ISOLATION, PARTIAL PURIFICATION, CHARACTERIZATION AND ANTIFUNGAL POTENTIAL OF CHITINASE FROM SEEDS OF *SPHENOSTYLIS STENOCARPA* (AFRICAN YAM BEANS)

BY

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ABSTRACT

The utilization of chemical fungicide has attracted increased scrutiny since they cause environmental contamination and induce pathogen resistance. These limitations calls for harmless alternative control strategy to prevent plant diseases for sustainable agriculture. This work was designed to partially purify,characterize and tested for the antifungal potentials of chitinase from seeds of African Yam Beans. Chitinase (EC.3.2.14) was isolated from *Stephynostylis stenocarpa,* by precipitation using 80% Ammonium Sulphate Saturation and Gel filtration on Sephadex G-75 column to 8.04 fold with a yield of 46.35% and a final specific activity of 4.45µmol/min. SDS-PAGE of the enzyme showed a molecular weight of 32KDa. The enzyme had an optimal temperature and pH of 55^{o}C and 5.0 respectively. It had temperature and pH stability within the range of 30-60^{o}C and 4-7 respectively. Initial velocity studies for the determination of kinetic constants with colloidal chitin as substrate revealed a K_M and V_{max} of 1.2mMand 12.29µmol/min. The Chitinase activity was inhibited by Pb^{2+}, Hg^{2+}, K^{+} and other reagents like EDTA and glucose but Na^{+} and Zn^{2+} showed little or no effect on the enzyme activity. The antifungal potentials of this Chitinase was tested against *Fusarium oxysponium, Fusarium solani,* and *Alterneria alternate.* It had no effect on the *Fusarium* species but inhibited the growth of *Alterneria alternate* as seen from clear Zones of inhibition (14.00mm-17.00mm) for the crude extract and (9.00mm-11.00mm) for the purified using agar diffusion method. This indicates that the enzyme can be used to combat such diseases triggered by the organism e.g. leaf blight, hence servingas a tool for crop protection in the agricultural sector.

CHAPTER ONE

1.0 INTRODUCTION

Plants like any other living organism are exposed to many pathogens including fungi and bacteria during their growth period. They do not possess an immune system, thus are vulnerable to pathogens e.g. fungal and bacterial infection. This can result in serious damage to plants such as legumes (Wang *et al.,* 2012). However, a series of defense compounds are synthesized by plants to protect themselves from pathogenic organisms. One class of these defense compounds is antifungal proteins. To date, many different types of plant antifungal proteins are known and they include glucanases, chitinases, protease inhibitors, thaumatin-like protein, miraculin-like proteins, Cyclophilin-like proteins, allergen-like proteins. Sometimes a combination of antifungal proteins are found in a single species (Xiangli *et al.,* 2011).

Chitinases (EC 3.2.1.14) are a class of antifungal proteins which are of particular interest in studies of defense compounds owing to their resistance both against predators such as insects as well as pathogens such as fungi (Wang *et al.,* 2012). The natural substrate of Chitinases, chitin, is the second most abundant polysaccharide in nature next to cellulose. Chitinases are enzymes that catalyze the hydrolysis of chitin, a linear homopolymer of β-1, 4-linked N-acetyl glucosamine (GlcNAc) residues (Zhang *et al.,* 2013). The types of Chitinase that are most extensively studied are classified into two: exochitinase and endochitinase based on mode of action. The endochitinase, randomly hydrolyze internal β-1,4-linkages of chitin to release N,N′ diacetyl-chitobiose and cut them into shorter segments Wang *et al.,* (2012) and the exochitinase which release chitobiose from chitin. They catalyze the hydrolysis of terminal 1, 4-β-linkage of N-acetyl-D-glucosamine (GlcNAc) polymers of chitin and chitodextrin. They cleave off two subunits from reducing or non- reducing ends of the chitin chain. Chitinases are further divided

into seven classes of classes I through VII, distinguished by their amino acid sequences, structure and mechanism (Wang *et al.,* 2008). However, most leguminous Chitinases belong to classes I through IV. Information pertaining to other three classes is lacking. During the previous decades, Chitinases have received increased attention because of their wide range of applications. Practical applications of Chitinases include use in the preparation of protoplasts from fungi, as a protective agent against plant-pathogenic fungi (Wang *et al.,* 2009; Wang *et al.,* 2007; Ye and Ng, 2005 and Taira *et al.,* 2005).

Chitin is a major component of the exoskeleton of insects and of the cell wall of most fungi (the Ascomycetes, Basidiomycetes and Deuteromycetes). In fungi, chitin constitutes 3-60%of the cell wall (David *et al.,* 1993**).** Chitin is a β-1,4-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc) that is widely distributed in the green algae, fungi, protozoan, crustaceans, molluscs and coelenterates (Konagaya *et al..,* 2006). It is often present in fungi hyphae as the main component of fungal cell wall but is absent in plants. Although plants lack endogenous chitin, they do express Chitinase. The function of Chitinase in plants appears to be a defense against attack by chitin containing fungal pathogens and insect pests. Chitinases, together with β-1, 3-glucanases, break down fungal cell, thereby inhibiting fungal growth (Collinge *et al.,* 1993). Some plants containing Chitinase includes some varieties of kidney beans such as French beans, Anasazi beans, Pinto beans, Haricot beans, Mung beans, Pine apple, tomatoes, Guava (Kumar *et al.,* 2013).

The African yam bean (*Sphenostylis stenocarpa*) also known as Okpodudu in Igbo, Sese sheshe in Yoruba and Bitei in Obudu is a perennial climbing plant, 1-3 m high and is generally grown as an annual crop. Its leaves are trifoliate with oval leaflets (2.7 to 13 cm long and 0.2 to 5.5 cm broad). *Sphenostylis stenocarpa* is cultivated for its edible tubers, which look like elongated

sweet potatoes, and for its seeds, which are enclosed in hard and tough, 20-30cm long pods. It is mainly used as food but can be used as animal feed (Heuze and Tran, 2013).

1.1 Statement of Research Problems

Plants are exposed to a large number of pathogenic fungi, but they do not possess an immune system, thus they are vulnerable to pathogenic fungal and bacterial infection which can result in serious damage to plants such as legumes, therefore there is need to synthesize antifungal proteins/peptides to combat fungal pathogen attack (Wang *et al.*, 2012).

A large number of environmental issues are linked with the eradication of plant diseases with chemical compounds. This has raised a lot of public concern over the harmful effects of these chemical compounds on the environment and human health thus the need to search for safer, environment friendly control alternatives or strategies (Sharma and Giordanengo, 2011).

One of those strategies is to control pest and pathogen populations by inhibiting their growth and development via the use of antifungal biologically active compounds in place of these chemical compounds (Saguez*et al.*, 2008).

1.2 Justification

Bio-control agents are biodegradable unlike chemical insecticides that can act as food contaminants which are harmful to humans thus the use of safe biological agent will reduce the large number of environmental issues which are linked with the eradication of plant diseases with chemical compounds (Sharma *et al.*, 2011).

Chitinase from plant serve as a better bio-control agent against plant fungi whereas those from microorganism serves as a better bio-control agent against soil and marine fungi. Since plants do

not possess immune system, they can be considered as a tool to strengthen their response against variety of pathogens (Sharma *et al*., 2011).

There is need to isolate Chitinase with a broad spectrum which is able to inhibit growth of many fungal species by causing lysis of the hyphal tip which occurs presumably through the hydrolysis of chitin in fungal cell wall (Zhang *et al.,* 2013).

1.3 Aim and Objectives

1.3.1 **Aim:**

This research aims at isolation, partial purification, characterization of the chitinase from seeds of ***Sphenostylis stenocarpa*** (African yam beans) with a view to determine its antifungal potentials.

1.3.2 Specific objectives are:

1. To Isolate and partially purify the chitinase from ***Sphenostylis stenocarpa***.
2. To characterize the purified chitinase.
3. To test for the antifungal potential of the crude and purified chitinase.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of African Yam Beans

The African Yam bean is a vigorous, herbaceous, climbing vine, reaching 1-3m in height, with trifoliate leaves, the leaflets being up to 14 cm in length and 5cm broad. The conspicuous flowers are mauvish-pink, purple or greenish-white in color, about 2.5cm in length and borne on stout auxiliary peduncles. The glabrous seed pods are linear, flat, with both margins raised, 25-30cm long and 1-1.5cm broad, containing 20-30seeds which may be ellipsoid, rounded or truncated, and show considerable variation in size and color; the largest are usually about I cm long and 0.7 cm wide. Seed color may vary from creamy-white or brownish-yellow to dark brown, sometimes with black marbling, and there appear to be a number of 'types' according to seed color. The plant produces small spindle-shaped tubers, about 5-7.5cm long. There is some evidence that yields of seeds and tubers are inversely related (Heuze and Tran, 2013).

Plate III: African Yam Beans Plant (*Sphenostylis stenocarpa*)

2.1.1 Distribution of African Yam Beans

Sphenostylis stenocarpa is native to tropical west and central Africa and is cultivated in southern and eastern Africa (Ecoport, 2009). Both wild and cultivated types now occur in tropical Africa as far south as Zimbabwe, throughout West Africa from Guinea to southern Nigeria. It thrives on deep, loose sandy and loamy soils with good organic content and good drainage. It grows better in regions where annual rainfalls range between 800-1400mm and where temperatures are between 19-27°C (Ecoport, 2009). The plant flowers after 90days and the pods mature in 140 to 210days. The tubers are ready to harvest 150 to 240days after sowing.

Plate I: Seeds of African Yam Beans (*Sphenostylis stenocarpa*)

PLATE II: Tubers of African Yam Beans (*Sphenostylis stenocarpa*)

2.1.2 Cultivation conditions for African Yam Beans

Small-scale cultivation is practiced throughout tropical Africa. The plant is especially suited to lowland conditions, though it can be grown up to 1800 m above sea level. Climates ranging from savannah to rainforest are tolerated provided there is a combination of adequate rainfall (100cm or more during the growing season) and reasonably good drainage. It is often planted along with yams and beans, using the same stakes as the yam for support, though sometimes left to trail on the ground. It is sometimes stated that the plants perform better when inter planted than when grown alone (Akinmutimi *et al.,* 2006).

2.1.3 Pests and diseases

Fungal diseases that are known are powdery mildew due to *Oidium sp*., which is parasitised by *Cincinnoboluscesati*, leaf spot caused by *Phoma sp*. and stem rust caused by *Aecidium sp.* Virus

mosaics have also been reported. Pests have not been defined in detail but include Orthopterous and Lepidopterous insects. Leaf rolling caterpillars and leaf miners have been described as causing serious damage to the foliage, and damage the flowers. Nematodes may attack the root system leading to reduction in yield.

2.1.4 Potential constraints in usage of seeds

The seeds contain tannins, trypsin inhibitors, hydrogen cyanide, saponins and phytic acid (Akinmutimi *et al.,* 2006). Processes such as heating, soaking or fermenting can be used to decrease antinutritional factors and improve the nutritional value of *Sphenostylis stenocarpa* products and its by-products (Onyeike *et al.,* 1995).

2.1.5 Nutritional attributes

The proximate analysis, determination of nutritionally valuable minerals and the functional properties of the seed flour of African yam bean (*Sphenostylis stenocarpa*) had been investigated. Three different colour varieties of whole seeds and their cotyledons from the same source were identified and processed for the study. The average composition of the seeds (whole grains) was as follows: protein 20.51%, fat 12.20%, carbohydrate 50.24%, ash 2.60%, fibre 6.00% and moisture 8.36%. The cotyledons contained protein 23.93%, fat 3.65%, carbohydrate 62.40%, ash 2.25%, fibre 2.07% and moisture 5.62%. The seeds are rich in potassium and phosphorus (625.43 mg/100g) and (206.35 mg/100g) respectively for whole grains while values for cotyledons were (553.6 mg/100g) and (234.161 mg/100mg) respectively. The functional properties, that is, protein solubility (PS), water absorption capacity (WAC), fat absorption capacity (FAC), fat emulsion stability (FES), lowest gelation concentration (LGC), foaming capacity (FC) and foaming stability (FS) were investigated. Heat treatment (for the preparation of cotyledons) reduced the protein solubility, fat emulsion capacity, foaming capacity, foaming

stability and lowest gelation concentration while water absorption capacity, fat absorption capacity and fat emulsion stability were slightly increased (Adeyeye *et al.,* 1994).

2.2 Chitin $(C_8H_{13}O_5N)_n$

Chitin, a linear polymer of β-1, 4-N-acetylglucosamine (GlcNAC), is the second most abundant biopolymer on the planet (Shahidi and Abozaytoun, 2005). Chitin is found in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, and lobsters, and in the internal structures of other invertebrates (Bhattachrya *et al.,* 2007). The overall weight of shellfish (e.g. crab, krill and shrimp), which is disposed as waste, is approximately 75%, and chitin consists of 20-58% of that dry weight (Wang and Chang, 1997). Amid a broad array of applications, chitin has its use in order to boost up the formation of extracellular chitinase. Chitin and its associated materials have a broad usage in drug delivery, wound healing, dietary fiber, and in waste water treatment (Muzzarelli, 1999). Chitin is a white, hard, inelastic polysaccharide, and is a major contribution to pollution in coastal areas (Zikakis, 1984). Chitin has a high percentage of nitrogen (6.89%), which makes it a useful chelating agent (Muzzarelli, 1997). Chitin exists in 2 allomorphic forms i.e. α-chitin and β-chitin. These 2 forms of chitin vary in packing and polarities of adjacent chains in the succeeding sheets (Bussink *et al.,* 2007). Chitin can be degraded by chitinase. The catabolism of chitin takes place in 2 steps, involving the initial cleavage of the chitin polymer by chitinase into chitin oligosaccharides and further cleavage to N-acetyl glucosamine, and monosaccharides by chitobiases (Chen *et al.,* 2010).

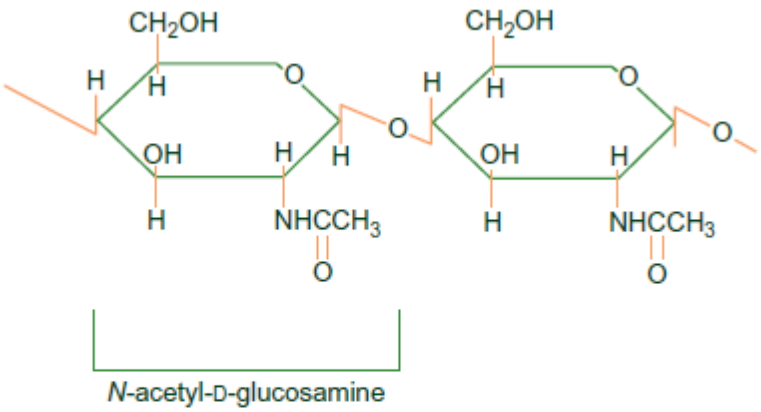

FIGURE 2.1 Chemical structure of chitin showing its monomer: *N*-acetyl-D-glucosamine.

Chitin is a modified polysaccharide that contains nitrogen; it is synthesized from units of *N*-acetyl glucosamine (2-acetylamino-2-deoxy-D-glucose). These units form β-1, 4 glycosidic linkages. Chitin may be described as cellulose with one hydroxyl group on each monomer replaced with an acetyl amine group (Hofmann, 2008). This allows for increased hydrogen bonding between adjacent polymers, giving the chitin-polymer matrix increased strength. .In its pure form, chitin is translucent, pliable, resilient, and quite tough. In arthropods, however, it is often modified, becoming embedded in sclerotin, a tanned proteinaceous matrix, which forms much of the exoskeleton. In its pure form, chitin is pliable and leathery in texture, but in most invertebrates it occurs largely as a component of composite materials. Combined with calcium carbonate, as in the shells of crustaceans and molluscs, chitin produces a much stronger composite. This composite material is much harder and stiffer than pure chitin, and is tougher and less brittle than pure calcium carbonate (Campbell, 1996). Another difference between pure and composite forms can be seen by comparing the flexible body wall between the segments of

a caterpillar (mainly chitin) to the stiff, light elytron of a beetle (containing a large proportion of sclerotin) (Lawrence, 2009).

2.2.1 Uses of chitin

2.2.1.1 Use in Industrial

Chitin is used in industry in many processes. Examples of the potential uses of chemically modified chitin in food processing include the formation of edible films and as an additive to thicken and stabilize foods (Shahidi *et al*; 1999) and pharmaceuticals. It also acts as a binder in dyes, fabrics, and adhesives. Industrial separation membranes and ion-exchange media can be made from chitin. Processes that involves paper strengthening employ the use of Chitin and Chitosan (Gaellstedt*et al;* 2005). Researchers have developed a method for using Chitosan as a reproducible form of biodegradable plastic and as a promising substrate for engineering human tissues by use of three-dimensional bioprinting (Lee *et al;* 2013).

