Purification and Characterization of Cellulase from *Aspergillusniger* Causing Soft Rot of White Yam in Three Yam-growing Environments in Nigeria

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Abstract- Aspergillus niger isolated from diseased yam in three yam-growing zones in Nigeria synthesized cellulase (EC.3.2.1.4) which caused soft rot of the yam within nine days of inoculation. Microscopic and molecular analyses revealed two isolates of A. niger, P1 and P2, from yam growing in tropical rain forest and southern guinea savannah produced cellulase enzymes in significantly different proportions. When the protein extracts from the infection were subjected to molecular exclusion chromatography, three peaks of absorption (A, B and C) were produced with only the components of peak A showing cellulase activity. Further fractionation of the components of peak A by ion-exchange chromatography produced two absorption peaks (Aa and Ab) with only component Aa showing cellulase activity. A. niger isolates, P1and P2 showed considerable differences in the intensity of cellulase production suggesting that multiple strains of A. niger in the soil in different yam-growing environments synthesized cellulase as transcriptional products in different manner underscoring the effect of physico – chemical properties of the soil on infectivity and virulence of the organism during yam rot.

Index Terms- Aspergillus niger, soil, cellulase, gel electrophoresis, Yam

I. Introduction

Food security is considered very essential for human security. Yams (*Dioscorea spp*) are most important food crop in many tropical countries where it provides staple food for majority of the people [1]. *Dioscorea rotundata* is the most important of the various species cultivated in Nigeria and it is currently an export crop providing foreign exchange. As important as yam is in the economy, out of the world production of about 52 million tons, about 10 million tons (about 20%) are lost annually through deterioration in storage [2] endangering food security and potentials for export. The black mould rot of yam is caused by *Aspergillus niger*. The infection is characterized by decay of entire tuber during which the cells collapse with loss of integrity as a functional unit. In host-pathogen interaction, the ability of the pathogen to produce extracellular enzymes capable of degrading the host tissues is one of the more obvious properties influencing virulence [3,4,5]. *A niger* is abundant in most tropical soils and, invariably, on the surface of yam tubers while still attached to the plant and on the root hairs during harvesting or storage. Naturally, the peridermic surface of the tubers function to exclude pathogen but damage caused by accidental incision or cut surface during weeding, insect attack and harvesting provide avenue for the infection. According to [6], chemo – taxonomic characteristic is a virulence factor with variation arising from a multiplicity of factors including the nature of soil ecology and production of primary and secondary metabolites such as enzymes, aflatoxins and phytoalexins.

A.niger is able to synthesize cellulase enzyme complex that breaks down cellulose of the cell wall components into glucose [7] giving the organism access to utilize the tissues for its metabolism causing the collapse and disintegration of the cellular structure thereby aiding the pathogen in the propagation of the disease.

The environment in which a pathogen grows dictates to a large extent, the quality and nature of enzymes involved in the infection and degradation of the host tissues. This study examined the pattern of synthesis of cellulase as transcriptional product of *A.niger* causing soft rot of yam in three tropical rain forest and Southern Guinea Savanna of Nigeria.

II. Materials and Methods

2.1. Organism and Culture Condition

A.niger was obtained from diseased yam from three yam growing zones in the Southern Guinea Savanna and tropical rain forest zones of Nigeria. It was grown in Petri dishes containing Potato Dextrose Agar (PDA) medium in aseptic conditions. The plates were incubated at ambient temperature and then sub-cultured on the same media plates and the fungi spores from 72hr – old culture was used to inoculate healthy yam tubers.

2.2. Extraction and Purification of Enzymes

Inoculated and uninoculate tissues of yam in the three environment were extracted for enzyme activity every 24hr. The extractant was 0.5N NaCl in 0.02M citrate phosphate buffer (pH 5.0).

2.3. Precipitation and Dialysis of Enzyme

The enzyme was partially purified by ultracentrifugation followed by ammonium sulphate precipitation and dialysis for 24

hours at 40C against the buffer (pH 6.0) using acetylated cellophane tubing [8,9]. The protein content was determined by the method of [10].

2.4. Cellulase Assay

The assay for cellulase was determined by the modified Dinitro Salicylic Acid (DNSA) method of [11, 12]. Glucose was used as standard and one unit of cellulase activity was defined as the amount of enzyme in 1ml of the reaction mixture required to liberate reducing sugar equivalent to 10mg glucose in one minute under the specific condition of the reaction on application of enzyme.

