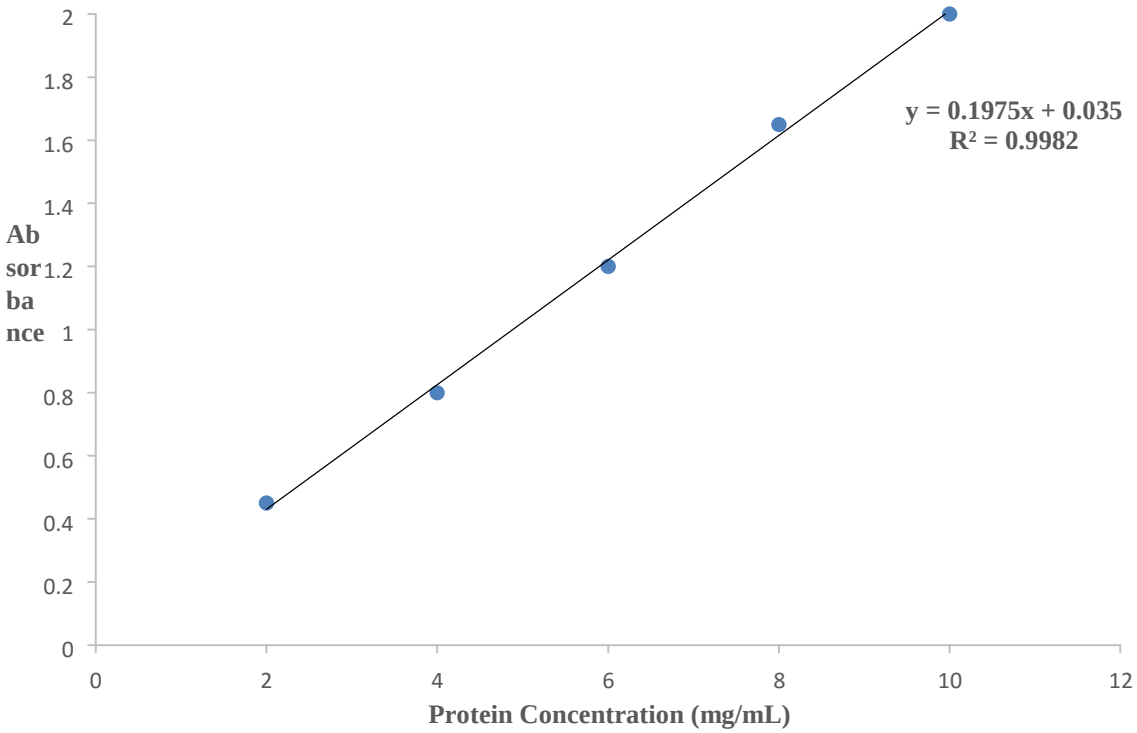
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6975.801	4	1743.95	87.016	8.27E-05	5.192168
Within Groups	100.2086	5	20.04172			
Total	7076.01	9				

Appendix III: Standard Curve of D-Galacturonic Acid



Appendix IV: Standard curve of Bovine Serum Albumin (BSA)



Appendix v:Orange peels collection

Appendix vi: Dried Orange peels

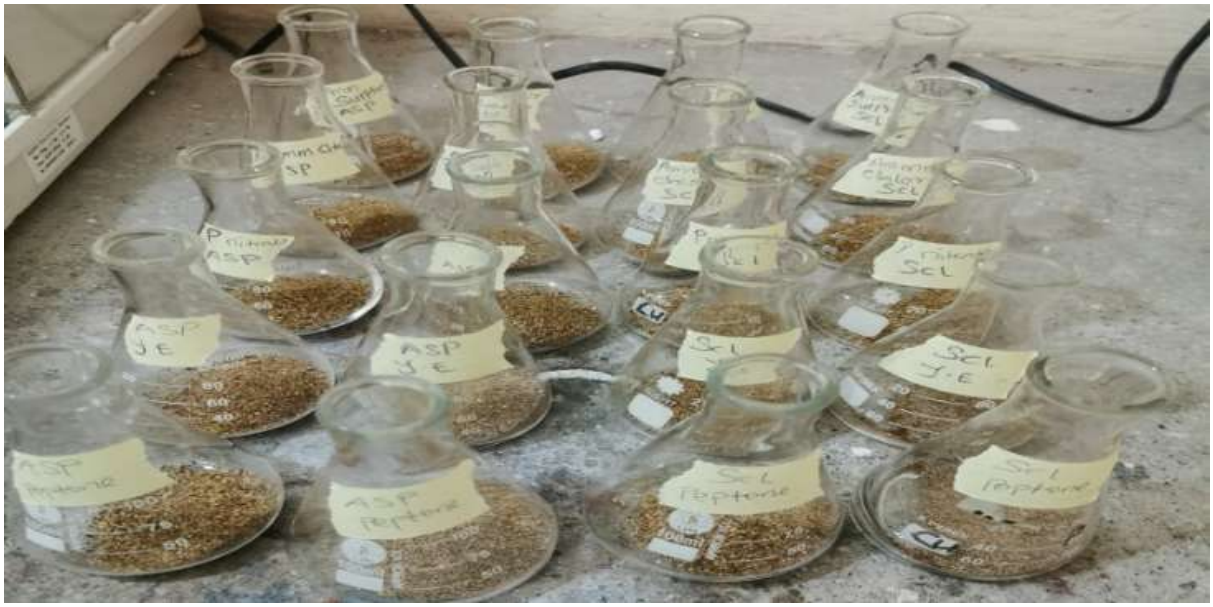
Appendix vii: Pulverized orange peel


Appendix viii: Proximate Analysis of Orange Peels


Appendix ix: Screening of Isolates for Pectinase Production

Appendix x: F: Assay for Pectinase Activity

Appendix xi: Orange Peel Powder



Appendix xii: Pectinase production by *Sclerotium rolfsii* and*Aspergillus niger*

Appendix xiii: *Sclerotium rolfsii*and *Aspergillus niger*

Sclerotium rolfsii

Aspergillus niger

Appendix xiv: Gel Filtration and Ion Exchange Chromatography