2.2.1.2 Use in Medicine

Chitin's properties as a flexible and strong material make it favorable as surgical thread. Its biodegradability means it wears away with time as the wound heals. Moreover, chitin has been reported to have some unusual properties that accelerate healing of wounds in humans (Fabrizi *et al.,* 2012). Occupations associated with high environmental chitin levels, such as shellfish processors, are prone to high incidences of asthma. Recent studies have suggested that chitin may play a role in a possible pathway in human allergic disease. For example, mice treated with chitin develop an allergic response, characterized by a build-up of interleukin-4-expressing innate immune cells. In these treated mice, additional treatment with a Chitinase enzyme abolishes the response (Tiffany *et al;* 2007).

2.3 Chitinase

Chitinase was described for the first time in 1911 by Bernard in orchid bulbs in which it behaves like a thermosensitive and diffusible antifungal factor. In animals the presence of Chitinase was marked in snails by Flach *et al.,* (1992). Since then these molecules are unanimously considered as a tool to strengthen plant immune response against a variety of pathogens by various workers owing to its property to lyse fungal cell wall and components of insect exoskeleton. Besides this, dramatic increase in Chitinase levels by numerous abiotic agents (ethylene, salicylic acid, salt solutions, ozone, UV light) and by biotic factors (fungi, bacteria, viruses, viroid, fungal cell wall components and oligosaccharides) also proved their role in plant defense response (Punja and Zhang, 1993; Gupta *et al.,* 2010b).

Chitinases catalyses the hydrolysis of chitin, a linear homopolymer of β-1, 4-linked N-acetyl glucosamine (GlcNAc) residues. The types of Chitinase most extensively studied in plants are endochitinase (EC 3.2.1.14) which randomly hydrolyze internal β-1, 4-linkages of chitin-releasing oligosaccharides of GlcNAc (Boller *et al.,* 1983). In addition, exochitinase activity measured by the release of GlcNAc has been reported from a number of plants (Wadsworth and Zikakis, 1984). Many purified plant endochitinase also show some degree of lysozyme activity (EC 3.2.1.17), i.e. they can hydrolyse β-1, 4-linkages between N-acetyl muramic acid and GlcNAc residues in peptidoglycan (Majeau *et al.,* 1990). Likewise, most plant lysozymes show a high level of chitinase activity (Audy *et al.,* 1988). Very little attention has been given to the possibility that plant chitinase may also be able to catalyse trans glycosylation reactions as recently shown for two fungal chitinases and hen egg lysozyme (Usui *et al.,* 1987, 1990). A unique protein with both a-amylase inhibitor activity (against a locust enzyme) and endochitinase activity has been purified from the grass Job's tears *(Coixlachrymajobi)* (Ary *et al.,* 1989).A

number of assays can be applied to measure both endo- and exochitinase enzyme activity. These include colorimetric, radiochemical and gel electrophoresis based assays (Boller *et al.,* 1983).

2.3.1 Classification and Structure of Chitinases

Three classes of plant chitinase have been proposed based on the primary structure (Shinshi *et al.,* 1990**). C**lass I chitinase are enzymes with a N-terminal cysteine-rich domain of approximately 40amino acids and a highly conserved main structure, separated by a variable hinge region (Figure 1**).** Class II chitinase lack the N-terminal cysteine-rich domain but have a high amino acid sequence identity to the main structure of class 1 chitinase. Class 111 chitinase show no sequence similarity to enzymes in class I or II

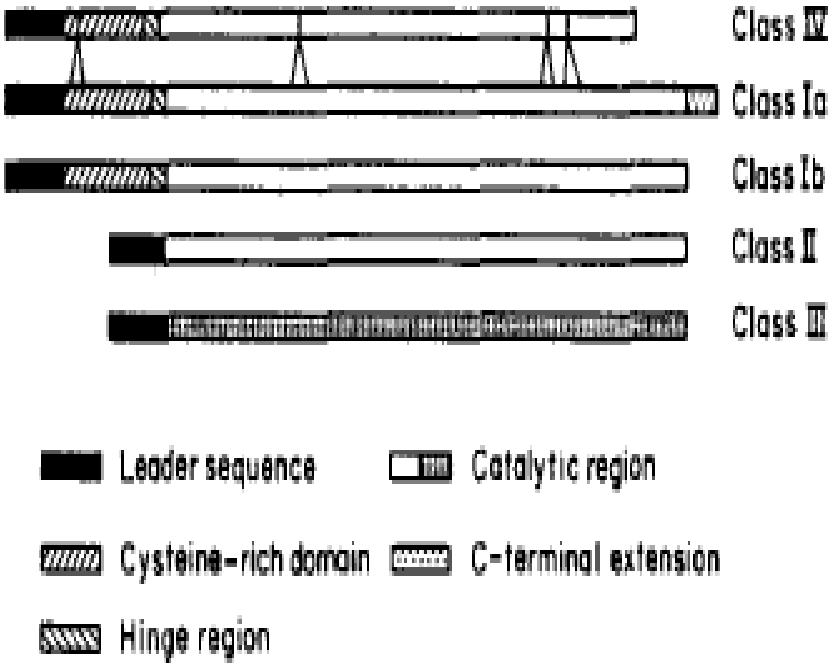

Figure 1. The structure of chitinase proteins.

Chitinase attack chitin molecules which are the main structural component in fungal cell wall and insect's skeleton. In nature chitin is found to be in the form of complex with other biomolecules such as carbohydrates and proteins. In Sponges it forms a complex with silica (Ehrlich *et al.,* 2007). In arthropods, chitin is an integral part of skeleton (Merzendorfer and Zimoch, 2003) and digestive tract lining. It is also known to be present in the eggshell (Mansfield *et al.,* 1992) and microfilarial sheath of nematodes.

In these organisms chitin metabolism is directly controlled by the activity of Chitin Synthases (CS) and Chitin Hydrolase (CH). Three types of chitin synthase were observed in *Saccharomyces cerevisiae:* CSI which is involved in repair functions at the end of cytokinesis; CS II, participating in the synthesis of primary septum and CS III, responsible for the formation of the ring or bud scar (Henar *et al.,* 1999). Besides these enzymes, the recycling of cell wall components also depends upon the activities of a range of hydrolases characterized belong to chitinase, glucanase and transglycosylase family (Adams, 2004). It is generally believed that Chitinases are commonly found in organisms which are possessing chitin as a structural component.

The enzyme Chitinase (EC 3.2, 1.14) hydrolyzes the chitin polymer into to N-acetyl glucosamine by either endo or exocleavages of the 1-3 and 1-4 bond (Van Aalten *et al.,* 2000). The enzyme is classified into several categories on the basis of their isolation, structural and functional characteristics. It belongs to families 18 and 19 of glycosyl hydrolases (Davies, 1997) which are key enzymes for carbohydrate metabolism. Most of the prokaryotic and eukaryotic Chitinases are grouped in family 18 whereas Chitinase of higher plants and some of the Gram positive bacteria like *Streptomyces* are included into Family 19 (Cohen-Kupeic and Chet, 1998). These two families contain both endo and exochitinase. Endochitinase cleave randomly in the chitin

chain while exochitinase cleave off chitobiose $(GlcNAc)_2$ or chitotriose $(GlcNAc)_3$ from the reducing or non-reducing end of the chitin chain (Suzuki *et al.,* 1999). In addition to endo- and exochitinase, chitin degrading organisms contain chitobiose (N-acetyl β-glucosaminidases), a third class of chitinolytic enzymes that convert GlcNAc dimmers into their monomers.

2.3.2 Source of Chitinase

Chitinolytic microbes occur widely in nature and are preferred source of Chitinase because of their low production cost, easy availability of raw materials for their cultivation. The ability of a microbial community to degrade chitin is also important for the recycling of nitrogen in the soil (Chandran*et al.,* 2007). Bacteria like *Serratiamarcescens, Xanthomonasmaltophilia, Stenotrophomonasmallophilia* and *Paenibacillusillinoisensi* have been proved as potent chitinolytic bacterial biocontrol agents while *Myrothecium verrucavia,* and *Trichodera*sp. (Howell, 2003) were found as main source of Chitinase among fungi. In insects and nematodes chitinase were found to be involved in molting process during their development (Adam *et al.,* 1996). Chitinases were also reported in gastric juices of human being (Paoletti *et al.,* 2007) where they were being thought to be involved in catabolic activities. Further Chitinase activity was also detected in human serum and found very similar to plant Chitinases that are related in the process of inflammation and pathogen resistance (Chupp *et al.,* 2007).

2.3.3 Chitinase in Plant Defense

The exploitation of Chitinase with respect to plant defense can be done by a variety of ways. The enzyme can be used in free or immobilized form to kill fungi and insects in affected areas. The microorganisms producing Chitinase can also be used in soil as rhizobacterial population or alternatively the gene encoding Chitinase can be inserted in the native microflora of soil.

Chitinase of *Serratiamarcescens* was inserted into *Pseudomonas fluoresces* which is normally present as normal flora of soil and obtained resistance in radish plants against *Fusarium oxysporum infection (*Chernin *et al.,* 1997). They were also able to decrease the onset of *Rhizoctonia solani* infection in cotton plants by using recombinant *E.coli* in rhizosphere expressing Chitinase gene of *Enterobacter agglgmerans.*

Kirubakaran and Sakthive, (2007) also demonstrated a broad-spectrum antifungal activity in *Escherichia coli* expressing chitinase gene of barley against *Botrytis cinerea* (Blight of Tobacco), *Pestalotiatheae* (Leaf Spot of Tea), *Bipolarisoryzae* (Brown Spot of Rice), *Alternaria sp.* (Grain Discoloration of rice), *Curvularialunata* (Leaf Spot of Clover) and *Rhizoctonia solani* (Sheath Blight of Rice).

Besides these, the phenomena of synergism (the interaction of organisms or proteins or elements that when combined, produce a total effect that is greater than the sum of the individual element contributions) can be meaningful to fasten the killing of pathogenic organism.

Lorito *et al.,* (1993a) demonstrated that chitinolytic enzymes from *Trichoderma harzianumrifai* and the closely related fungus *Gliocladiumvirens* act synergistically to inhibit the growth of a variety of plant pathogenic fungi. Lorito *et al.,* (1993b) also demonstrated that bacterial biocontrol strains may also act synergistically with chitinolytic enzymes to inhibit plant pathogenic fungi. They determined strong synergism between Enterobacter cloacae and chitinolytic enzymes of *Trichoderma harzianum* and found that chitinolytic enzymes enhance the bacterial growth and their ability to bind to the fungal hyphae (Mauch *et al.,* 1988) observed rapid killing of fungal cells by combining chitinases with β-glucanases. In addition to above

synergistic effects of a β-1, 6-glucancase and chitinase from *Trichoderma harzianum* on the hydrolysis of fungal cell walls had also been reported (Misra and Gupta, 2009).

Alternatively, the production of transgenic plants over expressing chitinase gene had also been demonstrated to get resistance against pathogens. It was achieved by manipulating the activity of extracellular enzymes through construction of over producing mutants, enzyme negative mutants or even transgenic plants expressing the enzyme (Brogue *et al.,* 1991). It showed an increased ability to survive in tobacco seedlings by expressing chitinase. It was also used to confirm the reduction in occurrence of *Rhizoctonia solani* infection in transgenic tobacco plants in which two bacterial chitinase gene were over expressed at high levels. Besides the immunity against fungal pathogen, overexpression of chitinase was also found effective to raise resistance plants against bacterial pathogens, salinity stress and heavy metals stress (Dana *et al.,* 2006).

It is not only the microbial Chitinase but also the plant Chitinase that had been used to improve plant health by various workers. In oil seed rape (*Brassica napus*var.oleifera) the importance of Chitinase was also shown by various researchers. Grison *et al.,* (1996) were able to increase tolerance in these plants against *cylindrosp; oriumconcentricum, Phoma lingam*, *Sclerotinia sclerotiorum* infection by expressing Chitinase gene. Transgenic wheat plants expressing Chitinase of plants were also raised. (Oldach *et al.,* 2001). In another experiment the transgenic wheat lines carrying a combination of a wheat β-1, 3-glucanase and Chitinase exhibited delayed symptoms of *Fusarium* Head Blight (Anand *et al.,* 2003).

Chitinases were also used as a method to control insect and pest population which indirectly suggests its role in plant defense. Lawrence and Novak (2006) showed that the expression of poplar Chitinase in tomato led to inhibition of development in Colorado potato beetle. Lipmann

et al., (2009) investigated the secretome of a tobacco cell suspension by a combined proteomic and metabolomics approach and identified Chitinase along with peroxidase and beta-1,4,-xylosidase among the major defense protein. Wasano *et al.,* (2009) observed that the presence of 56-kDa defense protein consisting of chitin like domain in mulberry latex was responsible to provide strong insect resistance to lepidopteran caterpillars, including the cabbage armyworm, *Mamesrabrassicae* and the Eri silkworm, *Samiarieini*. Similarly, Kitajima *et al.,* (2010) also reported two Chitinase-like proteins LA-a and LA-b (latex abundant) from the latex of mulberry (*Morus sp*) and found them associated with insecticidal activities against larvae of Drosophila melanogaster.

2.3.4 Role in Agriculture

Chitinases may be used to convert chitin-containing biomass into useful (depolymerized) components. Chitinases can be exploited for their use in control of fungal and insect pathogens of plants (Hamid *et al.,* 2013). Fungal protoplasts have been exploited as a very efficient experimental means to study the synthesis of cell wall, enzyme synthesis and secretion and strain improvement for biotechnological applications (Hamid *et al.,* 2013). Chitinase activity also acts as an indicator showing the activity of fungi in soil. It has been reported that there is a strong relationship between chitinase activity and fungal population in the soil. Therefore, it appears that chitinase activity acts as a suitable indicator of the actively growing fungi in the soil .Miller *et al*.,(1998) by making use of specific methylumbelliferyl substrates reported the correlation of chitinase activity with the content of fungus-specific indicator molecules 18:2ωb phospholipid fatty acid and ergosterol.

2.3.5 Medicinal Functions

Chitooligosaccharides have an enormous pharmaceutical potential. They are involved in root nodule formation, act as elicitors of plant defense and also have a potential to be used in human medicines (e.g., anti-tumor activity is shown by chitohexaose and chitoheptaose). It was reported by Murao *et al*., (1999) that chitotriose from colloidal chitin have been prepared using a chitinase from *Vibrio alginolyticus*. Kobayashi *et al*., (1997) have reported the use of *Bacillus* chitinase for the production of chitobiose by combining GlcNAc and a sugar oxazoline derivative. GlcNAc itself is an anti-inflammatory drug, and in the human body, it is synthesized from glucose, then incorporated into glycoproteins and glycosaminoglycans. The GlcNAc administered by oral routes, intravenous (IV), and intramuscular (IM) has been reported to be effective as an anti-inflammatory drug, useful in the treatment of ulcerative colitis and other gastrointestinal inflammation disorders (Aloise *et al.,* 1996). Horsch *et al*., (1997) recommended that N-acetyl hexosaminidase can be explored for its use as a target for designing antifungals with low molecular weight. According to Laine and Lo, (1996), chitin and chitin binding proteins can be explored for the recognition of fungal infections in humans.

Chitinases have a significant function in human health care. An important medical use for chitinase has also been recommended in augmenting the activity of anti-fungal drugs in therapy for fungal diseases (Orunsi and Trinci, 1985). Due to their topical applications, they have a prospective use in anti-fungal creams and lotions. A number of artificial medical articles such as contact lenses, artificial skin, and surgical stitches have been formed from chitin derivatives. These derivatives have an extensive medical use because they are known to be non-toxic, non-allergic, biocompatible, and biodegradable (Muzzarelli, 1997).