2.5. G-75 Column Calibration and Gel Filtration

A vertical glass tube chromatography column (640 x 25mm) was calibrated with proteins of known molecular weights according to the method of [13], and employed for the fractionation of the enzyme. 10ml of dialysed enzyme concentrate was applied to the column and eluted with 0.02M citrate phosphate buffer (pH 6.0). Fractions were collected (5ml per tube) and protein content determined at 280nM.

2.6. Further Fractionation of Enzyme by Ion-exchange Chromatography

Eluted fractions which showed appreciable enzyme activity after gel fractionation was combined and 10ml of enzyme concentrate applied to the column (280 x 250mm) of Sephadex CM G-50 and the fraction were collected and measured for protein content and cellulase assay. The effect of physico-chemical factors of soil on purified enzyme (G-75 factor) was determined. The effects of temperature, pH and cations (k+, Na+ and Ca2+) on enzyme activity in the three yam growing zones were determine by the method of [14].

2.7. Morphological Identification

Each of the A. niger isolated from decayed yam in the three environments were identified using the manuals about the genus Aspergillus [15, 16]. Slides were stained with cotton blue and mounted in lactophenol. Photomicrographs were taken with digital canon camera (A550, 7.1 megapixels).

2.8. Preparation of Genomic DNA

The method of [18] was used for DNA isolation. Dissolved generic DNA samples from each of the A.niger isolates P1 - P6 were stored at - 200C.

2.9. DNA Amplification and Electrophoresis

Polymerase Chain Reaction (PCR) was carried out on the isolated DNA of A.niger from the different environments using the amplification kit and automated programmable PCR thermal cycler with ITS 1 and ITS 4 as primers at the DNA laboratory, Ungwan Sarki, Kaduna, Nigeria. Amplified fragments P1, P2, P3, P4, P5 and P6 were each separated in agarose gel in the electrophoretic chamber and Chemidoc was used to produce camera snaps of DNA band pattern.

III. RESULTS

3.1. Infection of Yam Tissues by A. niger

A. niger caused extensive degradation of healthy yam tissues at the point of infection irrespective of the source of yam. Extensive sporulation occurred at the point of inoculation. Tubers aseptically inoculated with sterile water lacked sporulation. Infection of the Yam tissues was very slow in yams placed on bare ground devoid of soil. Organism labelled P1 was isolated from yam growing in the Southern Guinea Savanna zone while both P1 and P2 were isolated from yam growing in the tropical rain forest zone.

When the organisms from rotted yam in the different yam-growing zones were sub-cultured on Potato Dextrose Agar (PDA), there were variations in the cultural appearance of conidiospores of A. niger appearing on agar plates. Structural morphologies of the isolates also showed some differential characteristics.

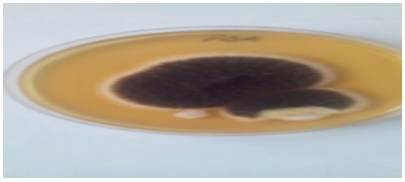

Plate I. Growth of A. niger Strain Isolate P1 from Infected Tubers on PDA Plates

Plate II. Growth of A. niger Isolate P2 from Infected Yam Tubers

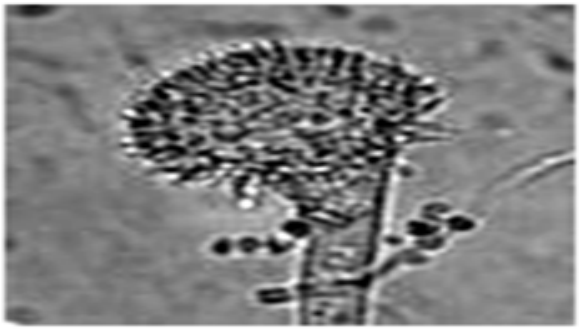

Plate III. Microscopic Features of A.nigerIsolate P1Stained withLactophenol in Cotton Blue (Mag.40x)

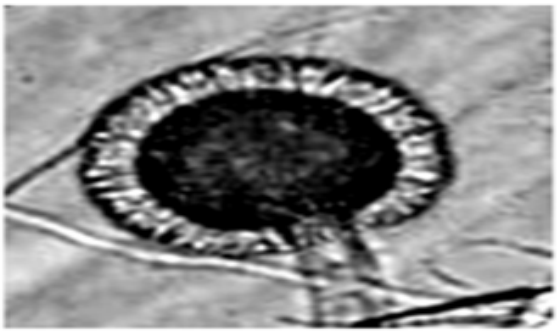

Plate IV. Microscopic Features of A. niger Isolate P2Stained with Lactophenol in Cotton Blue (Mag.40x)

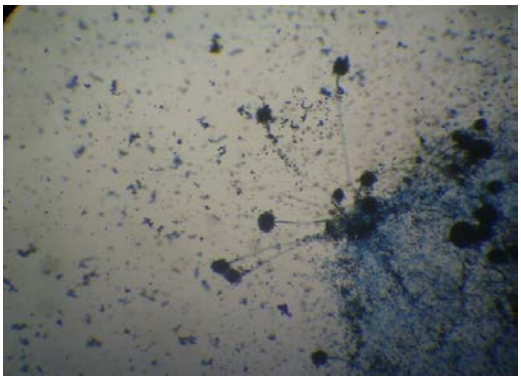

Plate V. Photomicrograh of *A.niger*Isolate P_1 Stained with Lactophenolin Cotton Blue

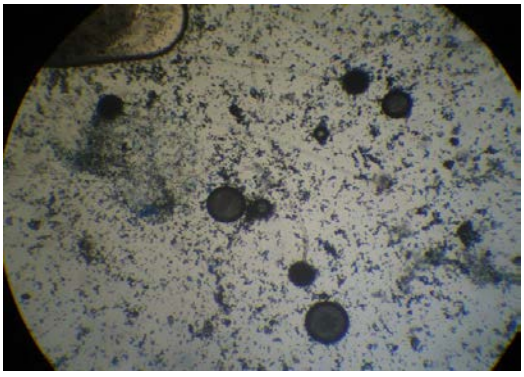

Plate VI. Photomicrograh of *A.niger*Isolate P_2 Stained with Lactophenolin Cotton Blue

3.2. Microscopic Examination of A. niger

Microscopy of P_1 and P_2 isolates showed a large black conidial that are arranged in a globose biseriate head arising from a spherical conidiophore. The black coloured colonies were identified as *A. niger* based on the structural morphologies as observed under the light microscope. It was observed that the isolates possessed distinct conidiophores terminated by a swollen vesicle bearing flask-shaped phialides. The black-coloured colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads similar to the observation of Ellis (2006). The organisms with the yellow basal felt (marked P_1 isolates) were isolated from Zone 1 Southern Guinea Savanna) while the ones with yellow and white basal felt (marked P_1 and P_2) were both isolated from Zones 2 and 3 (Tropical Rain Forest zones)

3.3 Macroscopic Characterization of P_1

Colonies on PDA plates attained 31mm in diameter in 7 days at 27^0C colony coloured brown to dark, reverse is light yellow and transparent (Plate I).

3.3.1.Microscopic Characterization of P1

The conidia head on PDA radiate, 80 – 100µ in diameter, conidiophores hyaline, smooth, 100 – 200µ long and 4-5µ wide with 1µ thick wall. Vesicle was globose and 10 – 18µ in diameter. Matulae was ampuliform 5 – 8µ by 1.5 – 2µwide. Phialides ampuliform, 5-7µ by 1.5-2µ wide. Conidia were globose and 1.5 – 2.5µ in diameter (Plate VII). The conidia head was compact columnar, 80-160µ long by 1.5 – 2µ wide and phialides ampuliform 5-8µ by 1.5 – 2µ wide. Conidia were also globose, 1.6 – 2.5µ in diameter

3.4 Macroscopic Characterization of P_2

The conidia based on PDA plates attained 55mm after 7 days at 30ºc colony colour was black (Plates II and III). The reverse side was mostly hyaline to light yellow.

3.4.1Macroscopic Characterization of P_2

Conidial heads on PDA plates radiate conidiophores that are 300-400µ long, 8-12µ wide with 1.5-2.5µ thick wall. Vesicles are globose and 3.5 -4µ in diameter. Conidial heads are also globose 3.5 – 4µ in diameter. Phialides are ampuliform and metulae club shaped.

3.5 Molecular Characterization

The two different isolates of *A. niger* identified through their cultural, and morphological characteristics, were further characterized molecularly. When the DNA of the two isolates ($P_1$and P_2) were prepared, quantified and amplified for agarose-gel electrophoresis in each case, six distinct bands were observed suggesting that there are differences in the nucleotide base sequences coding in the two *A. niger* isolates for enzyme production in different isolates. The molecular detection and amplification of the gene coding for *A. niger* cellulase enzymes associated with yam rot was shown in the gel electrophoresis of the DNA fragments of *A.niger* isolates from different yam-growing environments. This is evident with the appearance of bands on the extracted DNA of *A.niger* as shown in Plate VII. The

extent of yam rot may depend on the sample of the *A.niger* associated with the different soil or environment. The strains of *A. niger* may vary depending on their location and the arrangement of the nucleotide sequences in the genome of the organism. This results suggest that cellulase, is associated with yam soft rot as observed from different environmental conditions and that the extent of rot is also dependent on the strain of *A. niger* that confers differences in pathology.