2.4 Pathogenic Fungi

2.4.1 *Alterneria alternate*

Strains of *A. alternata* include some of the most destructive plant pathogens that affect a wide range of host plants, causing leaf spots, blights, blossom rots, and fruit rots. More than 380 hosts, including lilacs, have been recorded in the USDA Systematic Botany and Mycology Fungus-Host Distribution. Some isolates of *A. alternata* cause severe diseases in different ornamental crops, including trees and shrubs (Jones and Benson; 2001; Pryor and Michailides, 2002). However, only a few studies have been conducted on trees and shrubs (Sinclair *et al.*, 1987). The leaf-blight disease caused by *A. alternata* was first observed in 1996, and it was reported as one of the most serious diseases of lilac in middle Tennessee, USA (Mmbaga and Sheng, 1997; Mmbaga *et al.*, 2005). Out of fifty six cultivars within ten Syringa species evaluated in field environment, only ten cultivars were consistently resistant to this disease (Mmbaga *et al.*, 2005). Members of the genus *Alterneria alternate* frequently cause quiescent infections in which the fungus enters the tissue and remains dormant until changes in environmental conditions favor disease. *Alterneria* blight symptom development in lilac is consistent with that from quiescent infections. This disease has remained a persistent problem over multiple years, and healthy-looking plants can suddenly develop disease in late June to early July resulting in severe defoliations. The mode of infection are typical of plant pathogens that persist between seasons on infected plants, with symptoms starting on the upper or middle part of susceptible plants and subsequently spreading over the entire plant (Mmbaga *et al.*, 2005). Infections by *Alterneria* species typically cause the formation of necrotic lesions, which sometimes have a target-like appearance surrounded by an un-invaded chlorotic halo. This halo is created by the diffusion of fungal metabolites, which act as toxins (Agarwal *et al.*, 1997).

2. 4. 2 Mycotoxin Produced by *Alterneria alternata*

A. alternata produces a number of mycotoxins, including alternariol (AOD), alternariol monomethyl ether (AME), altertoxin I and tenuazonic acid (TeA). Extensive studies on *A. alternata* growth and mycotoxin production were carried out on wheat matrices (Megan *et al.*, 1984). Studies of *A. alternata* strains and production of AOH and AME on irradiated soya beans were done by Oviedo *et al.,* (2010). They found that optima for AOH varied with strains but were generally at around 25℃ and 0.98 a_w with minima around 0.92 a_w over the temperature range tested (15-30℃). For AME the production by two strains of *A. alternata* was more consistent with optima around 25℃ and 0.98 a_w, with a_w minima across the whole temperature range (15-30℃) of around 0.96 a_w. Interestingly, AME was not produced at > 30℃, while AOH was. Profiles in relation to the production of TeA have also been developed for *A. alternata* strains from soya bean-based media (Oviedo *et al.,* 2009). The profiles for TeA suggest that optimum concentrations are produced at 0.98 a_w and 25-30℃. This could be due to ecological differences or regional differences between strains. (Magan and Baxter, 1994) found that strains of *A. alternata* isolated from sorghum had different production patterns for TeA. TeA was produced over the range 0.99-0.93a_w over 28days growth periods. Even at 0.93 a_w TeA was produced with most biosynthesis occurring after 28 days, reaching levels similar to that at other more conducive a_w levels (< 0.95 a_w).

2.4.2.1 Harmful Effect of *Alternaria* Mycotoxins

Acute toxicity: Experimentally, when sodium tenuazonate (TeA salt) was administered to mice and rats, oral LD_{50} values were reported to be 81 and 186mg/kg in male and female mice, respectively and 168 and 180 mg/kg in male and female rats, respectively (Pero *et al.,* 1973).

Repeat dose toxicity: Airborne *Alternaria* allergens were detected in six mechanically ventilated air-conditioned non-industrial buildings in Montreal, Canada. *Alternaria* allergens were identified in the offices and in ventilation systems supplying the offices and of 214 workers, half reported frequent work-related respiratory symptoms. All workers underwent skin prick allergy testing for *Alternaria* allergens and those that had positive reactions reported significantly more respiratory symptoms than those that had negative skin reactions (Menzies *et al.,* 1998). *Alternaria* spores at natural exposure ranges have also been implicated in the pathogenesis of asthma. Seven mild asthma patients were bronchial challenged with whole spores or spore extracts, which resulted in immediate-type asthma during challenge with spore extract, while whole spores induced delayed type asthma only (Licorigh *et al.,* 1985).

Carcinogenicity and mutagenicity: *Alternaria* species have been found to have a role in the development of oesophageal cancer. AOH and AME have been isolated from *A. alternata* species which was the main contaminating fungi from grain ingested in by the population of Linxian County in China, which additionally exhibits a high incidence of oesophageal cancer (Liu *et al.,* 1991). Extracts of *A. alternata* and also food mildewed by the extracts are tumorigenic in nude mice and are able to induce stomach tumours in rats. Using *in vitro* studies, extracts of *A. alternata* induced reverse mutation in *Escherichia coli*, unscheduled DNA synthesis in cultured human amnion FL cells, chromosomal aberrations and sister chromatid exchange in human peripheral blood lymphocytes, mutation in V79 cells and transformation of NIH 3T3 cells in *in vitro* studies (Brugger *et al.,* 2006). This experimental study data showed *A. alternata* to be tumorigenic and taken together with findings from human epidemiology studies in the Linxian County of China led the authors to conclude that *A. alternata* is one of the causes of human oesophageal cancer (Liu *et al.,* 1991).

Reproductive and developmental toxicity: Experimentally, different doses of *Alternaria* species were administered to pregnant DBA/2 mice during gestation. The combined administration of AOH and AME (25 mg/kg each given together) on gestation days 9-12 led to an increased number of dead and resorbed foetuses, and runts per litter; an increase in the number of malformed foetuses was also reported. Administration of AOH at 100mg/kg on gestation days 13-16, resulted in an increase in malformed foetuses. However, administration of AME at 50 mg/kg on gestation days 13-16 had no such effect. Feotal toxicity was apparent at 100 mg/kg of AOH and it was suggested that synergism between these may be occuring when administered together (Pero *et al.,* 1973).

2.4.2.2 Mycotoxin Produced by *Fusarium Oxysporum*

The intra and extracellular extracts of *FusariumOxysporum* from mouldy mustard seeds, grown in Richard's medium, produced mycotoxins such as, diacetoxyscripenol, T-2 toxin and zearalenone, which produced toxicity in albino rats and rabbits. They caused a deep scab on the shaved skin of albino rats and rabbits.

The chloroform extract healthy of mustard seeds was without any toxic effect on either of these animals. They were found to be sedated, non-agressive, their motor power was normal but equilibrium was lost (impaired performance on rota-rod); pain sensitivity was considerably reduced (response to pain-stimuli). Significant changes were observed in the body weight, feed consumption, and in their fecal matter. (Jimennz *et al..*, 1997).

2.4.2.3 Mycotoxin Produced by *Fusarium Solani*

Trichothecenes are a very large family of chemically related mycotoxins produced by *Fusariumsolani.* They include T-2 toxin, HT-2 toxin, diacetoxyscirpenol which are of special

interest because they are more toxic than the other foodborne trichothecenes i.e. type B group which include deoxynivalenol, nivalenol, 3- and 15-acetyldeoxynivalenol. Trichothecenes belong to sesquiterpene compounds. The most important structural features causing the biological activities of trichothecenes are: the 12, 13-epoxy ring, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus and the structure and position of the side-chain (Chakrabarti and Ghosal, 1986).

2.4.2.4 Harmful Effect of *Fusarium Solani* Mycotoxins

Trichothecenes are powerful inhibitors of protein synthesis. They do this by reacting with components of the ribosomes: the structure within the cell where proteins are made. The specific site of action of T-2 toxin, which is a reaction with a critical site on the ribosomal RNA (rRNA), is known. Protein synthesis is an essential function in all tissues, but tissues where cells are actively and rapidly growing and dividing are very susceptible to the toxins (Miller, 2003). Trichothecenes are different from most other potential weapons toxins because they can act through the skin. Compared with some of the other mycotoxins such as aflatoxin, the trichothecenes do not appear to require metabolic activation to exert their biological activity. After direct dermal application or oral ingestion, the trichothecene mycotoxins can cause rapid irritation to the skin or intestinal mucosa. In cell-free systems or single cells in culture, these mycotoxins cause a rapid inhibition of protein synthesis and polyribosomal disaggregation. Thus, we can postulate that the trichothecene mycotoxins have molecular capability of direct reaction with cellular components. Despite this direct effect, it is possible to measure the toxicokinetics and the metabolism of the trichothecene mycotoxins (Miller, 2003).

When it comes to animal and human food, type A trichothecenes e.g. T-2 toxin, HT-2 toxin, diacetoxyscirpenol are of special interest because they are more toxic than the other foodborne

trichothecenes i.e. type B group e.g. deoxynivalenol, nivalenol, 3- and 15-acetyldeoxynivalenol. However, deoxynivalenol is of concern as it is the most prevalent trichothecene (Miller, 2003). The major effects of trichothecenes – related to their concentration in the commodity are reduced feed uptake, vomiting and immuno-suppression

2.4 .3 *Fusarium Oxysporum*

The fungal pathogen *Fusarium oxysporum* affects a wide variety of plant host such as tomato, tobacco, legumes, sweet potatoes and banana which are a few of the most susceptible plants, but it can also infect other herbaceous plants (Pan and Pestizid, 2010). They generally produce symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off. The most important of these is vascular wilt (Synder and Hans, 2003). *Fusarium* wilt begin to resemble vein clearing on the younger leaves and dropping of the older lower leaves, which is followed by stunting of the plant, yellowing of the lower leaves, defoliation, marginal necrosis and death of the plant. On older plants, symptoms are more distinct between the blossoming and fruit maturation stages (Agrios and George, 2005) but can sometimes be found in clusters or in short chains. They are round thick walled spores produced within or terminally on an older mycelium or in macro conidia. Chlamydospores unlike the other spores can survive in the soil for a long period of time.

Plate IV: A tobacco plant suffering from *Fusarium* wilt caused by *Fusarium oxysporum*

Source: (Pan and Pestizid, 2010)

Fusarium oxysporum is a common soil pathogen and saprophyte that feeds on dead and decaying organic matter. It survives in the soil debris as a myceliumand all spore types, but is most commonly recovered from the soil as chlamydospores (Synder and Hans, 2003). This pathogen spreads in two basic ways: it spreads short distances by water splash, and by planting equipment, and long distances by infected transplants and seeds.

Fusarium oxysporum infects a healthy plant by means of mycelia or by germinating spores penetrating the plant's root tips, root wounds, or lateral roots. The mycelium advances intracellularly through the root cortex and into the xylem. Once in the xylem, the mycelium remains exclusively in the xylem vessels and produce micro conidia (asexual spores) (Agrios and George, 2005). The micro conidia are able to enter into the sap stream and are transported upward where the flow of the sap stops the micro conidia germination. Eventually the spores and the mycelia clog the vascular vessel which prevents the plant from up-taking and translocating nutrients, thus the plant transpires more than it can transport, the stomata closes, the leaves wilt, and the plant dies. After the plant dies the fungus invades all tissues, sporulates, and continues to infect neighboring plants.

2.4.4 Environment for *Fusarium Oxysporum* Growth

F. oxysporum is a common soil saprophyte that has the ability to survive in most soil—arctic, tropical, desert, cultivated and non-cultivated (Mace *et al.*, 1981). Though *Fusarium oxysporum* may be found in many place and environments; development of the disease is favored by high temperatures and warm moist soils. The optimum temperature for growth on artificial media is between 25-30°C, and the optimum soil temperature for root infection is 30°C or above (Dreistadt and Clark, 2004). However, infection through the seed can occur at temperatures as low as 14°C.

2.4.5 Management of *Fusarium Oxysporum*

Fusarium oxysporum is a major wilt pathogen of many economically important crop plants. It is a soil-borne pathogen, which can live in the soil for long periods of time, therefore rotational cropping is not a useful control method. It can also spread through infected dead plant

material,thus cleaning up at the end of the season is important. Biological control can work using antagonists approach. Systemic approach and soil fungicides can also be used (Booth, 1971).

One control method is to improve soil conditions because *F. oxysporum* spreads faster through soils that have high moisture and bad drainage. Other control methods include planting resistant varieties, removing infected plant tissue to prevent overwintering of the disease, using soil and systemic fungicides to eradicate the disease from the soil, flood fallowing, and using clean seeds each year. Applying fungicides depends on the field environment. It is difficult to find a biological control method because research in a greenhouse can have different effects than testing in the field. The best control method found for *F. oxysporum* is planting resistant varieties (Mace *et al.*, 1981).

Fusarium oxysporum can be controlled by using clean seed, cleaning up infected leaf and plant material and breeding for resistance. Fungicides can also be used, but are not as effective as the other methods of control because of field conditions during application. Fungicides can be used effectively by dip treating propagation material (Agrios and George, 2005).

Different species of*F. oxysporum*, cause Panama disease on banana and can be susceptible, resistant and partially resistant. It can be controlled by breeding for resistance and through eradication and quarantine of the pathogen by improving soil conditions and using clean plant material. Biological control can work using antagonists. Systemic and soil fungicides can also be used. The main control method for *F. oxysporum*, vascular wilt on tomato, is resistance. Other effective control methods are fumigating the infected soil and raising the soil pH to 6.5-7 (Snyder and Hans, 2003).

The most effective way to control *F. oxysporum* f. sp. *melonis* is to graft a susceptible variety of melon to a resistant root-stock. Resistant cultivars, liming the soil to change soil pH to 6-7, and reducing soil nitrogen levels also help control *F. oxysporum* f. sp. *Melonis* (Booth, 1971).

2.4.6 *Fusarium Solani*

Fusarium solani is ubiquitous plant pathogens and saprotrophs, it's a phytopathogenic fungus and an important causal agent of several crop diseases, such as root and fruit rot of *Cucurbita* spp., root and stem rot of pea, sudden death syndrome of soybean, foot rot of bean and dry rot of potato. *Fusarium solani*was first described by C.F.P. Von Martius in 1842 as *Fusarium solani* from rotted tubers of potato, *Solanum tuberosum*. The species was transferred to the genus *Fusarium* by the Italian mycologist Piers A. Saccardo in 1881. *F. solani* was emended by Snyder and Hansen in 1941 to comprise a complex group of species that are widely distributed in soils and cause tuber, root, and stem rots of plants worldwide. There are at least 50 subspecies lineages (Desjardins, 2006).

2.4.7 Host range and distribution: The predominant hosts for *Fusarium solani* are potato, pea, bean, and members of the cucurbit family such as melon, cucumber, and pumpkin. Some strains may cause infections in humans.

2.4.8 Symptoms caused by *Fusarium solani*

The first symptoms of root rot in beans are narrow, long, red to brown lesions on the stems, and lengthwise cracks often develop. Lesions extend down the main taproot, which may shrivel, decay and die. The symptoms in some cases extend up the hypocotyl to the soil surface. Clusters of fibrous roots (lateral roots or adventitious roots) commonly develop above the shriveled

taproot. Severe *Fusarium* root rot kills primary and secondary roots of beans, and most times only adventitious roots are visible (Zaccardelli *et al.,* 2008).

Fusarium crown and foot rot of squash and pumpkin is caused by *Fusarium solani* f. sp. *cucurbitae*. The first symptom is wilting of the leaves. Within several days, the entire plant may wilt and die. If the soil is removed from around the base of the plant, a very distinct necrotic rot of the crown and upper portion of the taproot can be seen. The rot develops first as a light-colored, water-soaked area which becomes progressively darker. It begins in the cortex of the root, causes cortex tissue to slough off, and eventually destroys all of the tissue except the fibrous vascular strands. Infected plants break off easily about 2-4 cm below the soil line. The fungus generally is limited to the crown area of the plant (Cho *et al.*, 2001).

PLATE V: Plant suffering from root rot caused by *Fusarium solani.*

(Source: Luginbuhl, 2010).

2.4.9 Common Plant fungal diseases

A plant becomes diseased when it is continuously disturbed by some causal agent that results in an abnormal physiological process that disrupts the plant's normal structure, growth, function, or other activities. This interference with one or more of a plant's essential physiological or biochemical systems elicits characteristic pathological conditions or symptoms (Nwoboshi, 1982).

Fungi cause a variety of symptoms including leaf spots, leaf curling, galls, rots, wilts, cankers, and stem and root rots. Fungi are responsible for "damping off" symptoms associated with seedlings. The causative organisms for damping off may include a number of saprophytic fungi in the upper layer of soil which may become pathogenic under unfavourable conditions for the plants. Some of them include *Pithium spp., Fusarium spp., Rhizoctonia solani* and *Sclerotium bataticola*. The most important in Nigeria include *Pithium spp*. and *Fusarium spp*. (Ukoima *et al.,* 2013). The life cycle of these pests are so short and follow each other so rapidly that it is not considered cost-effective to organize such remedial measures as aerial dusting or spraying (Robertson, 2002). Root rot which is becoming endemic in Nigeria, is caused by *Rigidoporus lignosus.* A survey reported that 11 to 13year old trees in parts of Olukemeji forest Nigeria had fallen over from root rot. Some parasites of trees especially fungi and bacteria, cause the development of galls by the host tree. A notable example here is *Phytolima lata* which attacks Iroko (*Chlorophora excels*) in West Africa. Plant parasites found in forest ecosystems may attack and destroy leaves, branches, boles, roots and even flowers and fruits of forest trees. Sometimes young seedlings are destroyed by damping off parasites particularly soon after seed germination. Damping off may also through root infection kill a tree after its stem had become woody (Nwoboshi, 1982).