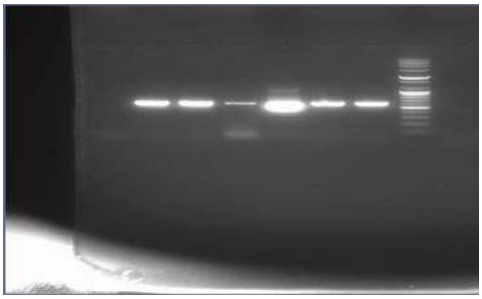

Plate VII: Agarose Gel Electrophoresis: Molecular Detection and Amplificationof DNA of A.niger Isolates fromRotten Yam

3.6. Infection of Yam by A. niger and Enzyme Production

Yam tissues infected with A. niger isolates exhibited cellulase activities (Figures 2, 3, 4, 5 and 6). Generally, the activity of each of the enzymes increased with the intensity of infection. Uninfected yam tissues lacked any appreciable decline in the infection of yam. In Zone 1 (Figure 2) the activity of cellulase increased progressively and continued until the eight day where it reached a maximum and in the ninth day when it started to decline. However, enzyme activity of A. niger from zones 2 and 3 could be noticed as from the second day of inoculation. Cellulase was the first to be detected in appreciable quantity. The activity of enzymes in yam Zone 2 continuously increased to the tenth day when it started to decline (Figure 2). An almost similar pattern of increase and decline was observed in yams infected with A. niger in yam Zone 3 (Figure 4).

The nature of the soil around which the tuber is grown or harvested and their physico-chemical properties was observed to contribute to the intensity of enzyme production, infectivity and tuber damage. Sporulation of A. Niger and yam rot was very slow when the yam was placed on bare ground. In some cases, sporulation was restricted to the zone of inoculation and rotting took a longer time than when the yam was damaged while attached to the plant or placed on the soil in ambient conditions after harvest underlining the influence of soil ecology on infectivity.

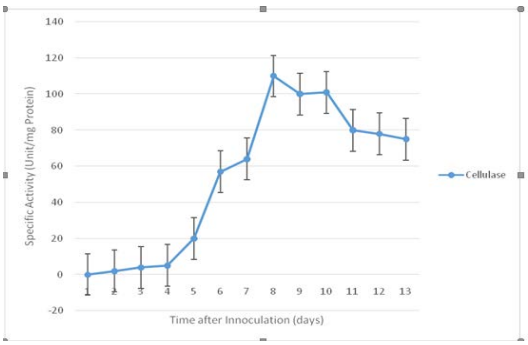

Figure 1. Enzymic Activities of Yam Tissues Infected by A. niger
(P1Isolate)in Env. 1(Southern Guinea Savanna) by Incubation Period

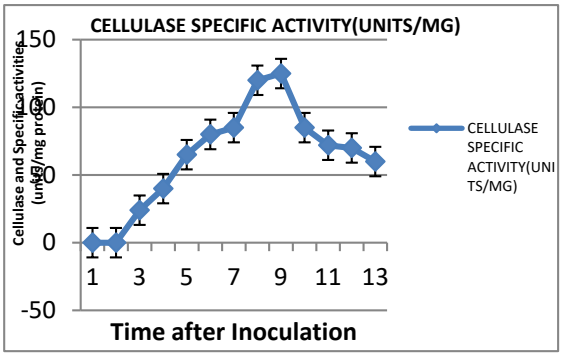

Figure 2. Enzymic Activities of Yam Tissues Infected by A. niger(P1 Isolate) Env. 2 (Tropical Rain Forest zone) by Incubation Period

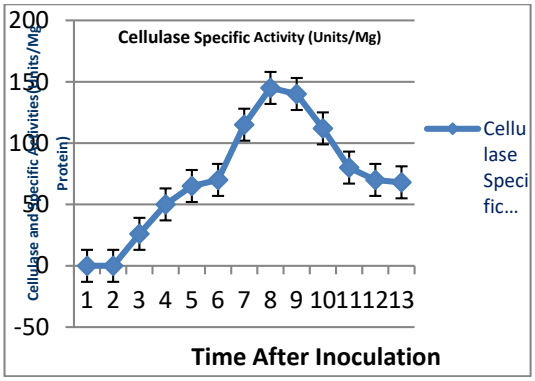

Figure 3. Enzymic Activities of Yam Tissues Infected by A. niger(P1 Isolate) Env. 3 (Tropical Rain Forest zone) by Incubation Period