According to Balasundaran (2002), in India plants are affected by a few serious diseases both in nurseries and plantations. Leaf spot caused by *Phomopsis* spp,*collectotrichum gloeosporoides, Alternaria* sppand *Curvularia* spp, leaf rust by *Olivia tectonae* and powdery mildew by *Uncinula tectone* are the major leaf diseases in nurseries in Kerala. During 1993 and 1994, 95% of thenurseries, leaf infection caused by *Phomopsis* sppincombination with C. *gloeosporiodes* were predominant. *Phomopsis* sppleaf necrosis, localized in patches almost year-round affecting 2 to 8 month old seedlings is more serious after the monsoon in nursery beds of high seedlings density. The characteristic symptom is the development of light brown necrosis at the margin of leaves, which gradually advances toward the midrib. The disease spreads to the upper leaves, petiole and ultimately to the terminal bud and stem top leading to the dying up of upper portion of the stem. At this stage a few secondary sprouts emerge from the top of the stump.

However, in most cases the affected seedlings die in the absence of control measures. Leaf spot caused by C. *gloesporiodes* is characterized by the development by the development of interveinal dark-brown spots which coalesce to form large necrotic areas. The disease occurs commonly mixed up with *Phomopsis* leaf necrosis. Leaves affected by these two leaf spots get dried up and defoliate (Ukoima *et al.,* 2013). If timely control measures are not taken, the diseases spread to the entire nursery, reducing the number of healthy seedlings available for planting, thus upsetting the planting programme considerably. Leaf rust, generally observed in August/ February also affects the production of healthy planting stock. A severe infection leads to extensive premature defoliation.

Other diseases which affect plant seedlings in the nursery include root rot caused by *Polyporous zonalis.* Pink disease fungus causes cankers and bark flaking. Powdery mildews caused by *Olivea tectoriae* and *Uncinula tectonae* leads to premature defoliation (Hedge, 2000).

2.5 Antifungal Properties of Chitinase

The effects of chitinase on the growth of agronomically important fungal pathogens *F. oxysporum* causing potato wilt, *A. solani* causing tomato early blight, *S. sclerotiorum* causing canola stem rot, *G. graminis* causing wheat take-all, *P. infestuns* causing potato late blight and nonpathogen *T. reesei* were examined (Quang *et al.,* 1992). The chitinase showed greater antifungal activity against *F. oxysporum, A. solani, and T. reesei*. It was found to inhibit the growth of these three fungi with as little as 0.5 μg.

Most chitinase function either as endochitinases or exochitinases and can cleave any portion of a chitin polymer with which they come in contact. They can access non-reducing termini of chitin as substrates, which may be difficult in intact fungal cell walls. Both exochitinases and endochitinase degrade chitin efficiently in standard chitinase assays, but these normally use purified, partially degraded chitin as substrate, and would contain numerous termini available for exochitinase attack. Schlumbaum *et al.*, (1986) also describes the inhibition of fungal growth by chitinase from bean leaves. In addition, these investigators found antifungal activity associated with commercial chitin-binding lectins from wheat-germ, tomato, potato, pokeweed and gorse, but only if the lectins were contaminated with the corresponding chitinases. These results, as well as that using grain chitinases, suggest that antifungal chitinases may be widely distributed throughout the plant kingdom, either in stems and leaves following induction by ethylene or pathogen attack Boller, (1985), or stored in seeds as a means of increasing the seeds' resistance to fungi in the soil (Powning and Irzykiewicz, 1965). Presumably, these chitinases, by acting directly on growing hyphal tips or in concert with other hydrolytic enzymes Schlumbaum *et al.*, (1986), help limit and define the fungal species that can successfully parasitize plants.

The organisms used in the course of this work were selected because they are among other organisms found in literature that commonly attack leguminous plants and other plants in general, and were readily accessible from the environment. They were isolated from legumes suffering from diseases, like damping off, root rot, leaf spot etc, that are caused by these organisms from Institute of Agricultural Research (IAR), Zaria. It was confirmed that these organisms pose a serious threat to the survival of plants in Nigeria and in the tropics as a whole.

Other common fungi in the tropics like *Aspergillus spp.*, *Penicillin* etc has been found to attack the seeds of African yam beans (Onyeke and Ugwuoke, 2011). The literature on antifungal properties of Chitinase in Nigeria are lacking rather works done were on antimicrobial activity using mainly ethanolic extraction and also on the nutritional aspect of the plant.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

The seeds of African yam bean were collected from a farm at Eluama Isukwuato L. G. A of Abia State. The seeds were authenticated by a taxonomist at the Herbarium section of Biological Sciences Department, Federal University of Agriculture Umudike in Abia State, Nigeria with Voucher no. S Nig; Barter 1804.

3.1.2 Chemicals and Reagents

Chitin, Coomassie Blue G-250, and Sephadex G-75 were purchased from Sigma Chemical Co. St. Louis, England. All other chemicals: Sodium acetate, 3,5-dinitrosalicylic acid, glucose, glycerol, tris, glycine, ammonium sulphate, potatoe dextrose agar , ethylenediaminetetraacatic acid (EDTA), dithiothreitol (DTT), 2-mercaptoethanol, sodium dodecyl sulphate, protein markers, N,N,N',N'-tetramethylethylenediamine (TEMED), chloroform are of analytical grade.

3.1.3 Microorganisms

The microorganisms (*Fusarium solani, Fusarium oxysporum, Alterneria alternata*) used were collected and identified at Agronomy Department, Institute of Agricultural Research, Ahmadu Bello University, Zaria.

3.1.3.1 Collection and isolation of *Alterneria alternate*

Leaves of onion (*Allium cepa* var. *aggregatum*) showing typical symptoms of dark blighted lesions caused by *Alternaria alternata* were collected and the fungus isolated by the following technique as shown by Ramjegathesh and Ebenezar, (2012).

The infected leaf bits along with some healthy portions were cut into small bits and surface sterilized using 1:1000 mercuric chloride solutions for 30 sec. The bits were washed thoroughly in sterile distilled water three times to remove traces of mercuric chloride. The molten warm potato dextrose agar (PDA) medium was poured in sterilized petri plates and allowed to solidify. The surface sterilized leaf bits were placed on PDA medium. The plates were incubated at room temperature 28±2°C and observed periodically for fungal growth. Colonies were developed from PDA slants. The slants were incubated at 28±2°C for sporulation for 10-18 days. Then such slants containing pure culture were used for further purification. The pathogen was identified up to species level based on their cultural and morphological characters.

3.1.3.2 Morphological characters

The morphological characters viz., conidia shape, size (length and width), number of cells /conidia, colour of conidia and sporulation time (days) were observed. Spores of *A. alternata* were taken from the pure culture and mounted on a clean glass slide. Spores were mixed with lactophenol thoroughly in order to obtain a uniform spread over and covered with cover slip. The morphological characters such as shape, size, and number of cells were measured in 50 conidia under high power objective using ocular and stage micrometers (Ramjegathesh and Ebenezar, 2012).

3.1.3.3 Collection and isolation of *Fusarium solani*

The soil samples collected from bean cultivated areas were mixed with deionized water and shaken. One gramme of soil sample was dissolved in 10ml of deionized water to give a concentration of 100mg/ml. Further concentrations of 60mg/ml and 20mg/ml were also prepared, then 1ml aliquot from each dilution was spread over modified Nash and Snyder's medium (MNSM) in petri dishes (Cho, *et al*, 2001). Plates were then incubated at room temperature and light. After 7 days, colonies of *F. solani* were then transferred to potato dextrose agar (PDA). The isolated fungal pathogens was identified using reference materials (Jurgen *et al.*, 1978, Barnett and Hunter, 1987 and Labbe and Garcia, 2001).

3.1.3.4 Collection and Isolation of *Fusarium oxysporum*

The base of the stem of a diseased plant was collected and cut to reveal the xylem just below the epidermis. The leaves and secondary roots were trimmed off leaving only the main stem and the hypocotyls and main root. Sterilization was carried out on the stem by soaking in 10% bleach solution for 5 minutes and was there after dried on paper towels. Using sterile technique, thin (2-4 mm thick) wedges were cut out of one side of the stem near the root/stem junction making sure to include xylem tissue with each wedge. 5-6 wedges were placed on PDA plates and the plates were incubated under fluorescent lights. Once grown sufficiently from the pieces, the isolates were transferred onto fresh PDA plates and the plates Incubated for 10-14 days. *F. oxysporum* produces colonies which are pigmented with a reddish purple color and surmounted by a pinkish white aerial mycelium (Synder and Hans, 2003).

3.2 Experimental Design

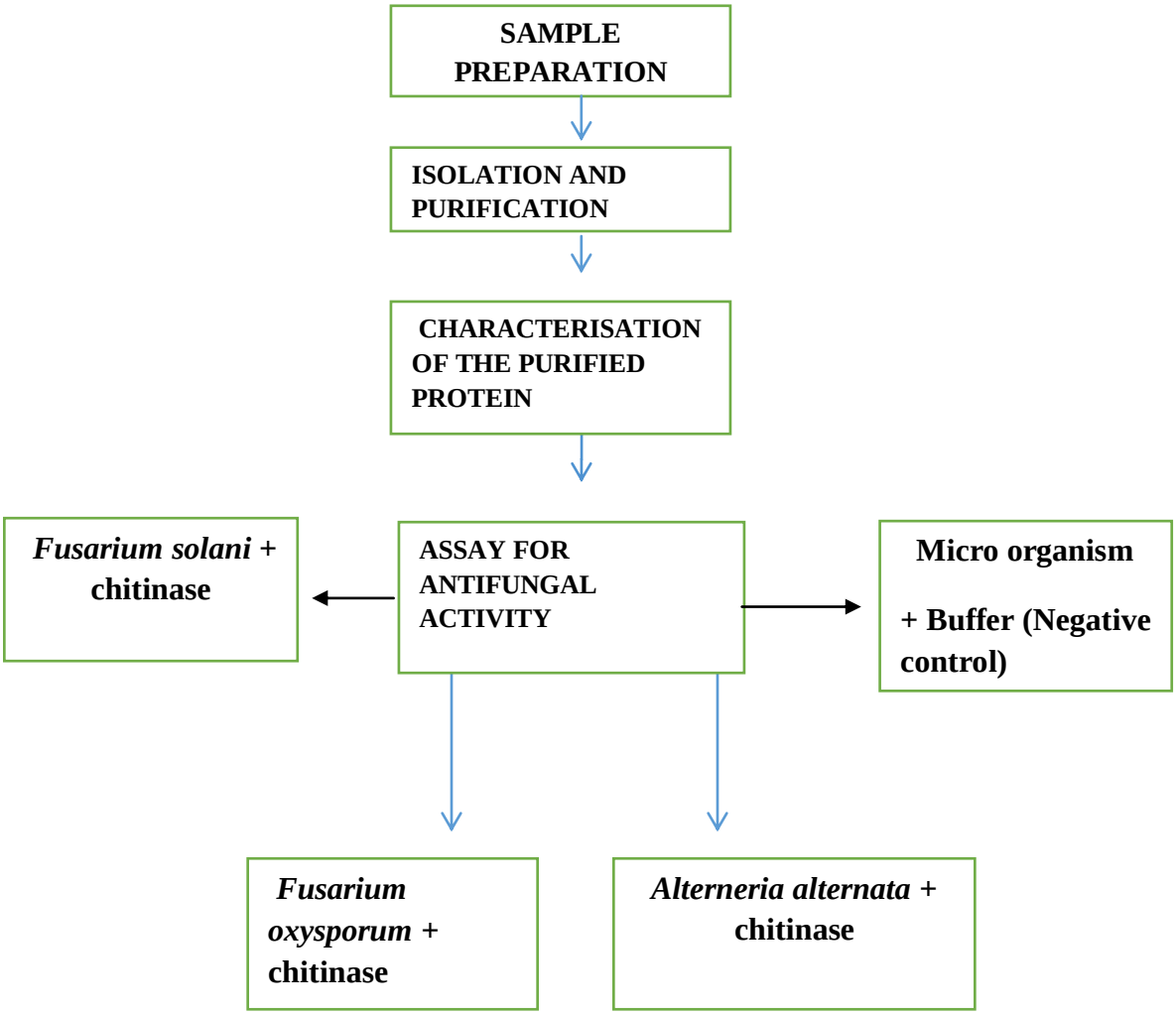

Fig 3: Flow chart showing the design of the experiment

3.3 Methods

3.3.1 Sample Preparation

Exactly 100g of African Yam bean seeds was soaked in 400ml distilled water for 24 h at 4°C and homogenized in 300ml of 0.2mol/L sodium acetate buffer (pH 5.4).The homogenate was centrifuged at 10,000 ×g for 20 min at 4 °C. The supernatant was designated as the crude extract and stored in the refrigerator at 4°C until required for further investigation. (Wang *et al.,* 2012).

3.3.2 Determination of Total Protein Content

The protein concentration was quantified according to the method described by Bradford (1976). The assay is based on the principle that the maximum absorbance of an acidic solution of Coomassie blue G-250 shift from 365nm to 595nm when binding of the dye to protein occurs.Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change.

Coomassie Brilliant Blue G-250 (25 mg) was dissolved in 12.5 ml 95% ethanol. To this solution, 25 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 250 ml. Protein solution (1 ml) was pipetted in test tubes. Five milliliters of protein reagent was added to the test tube and the contents mixed by vortexing. The absorbance at 595 nm was measured after 2 min and before I hour against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

3.3.3 Chitinase Activity (Wang *et al.*, 2012)

A reducing sugar in a basic solution forms an aldehyde or ketone. The aldehyde group of glucose ring in the chitin molecule converts 3,5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid, which is the reduced form of DNS. Water is used up as a reactant and oxygen gas is released during the reaction. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed, at wavelength 540 nm. The absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar.

3,5 dinitrosalicylic acid $\xrightarrow{\text{Reduced}}$ 3amino,5nitro salicylic acid

Chitinase activity was determined by measuring the reducing end group N-acetamino-glucose produced from colloidal chitin

The reaction mixture consisting of 1 ml enzyme solution, and 1 ml of 1% (w/v) colloidal chitin (pH 5.4) was incubated at 50°C for 60 min. The reaction was terminated by adding 2ml dinitrosalicylic acid reagent and heating in boiling water for 5 min, then cooled to room temperature, and centrifuged at 6000 ×g for 10 min. The reducing end group N-acetamino-glucose produced from colloidal chitin was determined by measuring the absorbance at 530nm. One unit of Chitinase activity was defined as the amount of enzyme that liberates 1μg N-acetamino-glucose per minute at pH 5.4 and 50°C.

3.3.4 Partial Purification of Protein Chitinase

3.3.4.1 Purification with Ammonium Sulphate

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. It is a general technique known as salting out. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out).

The principle is based on the solubility difference between the Chitinase and the ammonium sulphate, in which case the more soluble ammonium sulphate abstracts the water molecules thus making the protein-protein interaction to increase but decreasing the interaction of protein with the solvent, hence precipitating the protein.

Here, the solid ammonium sulphate was added slowly to the crude extract with constant stirring to80% until saturation is obtained. The sample was allowed to stand overnight at 4^{o}C. Thereafter, it was centrifuged at 10,000×g for 20minutes. The supernatant was discarded and pellet resuspended in the assay and lysis buffer which was 300 ml of 0.02 mol/L sodium acetate buffer (pH 5.4) and tested for Chitinase activity as described by Wang *et al*. (2012).

3.3.4.2 Gel filtration on Sephadex G-75

The principle is based on separating macromolecules according to their various sizes as they pass through gel filtration media in a column.

Here, the pooled fractions from the ammonium sulphate precipitation were loaded onto Sephadex G-75 column (1.6 cm ×12cm) previously equilibrated with 0.02 mol/L sodium acetate buffer (pH 5.4) . The column was eluted with the same buffer, 50 fractions of 3ml each were collected at a flow rate of 3ml/minute. Chitinase activity was determined in each of the fractions. Fractions containing the enzyme activity were pooled together and the protein content was estimated at 280nm.

3.3.4.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, or SDS-PAGE, is a widely-used technique for separating mixtures of proteins based on their size. SDS, an anionic detergent, is used to produce an even charge across the length of proteins that have been linearized by first loading them into a gel made of polyacrylamide and then applying an electric field to the gel. SDS-coated proteins are then separated. The electric field acts as the driving force, drawing the SDS coated proteins towards the anode with larger proteins moving more slowly than small proteins. In order to identify proteins by size, protein standards of a known size are loaded along with samples and run under the same conditions.

Electrophoresis under denaturing conditions was performed in 12.5% (w/v) acryl amide disc gel according to the method of Laemmli (1970) and Orstein (1964) using a tris-glycine buffer, pH 8.3. The electrophoresis was carried out on the *crude enzyme*, *ammonium sulphate precipitate* and on the *pooled active fractions from Sephadex G-75 column chromatography*. The protein bands were located by staining with Coomassie Brilliant Blue G-250. The proteins were separated according to their electrophoretic mobility, which is a function of the length of the polypeptide chain or molecular weight as well as higher order protein folding, posttranscriptional modifications.

3.3.4.4 Sample Preparation for SDS-PAGE

The sample was prepared by mixing in a test tube, 100µl of sample, one drop of 2-mercaptoethanol, which is a reducing agent that denatures the protein by reducing the disulphide linkages; one drop of bromophenol blue serves as tracking dye, one drop of 2% SDS, 2mM EDTA, and 50µl of Tris-HCL buffer, pH 6.8. The mixture was incubated at 100^{o}C for 5minutes, after which 3 drops of 20% glycerol was added to increase the density. The mixture was ready for loading on the gel (Laemmli (1970) and Orstein (1964)).

3.3.4.5 Preparing SDS Gel

From 30% acryl amide stock, gels of composition 12.5% acryl amide were prepared. The gel buffer stock (30ml) consists of 10% SDS (9.4ml), 1.5M Tris-HCL (7.5ml, pH 8.8) and distilled water (12.3ml). Polymerization was initiated by adding freshly prepared 10% ammonium persulphate (0.5 ml) to the mixture followed by N, N, N', N'-tetramethylethylenediaamine (20µl). Once the catalyst is added, the solution was swirled to mix and the mixture was poured into the gel tube immediately before polymerization occurs.

3.3.4.6 Loading, Running and Staining of the Sample

Hamilton syringe was used for loading samples into the tubes. The prepared sample (50µl) was dispensed into the gel tubes. The tubes were fixed in the disc Shandon apparatus. The upper and lower parts of Shandon tubes were filled with 125mM Tris-glycine buffer, pH 8.3 containing 4% SDS. The power was switched on and was switched off when the marker dye was a few milliliters away from the bottom. The gel was removed from the gel tubes and stained with Coomassie Brilliant blue G-250.

3.3 4.7 Molecular Weight Determination

The molecular weight was estimated by SDS-Polyacrylamide gel electrophoresis as described by Weber *et al*, (1972). The molecular weight markers for the SDS-PAGE include Myosin (200 KDa), Bovine serum (91 KDa), Glutamate (38 KDa), Myoglobin (26 KDa), Aprotini (9 KDa).

3.4 Characterization of the Purified *Chitinase*

3.4.1 Determination of Optimum Temperature (Zarei *et al.*, 2011)

The Chitinase activity of the enzyme solution was determined by incubating 0.1ml of the enzyme solution in 1ml of colloidal chitin as substrate at various temperatures: 30, 35, 40, 45, 50, 55, 60 and 70^{o}C for 30mins at pH 5.4. After 30 min of incubation, the residual enzyme activity was monitored as described by Wang *et al.* (2010).

3.4.2 Determination of Thermal Stability (Zarei *et al.*, 2011)

The temperature stability of the partially purified Chitinase was measured by preincubating 1.0ml of the enzyme at different temperatures: 50, 55, 60, 65, 70, 75, 80, and 85^{0}Cat pH of 5.4 for 30min. After 30min, the mixture were brought to room temperature and 0.1ml colloidal chitin was added to the reaction mixture and then in chitinase activity was carried out as described by Wang *et al.* (2010).

3.4.3 Determination of Optimum pH (Zarei *et al.*, 2011)

The effect of pH on the enzyme activity of the purified Chitinase were investigated at 50^{O}C within a pH range of pH 3.0 to pH 9.0 using 0.2mol/L sodium acetate buffer (pH 3.0, 4.0, 5.0 and 5.4) and 0.2mol/L sodium citrate buffer (pH 6.0, 7.0, 8.0, 9.0) activity of the enzyme was determined as a function of pH at the temperature of 50^{o}C with the assay mixture consisting of 0.1 ml of the enzyme solution and 1ml of colloidal Chitin. After 30 min of incubation, the residual activity of the enzyme was monitored as described by Wang *et al.*, (2012).

3.4.4 Determination of pH Stability (Zarei *et al.*, 2011)

The pH stability of the enzyme was measured by incubating 0.1ml of the enzyme in 1ml each of 0.2mol/L sodium acetate buffer for pH: 3.0, 4.0, 5.0, 5.4 and 0.2mol/L sodium citrate buffer for pH: 6.0, 7.0, 8.0, and 9.0 at 50^0C for 30 min. Later, the samples were brought to room temperature and colloidal chitin was added to the reaction mixture and Chitinase activity was performed as described by Wang *et al.,* (2012)

3.4.5 Effect of Metal Ions

The effect of the following metal ions: Pb^{2+}, Hg^{2+}, K^{+}, Fe^{2+}, metal chelator EDTA and sugar such as glucose on Chitinase activity were studied. The purified Chitinase was incubated with 2mM of the various metal ions and sugar like glucose for 30min. After incubation, the residual activities were measured under standard assay conditions. Activity without added metal ions was taken as control.

3.4.6 Effect of Substrate Concentration

The Michaelis-Menten constant (*Km*) and maximum velocity (*Vmax*) of the enzyme were measured for the activity on chitin at pH 5.4 and 50^oC for 60 min. Different concentrations of the substrate, 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml were prepared from a stock solution of 10mg/ml and the absorbance read at 540nm. A graph of ($1/V_{max}$) was plotted against (1/(S)) and the K_M and V_{max} determined. This is called double reciprocal (Linweaver-Burk) plot (Lineweaver and Burk, 1934).

3.5 Assay for Antifungal Activity

The antifungal properties of the crude and partially purified extracts were determined using the agar diffusion method of (Bookye-Yiadam, 1979). Seventy- two hour old broth culture of test organisms were swabbed onto sterile Potato Dextrose Agar in Petri dishes using sterile cotton

swabs. A sterile stainless steel cork borer of size 12mm in diameter was used to make wells on the plates. The holes were filled with 500µL and 1000µL of the crude extracts, while 500µL and 1000µL of the partially purified extract was used. Each well was labeled appropriately. Control experiments were also carried out where the holes were filled with sterile distilled water for the negative control and Benylate for the positive control. The sterile distilled water and the extracts were incorporated into the holes by the use of sterile 2mL syringes. The inoculated Petri dishes were left for an hour at room temperature for the extracts to diffuse before the growth of organisms commenced. The plates were incubated at room temperature for 72 hours after which the results were read by measuring the diameters of zones of inhibition around the wells with the aid of a metric rule and recorded.

CHAPTER FOUR

4.0 RESULTS

4.1 Purification Profile of *Sphenostylis stenocarpa* Chitinase

Results of the partially purified *Sphenostylis stenocarpa* protein Chitinase (EC 3.2.1.14) are summarized in Table 4.1. The crude protein contained approximately 2.77mg/ml total protein with a specific activity of 4.45µmol/min/mg. Precipitation of the crude protein with ammonium sulphate resulted to a purification fold of4.14 and a 71.64% yield. The result obtained following gel filtration on Sephadex G-75 column revealed a total yield of 46.35% with a specific activity of 35.75µmol/min/mg.

Figure 4.1 is a plot showing the elution profile of protein Chitinase after gel filtration on Sephadex G-75 column. The plot resulted to a two-peak fraction of the enzyme. From the plot, the first peak has about 0.28 mg/ml of protein concentration with a corresponding enzyme activity of 0.010µmol/min. The second peak with a reduced height has about 0.06 mg/ml of protein concentration and an activity of about 0.003µmol/min.

4.2 Molecular Weight of Protein Chitinase Isolated from *Sphenostylis stenocarpa*

The purity and molecular weight of the partially purified enzyme was determined by Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE). The electrophoregram of the sample under denaturing conditions is shown in plate 4.1. Bands of the protein sample were visualized against the standard on the gel and the molecular weight which was 32kDa was estimated from the plot of log of molecular weight of the standard marker proteins against their respective relative mobility (R_f).

Table 4.1: Purification Profile for Chitinase from *Sphenostylis stenocarpa*

Purification Step	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	Percentage Yield
Crude Protein (28ml)	2.77	12.34	4.45	1.00	100
80% Ammonium Sulphate Fractionation (5ml)	0.48	8.84	18.42	4.14	71.64
Gel Filtration on Sephadex G-75 Column (0.5ml)	0.16	5.72	35.75	8.04	46.35

One unit of activity is equivalent to the amount of enzyme required to hydrolyze 1 µmol *chitin* per min at pH 5.4 and temperature of 50^{O}C.

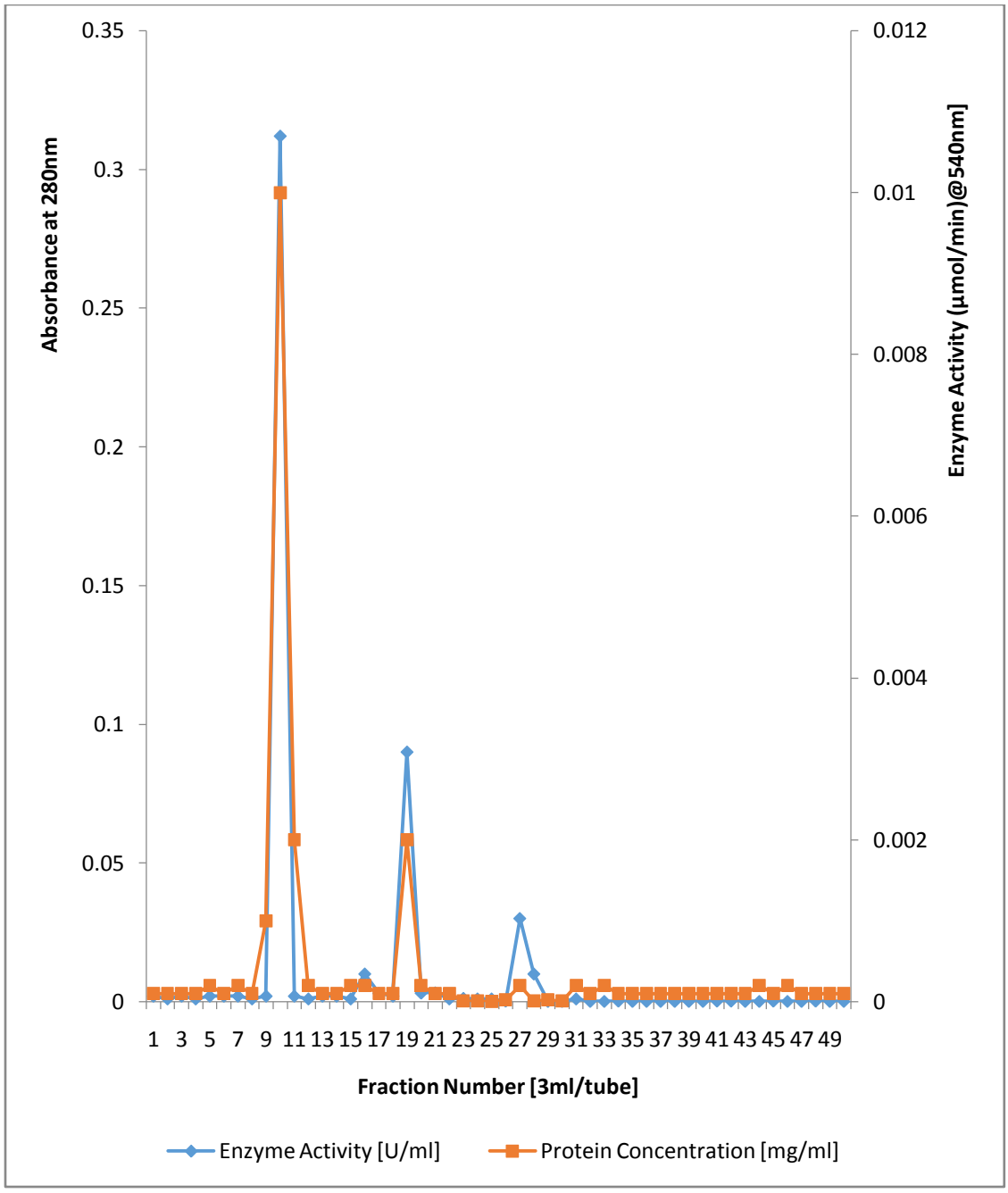

Figure 4.1: Elution Profile of *Chitinase* on Sephadex G-75 Column Chromatography. **(1.6 × 90cm)**.

Lane A Lane B Lane CMaker Protein

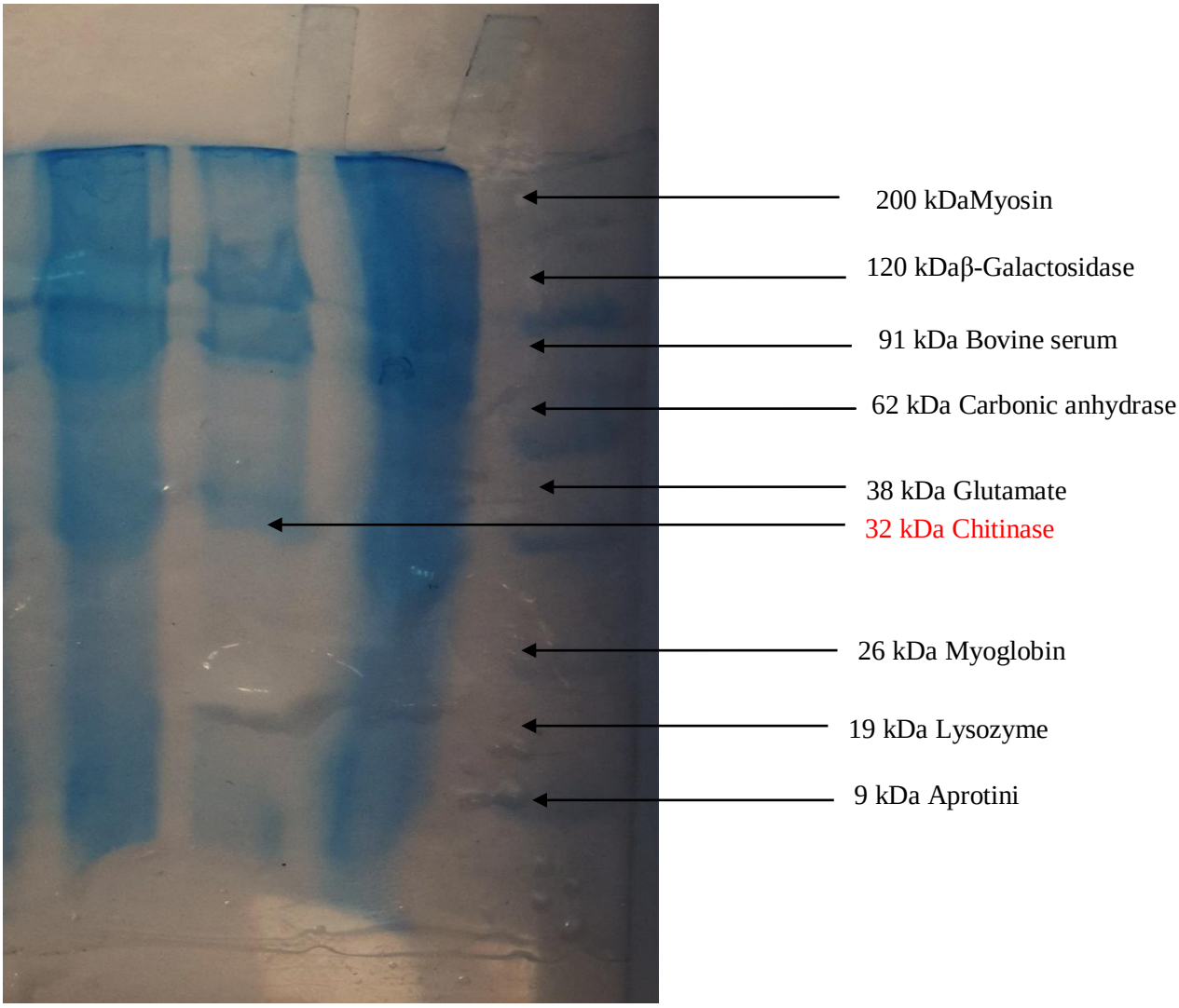

PLATE VI: Electrophoregram of *Chitinase* from *Sphenostylis stenocarpa* on Polyacrylamide gel using Coomassie blue staining.