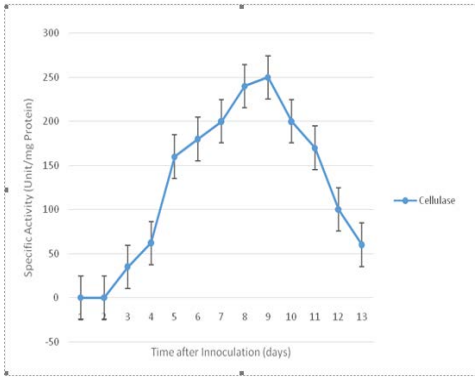

Figure 4. Enzymic Activities of Yam Tissues Infected by *A. niger*(P_2 Isolate) Env. 3 (Tropical Rain Forest zone) by Incubation Period

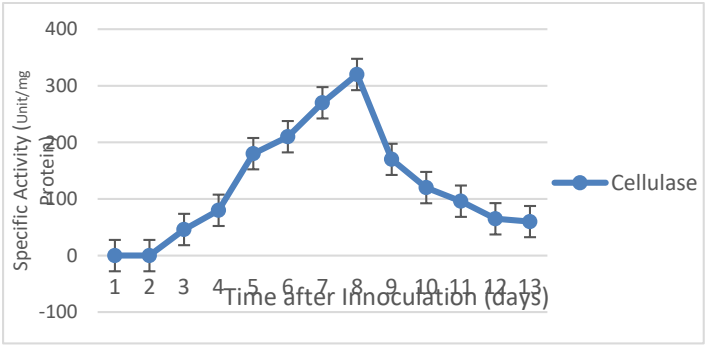

Figure 5. Enzymic Activities of Yam Tubers Infected by *A. niger*(P_2 Isolate) in Env. 3 (Tropical Rain Forest zone) by Incubation Period

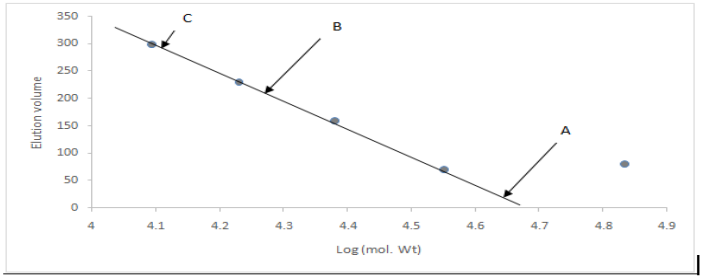

Figure 6. Elution Volume against log (M.WT) for proteins in Peaks A,B and C Calibrated by a Gel Filtration with Sephadex G-75

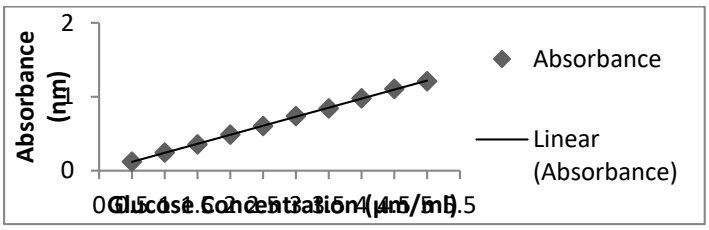

Figure 7. Standard Curve for Known Concentrations of Glucose at 540nm

3.7 Purification of Enzymes

3.7.1 Enzyme Separation on Sephadex G-75 and CM G-50

Fractionation of the enzyme concentrate on Sephadex G-75 produced three absorption peaks marked A, B and C. (Figure 8). The molecular weights of the components estimated from the calibration using their respective elution volumes are about 44,670 (Peak A), 17,780 (Peak B) and 12,590 (Peak C). The activities of cellulase was detected only in Peak A.

Further fractionation of Component A on CM-Sephadex CM-C50 gave two new absorption peaks marked (Aa) and (Ab) respectively (Figure 9). Similarly, the activity of cellulase was detected only in Peak (Aa).