(Lane A; Band from crude Protein, Lane B; Band from Sephadex G-75 purification step, and Lane C; Band from Ammonium sulphate precipitation, Lane D; Marker proteins). The Band on lane B represents Chitinase with an estimated molecular weight of **32kDa**

4.3 Characterization of Protein Chitinase

4.3.1 Optimum Temperature and Thermal stability.

The enzyme activity was measured at different temperature and the optimum temperature for activity was determined. The enzyme was most active at temperature of 55^0C (Figure 4.2). The thermos stability of chitinase activity was determined in the temperature range of 30 -70^0Cas shown in figure 4.3. The enzyme was highly stable up to 60^0C after heating at 50 ^{0}C for 30min, thereby retaining about 65% of its activity up to 60 ^{0}C for 30min, and was without activity within 30min at temperatures above 70 ^{0}C. This indicates that the activity of this enzyme is relatively stable to heat treatment.

4.3.2 Optimum pH and pH stability

The effect of pH on chitinase activity is presented in Figure 4.3. The enzyme was most active at pH 5.0. Enzyme activity decreased below pH 5.0 and above pH 7.0. However, Chitinase activity was stable after incubation of the enzyme for 30 min within a pH range of 4.0 to 6.8 at room temperature, but stability decreased markedly after incubation for 30min at a pH below 3.0 at the same temperature (Figure 4.4).

4.3.3 Effects of Some Divalent Cations /Reagents on chitinase activity

The effect of some divalent cations and other reagents studied is shown in figure 4.6. It was observed that chitinase activity was inhibited by Pb^{2+}, Hg^{2+}, k^+ and other reagents like EDTA and glucose but Na^+ and Zn^{2+} showed little or no effect on the enzyme activity.

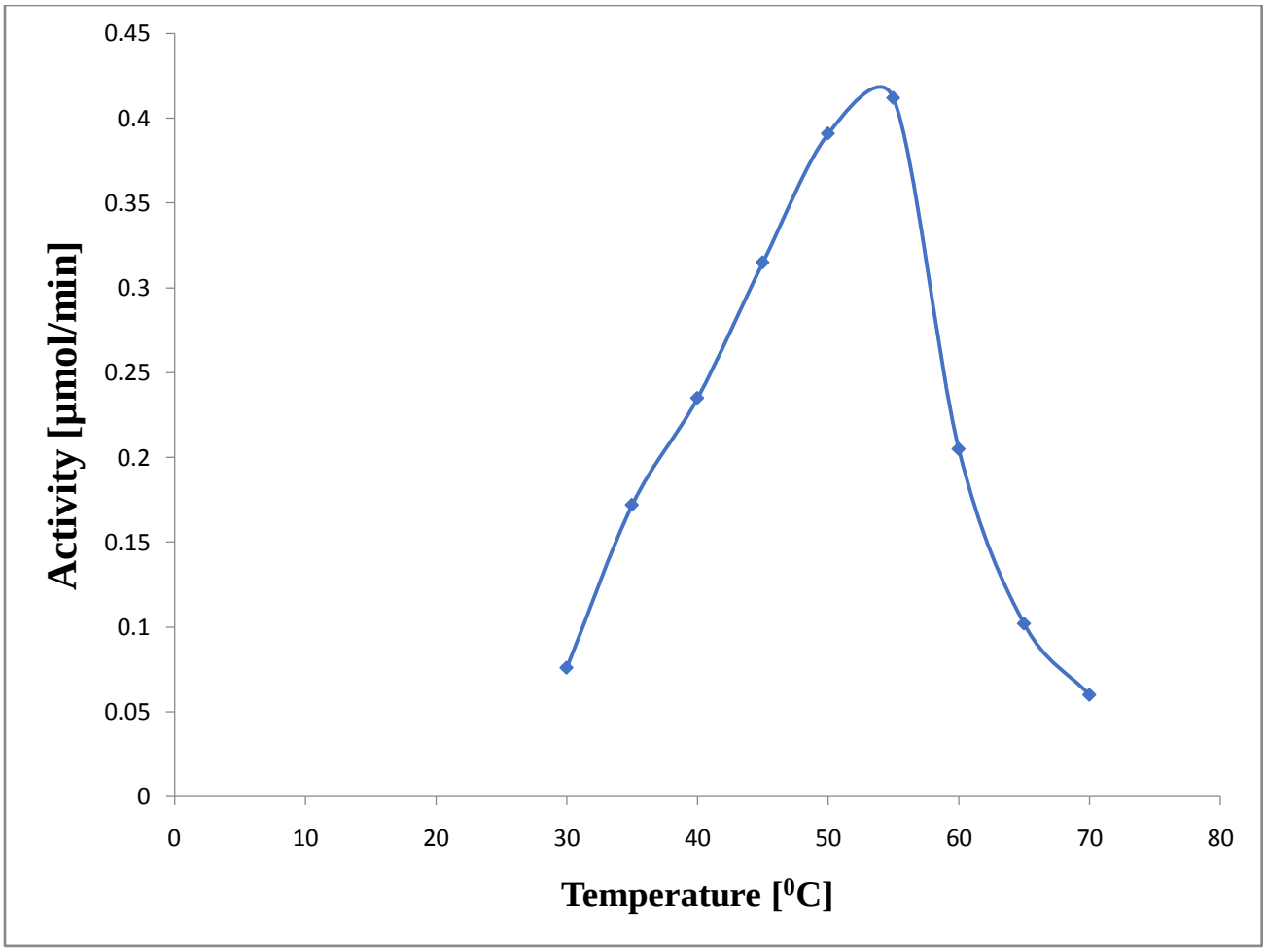

Figure 4.2: Effect of Temperature on Chitinase Activity from African Yam Beans

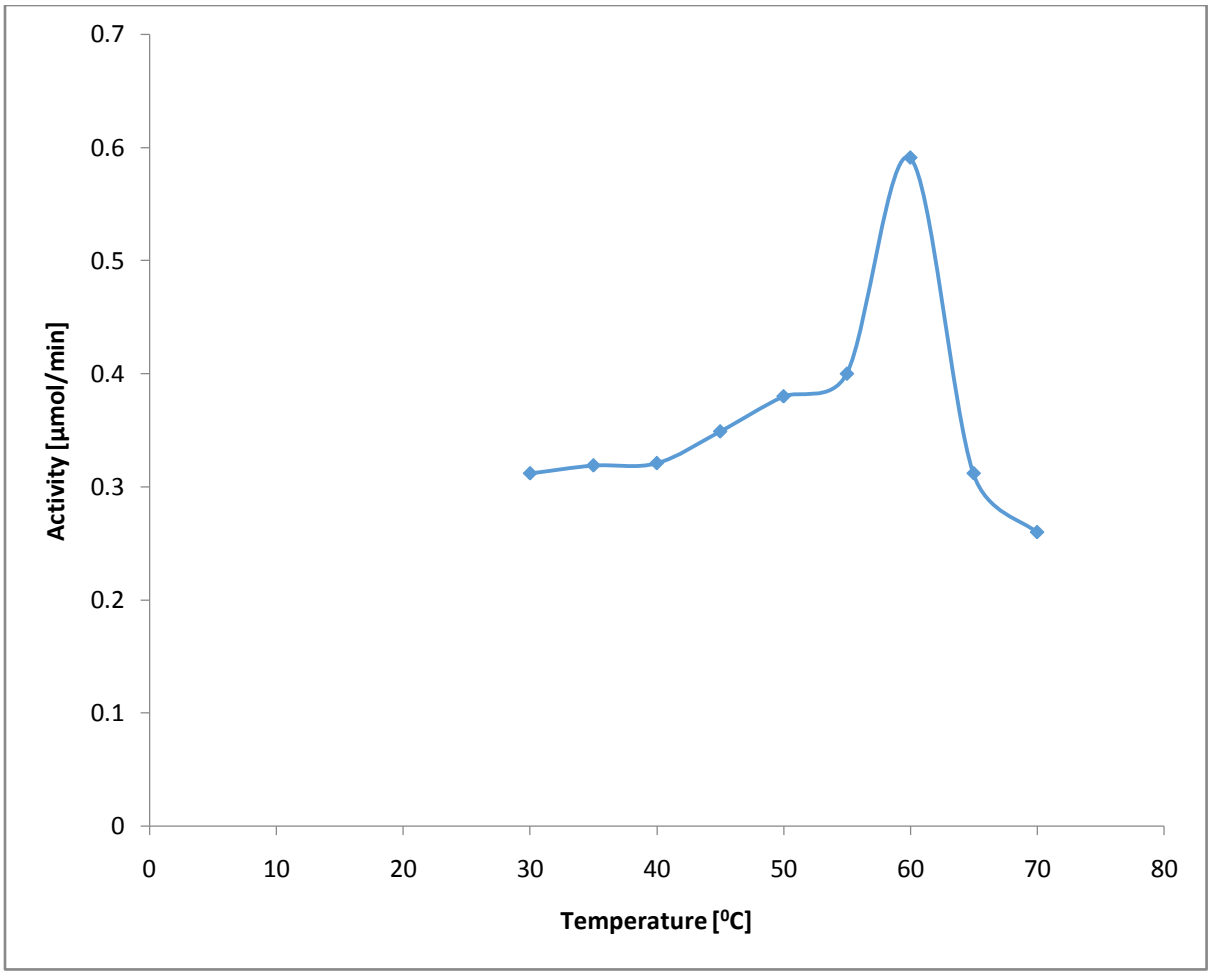

Figure 4.3: Temperature Stability of Chitinase Activity from African Yam Beans

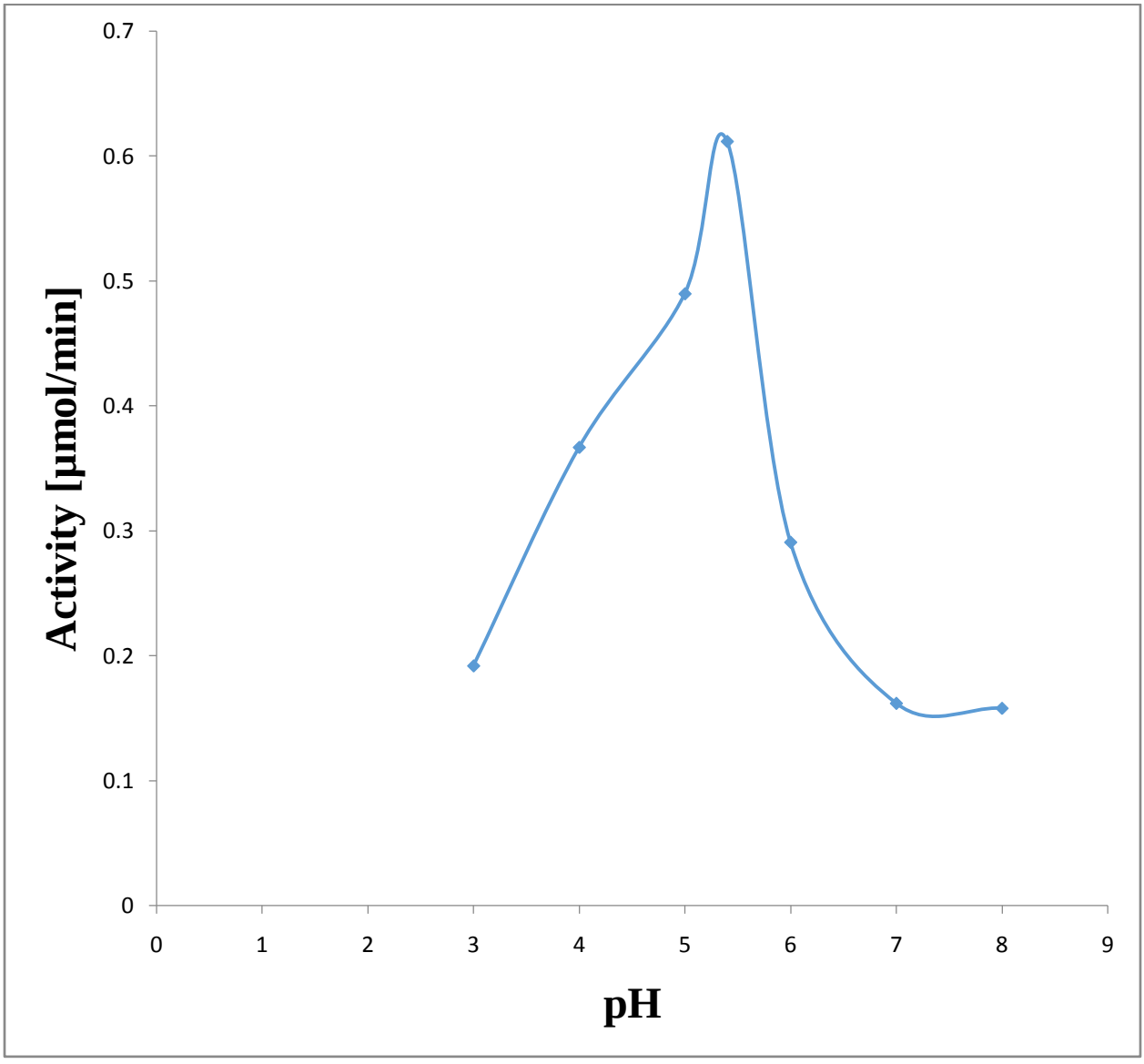

Figure 4.4: Effect of pH on Chitinase Activity from African Yam Beans

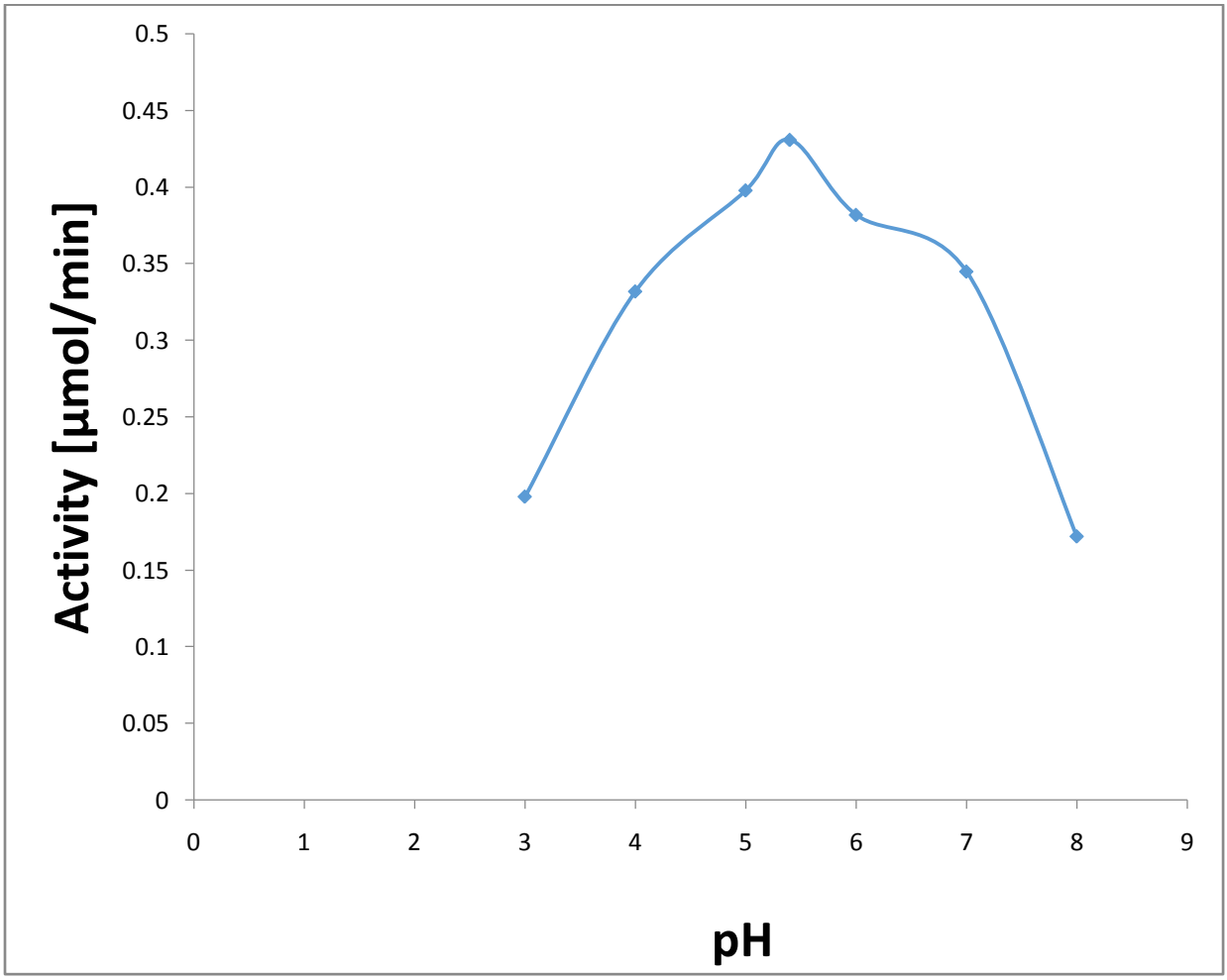

Figure 4.5: pH Stability of Chitinase from African Yam Beans.

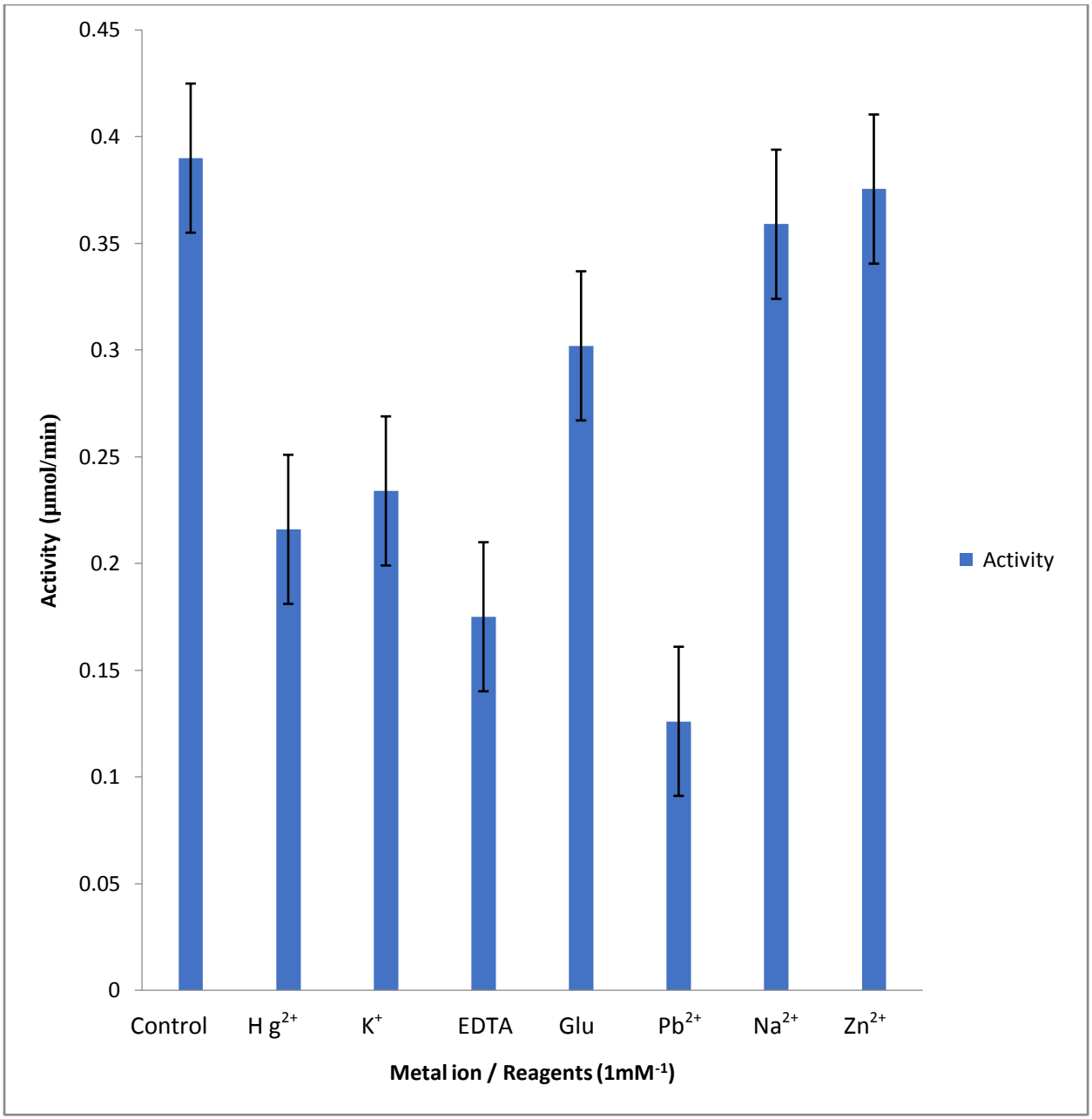

Figure 4.6: Effects of Cations/ EDTA and Glucose on Chitinase Activity

4.3.4 Determination of Kinetic Constants

The Michaelis-Menten constant and maximum velocity of the enzyme for the substrate hydrolysis presented in Lineweaver-Burk's plot in figure 4.7 revealed an estimated K_M of 1.2mM and V_{max} of 12.29μmol/min

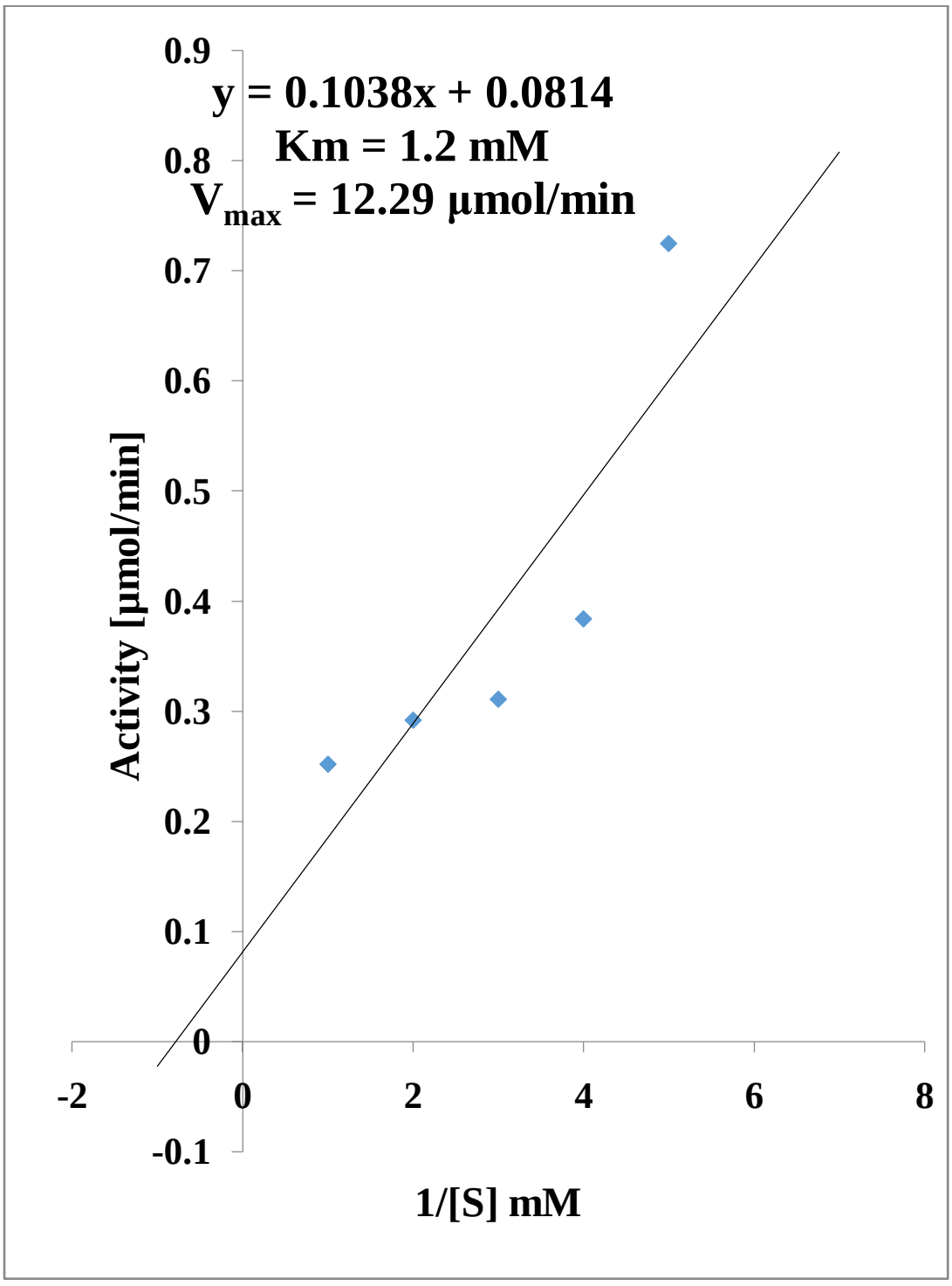

Figure 4.7: Lineweaver-Burk Plot for the Cleavage of *Chitin* by Chitinase

4.3.5 Antifungal assay

The results of the antifungal screening of the crude and purified extract against various fungal species are presented in the Table 4.2.The antifungal activity of Chitinase from African Yam Bean seeds were tested against different fungi, *Fusarium oxysporum*, *Fusarium solani* and *Alterneria alternata*. The protein had no effect on the two *Fusaria* species but inhibited the growth of *Alterneria alternate* in a dose dependant fashion as observed from inhibition zones measured.

Table 4.2: Effect of *Stephynostylis stenocarpa* on mycelial growth of test organism

Test Organism	**Diameter of zones of inhibition in (mm)/Conc. (µL)**				
	Crude		**Purified**		**Control**
Fusarium solani	0.00	0.00	0.00	0.00	0.00
Fusarium oxysponium	0.00	0.00	0.00	0.00	0.00
Alterneria alternate	14.00	17.00	9.00	11.00	20.00

CHAPTER FIVE

5.0 DISCUSSION

Chitinases (E.C 3.2.2.14) belongs to a group of pathogenic related proteins. In plants, Chitinases are believed to be involved in defensive roles. Due to this function, Chitinases possessing antifungal property received wide research attention (Kopparapu *et al.,* 2011; Wang *et al.*, 2012). They are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa. (Rifat *et al.,* 2013). They are present in a wide range of organisms such as bacteria, fungi, yeasts, plants, actinomycetes, arthropods, and humans. Chitinases have the ability to degrade chitin directly to low molecular weight chitooligomers, which serve a broad range of industrial, agricultural, and medical functions such as elicitor action and anti-tumor activity.

The enzyme Chitinase was found to have a molecular mass of 32 kDa both under native (gel filtration) and denaturing conditions (SDS-PAGF) (Plate 4.1). The molecular weight of Chitinase is closely similar to earlier reported plant Chitinases from *Itenmcasa hispida*, cranberry bean, maize, mung bean and pomegranate (Kopparapu *et al.*, 2011b; Wcissinger and Payne, 2004; Shih *et al.*, 2001; Wang, Wu. Rao, Ng and Ye, 2005 and Wang *et al.,* 2009). The molecular mass for most of the plant chitinases was reported to be within the range of 24-35 kDa (Moore *et al.,* 2004; Santos *et al.,* 2004; Wang *et al.*, 2005. 2009). But, some chitinases having molecular mass even up to 45 kDa was also reported from seeds, as in the case of pearl millet (Radhajeyalakshmi *et al.,* 2000).

African Yam beans exhibited its maximum enzyme activity at pH 5.0 (Fig. 4.4). Most of the plant Chitinases have their optimal activities at pH 3.0-5.0 However, plant Chitinases from *Ficusmicrocarpa.* Papaya, pine apple (chic), fava beans and oaf; exhibited (their optimal activities at pH 5.4-9.0 (Chen *et al.,* 2007; Sorensen *et al.,* 2010; Taira *et al.,*2005 and Wang *et*

*al.,*2012). The optimal pH of Chitinase from African Yam Beans is similar to Class I chitinases from pineapple (ChiA) and l*encuetia leufacephaia* (Kao-mek *et al.,* 2003; Taira *et al.,* 2005). Researchers have reported that plant chitinase exhibit broad pH stabilities ranging from 3.0-10.0. Chitinase exhibited broad pH stability ranging from pH 4.0-6.8 retaining more than 70% of its activity when incubated in different buffers for 30 min (Fig. 4.4) and this is similar to chitinase from fava beans which is stable only from pH 4.0-6.8 as reported by *Wang et al.,* (2012).

The pH stability of Chitinase was found to be different with other reported chitinases from pine apple (Taira *et al.,* 2005), *F. microcarpa* (Taira *etal*., 2005) and *Limoniumbicoior (Liu et al.,* 2010). Some plant chitinases are not stable in broad pH buffers, such as the chitinase from grapes, papaya and fava beans (Ano *et al.,* 2003; Chen *et al.,* 2007 and Wang *et al.,* 2012). This differences of the purified chitinase with regards to its stability and functionality could be because they are developmentally regulated and are tissue-specific. It could also be due to their amino acid sequence, were some have globular domains, some others could have 8 α-helices and 8 β-strands form. Being a class III Chitinase, it carries out the hydrolysis of the β-1, 4-glycosidic linkage by means of an inverting mechanism while some other class through a retaining mechanism (Fukamizo *et al.,* 2003). It is produced as pathogenesis-related proteins in plant self-defense in response to the attack of phytopathogens, or by contact with elicitors such as chitooligosaccharides or growth regulators such as ethylene (Gooday, 1996). There are some chitinases, which are expressed in response to environmental stresses, (i.e., high salt concentration, cold, and drought). There are also reports of some chitinases, which take part in vital physiological processes of plants, like embryogenesis and ethylene synthesis (Fukamizo *et al.,* 2003).

Chitinase from African Yam Beans exhibited an optimal temperature of 50 ^{0}C (Fig. 4.2). The optimal temperature of Chitinase is relatively higher than that of chitinase from maize, cranberry bean, fava bean and mung bean (Moore *et al.,* 2004; Wang *et al.,* 2005, 2012) and similar to those of chitinase from seeds of *Adeuanthera pavonina* L and rice (Mizuno *et al.,* 2008; Santos *et al.,* 2004). As shown in Fig. 4.3, Chitinase was highly thermostable and stable up to 60 ^{0}C. It retained 80% of its activity up to 60 ^{0}C when incubated for 30 min (Fig. 4.3). It displayed higher thermostability than the chitinase from grapes, L *bicolor,* fava bean and mung bean (Ano *et al.,* 2003; Liu *et al.,* 2010; Wang *et al.,* 2005, 2012). The chitinase from *A. pavonina I.* seeds was shown to be more stable than Chitinase from *S. stenocarpa* (Santos *et al.,* 2004). It can be said that the enzyme showed relatively, a good pH and thermostability. Nevertheless, the enzyme activity decreased drastically at temperatures above 70 ^{0}C. This decrease at high temperature could be as a result of thermal denaturation leading to conformational changes in the integrity of the enzyme which in turn affects activity. According to theory, the intramolecular bonds holding the structure of the enzyme in place were broken by heat leading to loss of active site specificity of the enzyme. Hence, it becomes denatured and cannot participate as a catalyst (Alyward and Haisman, 1969). The activity of chitinase was moderately inhibited *by* the metal ion K^+, Pb^{2+}, EDTA and sugar like glucose but was strongly inhibited by Hg^{2+}which is a common inhibitor for most of the chitinases. Zn^{2+} and Na^{2+} had little or no activity on the enzyme activity.

Chitinase exhibited antifungal activity against pathogenic fungus. It was found to strongly inhibit the growth of *Alterneriaalternate* (Plate VII). This indicates that it can serve as a good fungicides towards fungal diseases affecting leguminous plants such as Leaf blight and Leaf Spots.

Chitinase was able to inhibit the growth of *alterneria alternate* by distrupting its structural components. The fungal cell wall is composed of multiple layers where mannoproteins and

glucan make up more than 80% of the cell wall composition, while chitin represents less than 2% (Wagner and Sohnle, 1995). The Mannoproteins are predominantly expressed at the external surface while the plasma membranes of fungi are primarily composed of ergosterol (Wagner and Sohnle, 1995). They disrupt the cell membrane by interfering with the ergosterol biosynthesis pathway by inhibiting cytochrome P450-dependent 14-demethylase and blocking the oxidative removal of 14-methyl from lanosterol resulting into the structural changes in the lipid membrane. They are directed against lanosterol 14-alpha-demethylase, a cytochrome P-450 enzyme containing a heme moiety in its active site, in the ergosterol pathway. They can increase the permeability of the plasma membrane. They bind to fungal membrane sterol, resulting in the formation of aqueous pores through which essential cytoplasmic materials leak out and thereby destroying the proton gradient within the membrane (Vanden *et al.,* 1994 and Odds *et al.,* 2003) They also inhibit the synthesis of β 1, 3-glucan which is the major structural polymer of the cell wall (Balkis, 2002). They achieve this by inhibiting β-glucan synthetase which is responsible for the process.