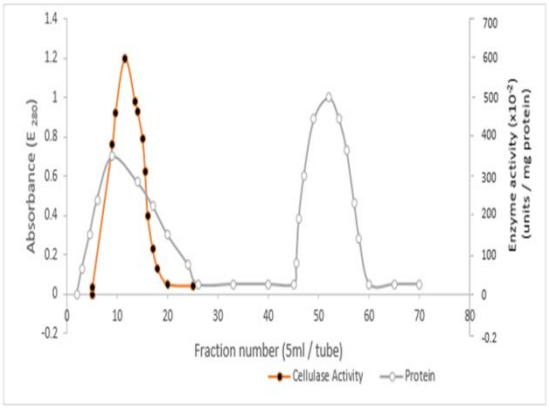

Figure 8. Figure 8.Separation of Protein in the Concentrated Extract of *A.niger* Infected Yam Tissues by Molecular Exclusion Chromatography and the Enzymic Activity of the Fraction towards Pectin and CM-cellulase

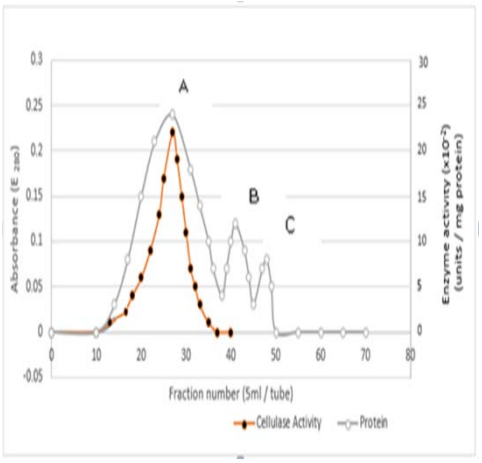

Figure 9. Separation by Ion-exchange Chromatography of High Molecular Weight Proteins (Fractions 12-50) Produced from *A. niger* Infected Yam Tissue Extracts by Gel Filtration and the Enzymic Activity of the Fractions towards Cellulase and Pectin.

3.7.2 Purification Table of Cellulase

Table 1 shows the purification levels of cellulase. The total activity of the crude enzyme at maximum yield was 29,400 units and specific activity of 73.3 units/mg protein. These mean activity values were derived from the replicates with the limit of standard error. When the proteins in the crude enzyme were subjected to

ammonium sulphate precipitation, the specific activity increased to 209.3 units/mg protein and a yield of 85.4% was obtained with 2.9 fold purification. Further fractunation and purification by molecular exclusion chromatography (Sephadex G-75) and ion-exchange chromatography (CM-Sephadex G-50) yielded much more purified enzyme up to 30.7 fold by gel filtration and 77.3 fold by ion-exchange chromatography. The specific enzyme activity increased to 2,215 units/mg protein and 5,581 units/mg protein by gel filtration and ion-exchange chromatography respectively. The partially-purified cellulase enzyme, G-75 fraction was used for further analysis

Table 1: Partial Purification of cellulose Obtained from Yam Infected with *A. niger*

Purification Step	Total Protein (mg)	Total Activity (Units)	Specific Activity (units/mg Protein)	Yield %	Purification (Fold)
Crude Enzyme	402 ± 1.0	29400 ± 1.7	73.3 ± 0.9	100	1.
$(NH4)_2S0_4$ Precipitate	120 ± 0.7	25120 ± 2.0	209.3 ± 1.0	85.4	2.9
Sephadex G-75 (Peak Aa)	8.4 ± 0.2	18610 ± 1.0	2215.5 ± 1.2	63.3	30.7
CM-Sephadex G-50	[2.9 ± 0.1	16185 ± 0.8	5581.0 ± 0.8	55.1	77.3

3.8 Effect of Soil Temperature

As shown in Figures 10, 11 and 12, optimum enzyme activity was observed at about 40ºC in yam rot caused by *A. niger* in all the three zones (Zones 1, 2 and 3). Temperatures lower than 25ºC and higher than 40ºC resulted in diminished enzymes activity and the enzyme was almost completely inactivated at 50ºC. This indicated that the quantity of cellulase activity was completely influenced by temperature. *Aspergillus niger* growing on yam tubers in the three ecozones achieved maximum sporulation and enzyme activity at ambient temperature of 40ºC.

In Zone 1, the enzyme activity of cellulase was very slow at temperature of 20^0Cand 30^0C. This might have contributed to the slow sporulation at the temperatures lower than 35^0C.

In Environment 2 (Tropical Rain Forest zone), there was a steady increase in the activity of cellulase as from20^0C until the activity of enzyme reached a maximum at 40^0C.

Enzyme activity of *A.niger* cellulase was affected by increasing soil temperature in Environment 3 (Tropical Rain Forest zone). The enzyme activity was optimum at 40^C after which increasing the temperature above resulted in a sharp decline of activity.

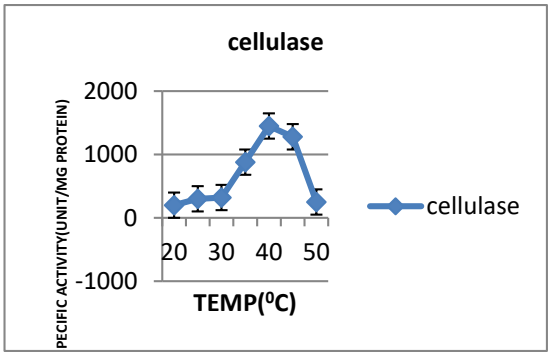

Figure 10. Effect of Soil Temperature on Cellulase Activity of ProteinExtracts of Infected Yam in Env.1(Guinea Savanna zone)

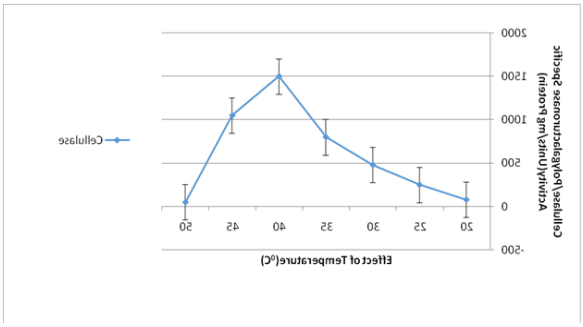

Figure 11. Effect of Soil Temperature on Enzyme Activity of ProteinExtracts of Infected Yam in Env. 2 (Tropical Rain Forest zone)

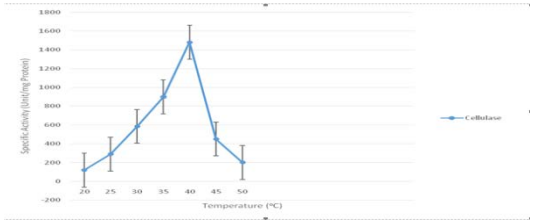

Figure 12. Effect of Soil Temperature on Enzyme Activity of Cellulase Extracts On Infected Yam in Env.3 (Tropical Rain Forest zone)

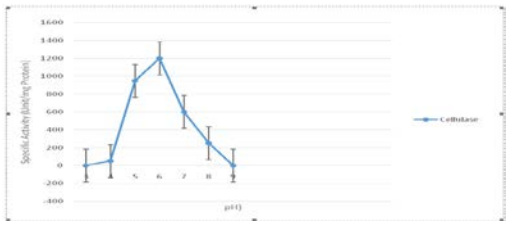

Figure 13. Effect of pH on Enzyme Activity Activity of Protein Extracts from YamInfected with A. niger in Env. 1 (Guinea Savanna zone)

3.9 Effect of Soil pH

Figures 14, 15 and 16 showed that the intensity of enzyme production is greatly influenced by changes in the hydrogen ion concentration (pH) of the soil. Optimum production occurred in slightly acidic medium of between pH 4.0 and 6.0 in the three environments.

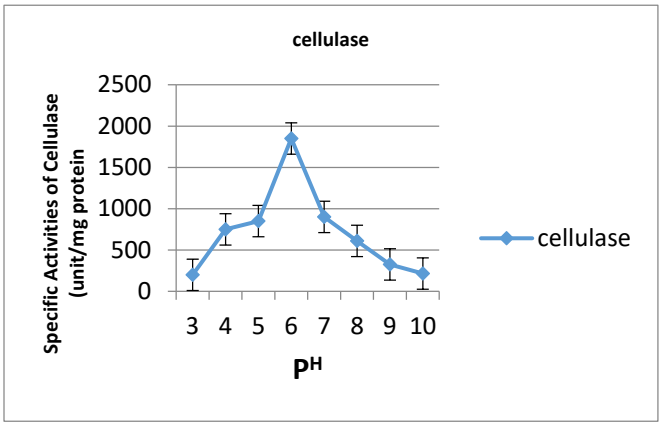

Figure 14: Effect of Soil pH on Enzyme Activity of Protein Extracts in Infected Yam from Env. 2 (Tropical Rain Forest zone)