The main biochemical and molecular mechanisms that contribute to antifungal resistance towards *fusarium oxysporum* and *fusarium solani* include reduced uptake of the drug, an active transport out of the cell or modified drug metabolic degradation of the cell, changes in the interaction of the drug to the target site or other enzymes involved in the same enzymatic process by point mutations, overexpression of the target molecule, overproduction or mutation of the target enzyme, amplification and gene conversion (recombination), and increased cellular efflux (Martinez-Rossi *et al.,* 2008, Sanglard and Bille, 2002).

Changes in the sterol and phospholipid composition of the fungal cell membrane and membrane fluidity may result into a decrease chitinase uptake by the fungal cell. Similarly, reduced

intracellular accumulation of the chitinase may occur due to increased active transport of the enzyme out of the cell. Resistance of the *Fusarium* species to Chitinase could also be as a result of gene mutation resulting in overexpression on proteins that acts as efflux pumps (Monk and Goffeau, 2008). It could also be as a result of point mutation in genes responsible for regulation of transcription. This mutations are called "gain-off mutations'' which activates downstream genes such ATP-binding cassette (ABC Transporter genes) and major facilitator super family genes(MFS Transporter genes). The products of this genes are efflux pumps that transports compounds out of the cell thereby reducing intracellular concentration to a sub-lethal level.

In summary, a chitinase with antifungal activity against *Alterneriaalternate* was isolated from African yam beans seeds. This is noteworthy since not all chitinases have been shown to possess antifungal activity. The purified chitinase was different from other reported chitinases from bean leaves and bean seeds in molecular mass, pH and temperature optimal. The observation that the purified protein showed antifungal activity toward *Alterneria alternate* will have important implications in agriculture.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

Protection of plants from disease produced by phyto pathogenic fungi is one of the most important challenges in agriculture. The total losses as results of plant diseases reach almost 50% of the crop in developing countries. One-third of this is a consequence of fungal infections. Therefore finding biological products that could be used as biocontrol agent is very important in agriculture. Recent studies demonstrated that chitinase from plants and microorganism are able to inhibit the fungal growth.

6.2 CONCLUSION

Chitinase isolated from *Stephynostylis stenocarpa*, was purified to 8.04-fold and a yield of 46.35% on Sephadex G-75 column with a final specific activity of 35.75µmol/min.

A novel Chitinase was partially purified from African Yam bean seeds which was suspected to be a monomer with a molecular mass of 32KDa from SDS-PAGE. The enzyme had broad temperature and pH ranges with optima of 55 ^{0}C and 5.0 respectively.

Chitinase exhibited maximum activity at pH 5.0 and 55 ^{0}C with broad pH and thermo stability.

The crude and partially purified samples were potent inhibitors of the growth of *Alterneria alternata*.

Chitinases with antifungal property can be used as biocontrol agents replacing chemical fungicides in protecting plants from *Altaneria alternata*.

6.3 RECOMMENDATION

i. Further research could be carried to improve the activity of the enzyme by carrying out further purification steps as well as cloning and expression studies.

ii. Gas chromatography mass spectroscopy GC-MS analysis of bioactive fractions of the enzyme can help in studying its antifungal property towards other pathogenic fungi as this can be helpful in agriculture and other industries.

iii. Chitinases can be exploited for their use as food preservatives, thereby increasing the shelf life of the foods. This research can be directed towards the identification of the active sites of chitinases and the novel functions associated with them. Protein engineering can be exploited for the production of chitinase with exclusive functions.

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APPENDICES

APPENDIX 1.0

CALCULATION FORMULA FOR THE PURIFICATION PROFILE

Total Protein = $\frac{\text{Total Activity}}{\text{Specific Activity}}$

Total Activity = Specific Activity * Total Protein

Specific Activity = $\frac{\text{Enzyme Activity (Total Activity)}}{\text{Total Protein}}$

Purification Fold = $\frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$

Percentage Yield = $\frac{\text{Total Activity of protein at a step}}{\text{Total Activity of Original Purification (Crude)}} * 100$

Enzyme Activity = $\frac{\text{Conc. Extrapolated from Std Curve * Vol. of Reaction Mixture}}{\text{Time in Min * Sample Vol.}}$

APPENDIX 2.0

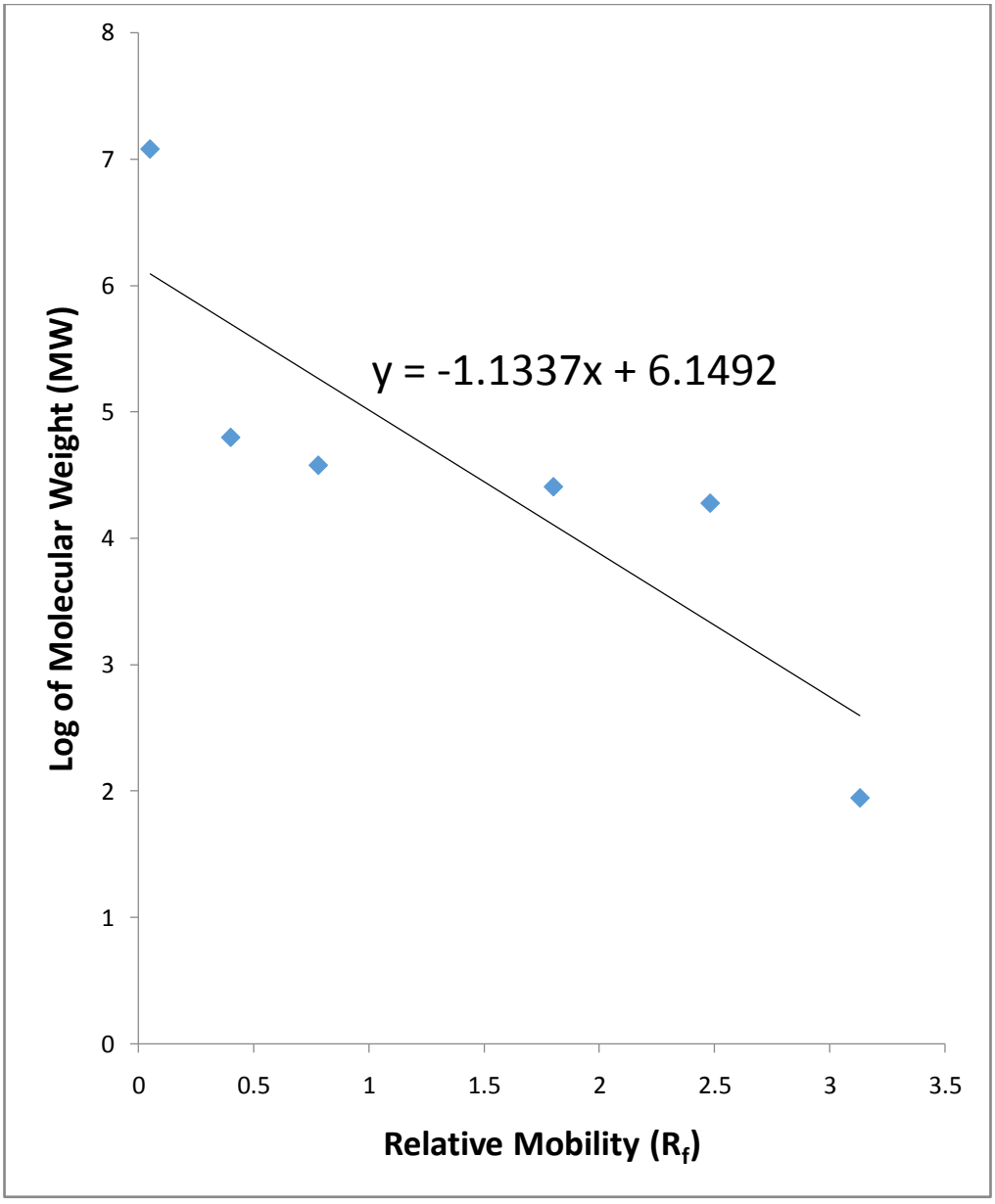
PREPARATION OF STANDARD OF REAGENTS

Preparation of SDS-PAGE Reagents

- **1.5 M Tris pH 8.8** exactly 181.65 grams of tris salt was weighed and dissolved in one litre of deionized water
- **0.5 M Tris pH 6.8** was prepared by weighing 60.5 grams of tris and dissolved in one litre of deionized water
- **10% SDS** was prepared by dissolving 10 grams of SDS in 100ml of deionized water
- **30%Bis-Acrylamide Solution** exactly 10 grams of Acrylamide and 2 grams of Bis were weighed and dissolved in 100ml of deionized water
- **Running buffer working solution** was prepared by weighing 1 gram SDS (1% SDS), 14.4 grams glycine(0.192 M glycine), and 3.03 grams tris (0.025M Tris pH 8.3) dissolved in one liter of deionized water
- **Loading buffer (×5)** this constitute the following; 2.5ml of 1 M Tris pH 6.8, 4ml of glycerol, 0.8 gram of SDS, 2ml of β-mercaptoethanol, Bromophenolblue and distilled water to final 10ml
- **Separating gel (12%)** was prepared by mixing the following: 3.35ml of deionized water, 2.5ml of 1.5 M tris pH 8.8, 0.1ml of 10% SDS, 4ml of 30%Bis-acylamide solution, 50µl of 10%Ammoniumpersulphate (APS) (0.1g/ml; made fresh) and 15µl of TEMED to a total volume of 10ml
- **Staking gel (4%)** this was prepared by mixing the following in a test tube; 3ml of deionized water, 1.25ml of 0.5 M Tris pH 6.8, 50µl of 10% SDS, 665µl of 30% Acrylamide, 25µl of 10% APS and 10µl of TEMED to a total volume of 5.0ml

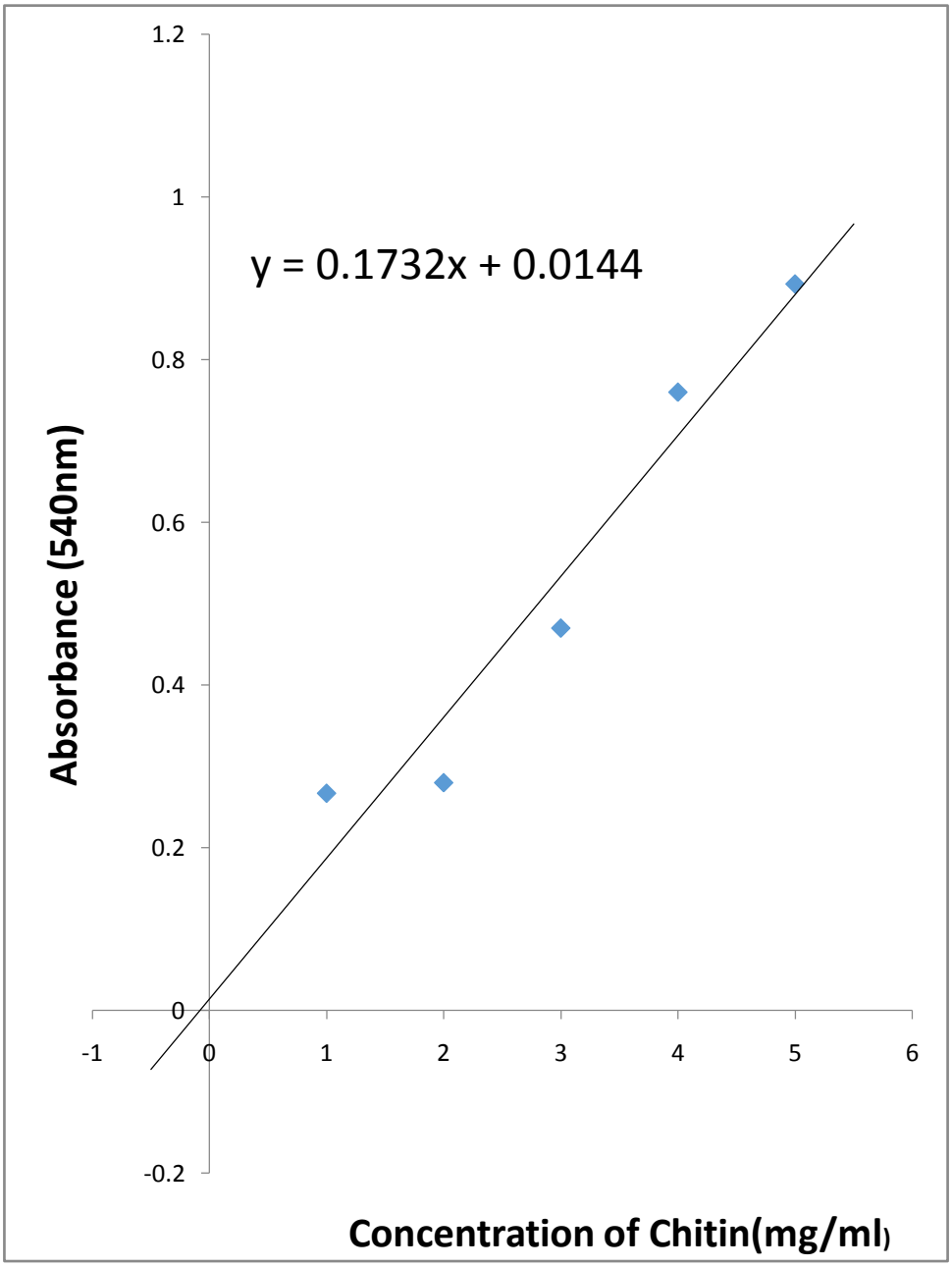
APPENDIX 3.0

A PLOT SHOWING THE LOG OF MOLECULAR WEIGHT AGAINST THE R_f OF THE MARKER-PROTEIN BANDS WHICH WAS USED TO ESTIMATE THE MOLECULAR WEIGHT OF THE PROTEIN SAMPLE, AND FOUND TO BE **31.501 KDa.**



APPENDIX 4.0

STANDARD CURVE FOR ENZYME ACTIVITY USING CHITIN



APPENDIX 5.0

STANDARD CURVE FOR PROTEIN CONCENTRATIONUSING BSA

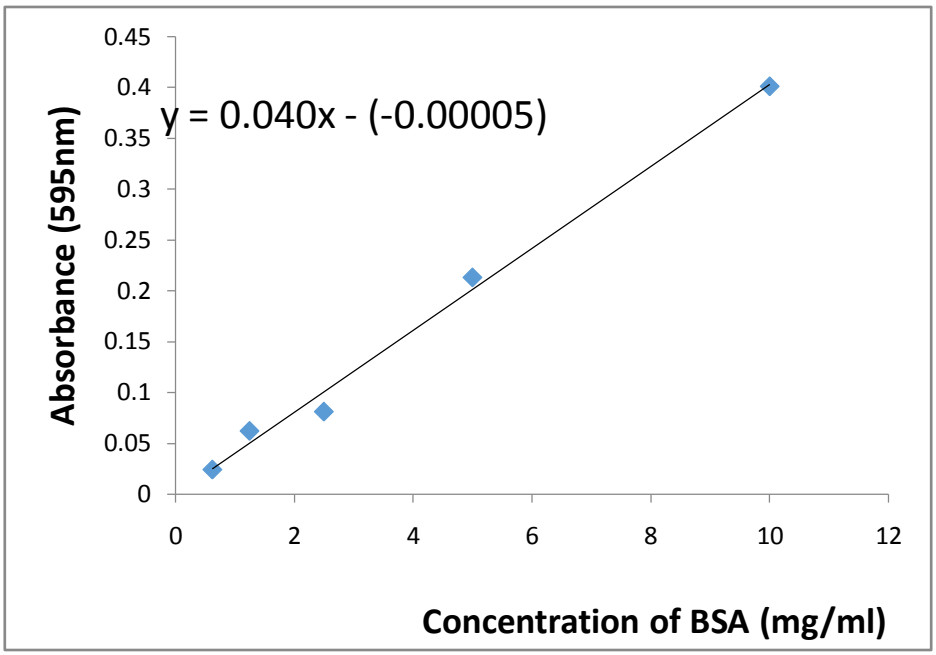


PLATE VII: Effect of chitinase from African Yam beans *Sphenostylis stenocarpa* on *Alterneriaalternata*

A = 500µL crude extract

B = 1000µL crude extract

C = **500µL purified extract**

D = 1000µL purified extract

PLATE VIII: Effect of protein chitinase from African Yam beans *Sphenostylis stenocarpa* on *Fusarium Oxysporum*

A = 500µL crude extract

B = 1000µL crude extract

C = **500µL purified extract**

D = 1000µL purified extract

PLATE IX: Effect of chitinase from African Yam beans *Sphenostylis stenocarpa* on *Fusarium solani*

A = 500µL crude extract

B = 1000µL crude extract

C = **500µL purified extract**

D = 1000µL purified extract

PLATE X: Effect of Benlyate on *Alterneriaalternata* (Control)

A= 500µL of Benlyate

B = 500µL of Sodium acetate buffer