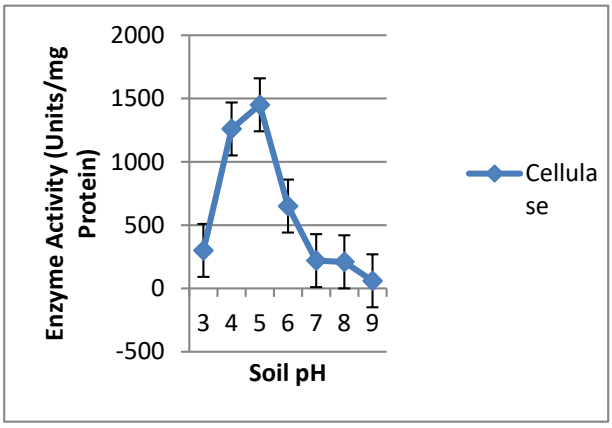

Figure 15. Effect of Soil pH on Enzyme Activity of Protein Extracts in Infected Yam from Env. 3 (Tropical Rain Forest zone)

Cellulase enzyme can tolerate high concentration of cations of up to 100mM for Ca^{2+},Na^{2+} and K^{+} with simulating effects before enzyme activity began to slow down gradually.

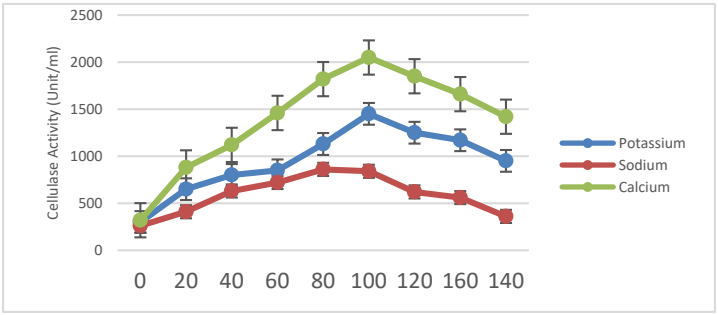

Figure 16. Effect of Cations on the Activity of Partially-Purified Cellulase (G-75 Fraction)

IV. DISCUSSION

The results showed that *A. Niger* caused extensive degradation of yam tissues after nine days of infection. The statistical significance is in agreement with the practical significance buttressed in the analysis of variance (ANOVA) of the mean of replicate values across the three environments which showed significant variations in the extent of cellulase produced. The sum of squares of between group was moderately high (23280.150 at 2,38, df respectively). The value of p = 0.48 is less than 0.05 indicating statistical significance in the pattern of cellulase production in the infection of yam by *A. niger*. The results of multiple comparisons also indicated that the mean difference in the intensity of enzyme production in the three yam – growing environments studied is significant at 0.05 level.

Soil ecology has also been observed to play a prominent role in the infection process as washed yams that were placed on bare concrete floor without soil on them took a much longer time to register decay of the yam tissues.

The increase in the rate of enzyme production and infectivity might be attributed to the virulence factor of the isolate of *Aspergillus niger* involved in the infection which has contributed to its pathogenicity.

Identified through their cultural, microscopic and morphological characteristics, the isolates of *A. niger* labelled P1 and P2 (isolated from infected yam) were the same organisms isolated from the soil of the yam-growing areas investigated.

When the DNA of the two isolates (P_1 and P_2) were prepared, quantified and amplified for agarose gel electrophoresis, six distinct bands were observed suggesting that there are possibly differences in the nucleotide bases sequences in the two *A. niger* isolates in the three environments. This may also be responsible for the variations in the production of cellulase as an aid to infection of yam in the three yam-growing environments studied. The mere fact that yam tubers placed on bare soil without the effect of the physico-chemical properties of the soil ecology playing a facilitative role failed to develop appreciable sporulation and decay at the point of infection emphasized the importance of the soil ecology on infection. Yam tubers grow in the soil and when harvested usually have soil particles attached to the root hairs. The knowledge of the understanding of the physico-chemical properties of the soil ecology is important to quantify the conditions that are favourable for infection and disease development.

This may provide insights into the development of measures that can be used to prevent or slow down the infection. This may also lead to the devising of mechanisms for preservation of the tubers while still attached to the plant in the soil and inhibit or slow down deterioration during storage. In a similar vein, having proper knowledge of the genetic blueprint for enzyme production by strains of *A.niger* will increase the understanding of the organisms metabolic pathway that can be used to slow down or prevent the infection process and increase the shelf-life of yam during storage. This of course, can be applied to other crops facing soft rot during storage.

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