*Original Research Article*

# Production of Fungal Pectin Lyase and Polygalacturonase from fruit wastes by solid state fermentation


## ABSTRACT

Polygalacturonase (PG or PGase) and Pectin lyase (PL) are depolymerase enzymes that split the α-1, 4-glycosidic linkages in the backbone of homogalacturonans. They are produced by microorganisms degrading pectin-containing substrates. PG and PL were produced in the solid-state fermentation (SSF) of beans testa (BT), mango peels (MP), Plantain peels (PP), and BT: MP: PP (1:1:1). Substrates were seeded with individual fungal strains namely *Aspergillus tamariii*, *Aspergillus terreus*, *Aspergillus piperis, Aspergillus parasiticus* and *Mucor piriformis.* PG production ranged from 0.0377 - 141.0095U/g with *A. tamarii*, *A. terreus* and *A. piperis* producing the highest on mango peels. PL production ranged from 50.50 - 10,852.50 U/g with *Mucor piriformis* producing the highest on plantain peels. The pH of the fermentation medium changed during growth, metabolism, and pectinase production. The best pH for pectinase production falls within the acidic range. Unconventional substrates such as PP are viable for pectinase production, and PL yield in SSF is improved by compositing substrates.



*Keywords:* Fungi, Polygalacturonase, Pectin lyase, Fruit waste, Solid State Fermentation


## 1. INTRODUCTION

There is a steady rise in demand for fruit and vegetables because of the increased awareness of their nutritional benefits. Consequently, global fruit production doubled from 401.61 to 887.03 million metric tons from 1990 to 2020 [1]. The resulting fruit waste imposes high health, environmental, and recycling challenges on nations. Fruit processing industries generate tons of waste, and about half of the fruits produced globally are wasted annually [2; 3]. Fruit wastes are rich in pectin and are known substrates for fungal pectinases production 4; 5].

Diverse fungal species degrade these pectin-rich substrates to meet their carbon and energy needs using depolymerizing pectinases like polygalacturonase (PG or PGase) and Pectin lyase (PL)[5]. Pectinolytic enzymes are classified according to their way of attack on the galacturonan part of the pectin molecule. The three types of pectinases are protopectinase, esterase and depolymerase. Depolymerases (PG and PL) split the α-1, 4-glycosidic linkages in the backbone of homogalacturonans by hydrolysis or trans-elimination [6].

Pectin lyase (or polymethylgalacturonate lyases) is a pectin depolymerase of the endo-type. It has great affinity for long, highly methylated chains and acts by β-elimination of methylated α-1, 4-homogalacturonan with the formation of $C_4 – C_5$ unsaturated oligo-uronides [6]. It is the only known pectinase capable of lysing α-1, 4-bonds of highly esterified pectin without

previous action of other pectinases [7]. This pectinase is produced by fungi and bacteria only [8].

PGase is a hydrolytic pectin depolymerase produced by microbes and in plant tissues [8]. It exists in three forms: Endo-PG, Exo-PG, and rhamno-PG. Both types act only on pectin with a degree of esterification of less than 50-60% [9]. Endo-PG acts randomly at the α-1,4-polygalacturonic backbone and results in a pronounced decrease in viscosity, Exo-PGs act at the non-reducing ends of the chain releasing small fragments from the chain and does not significantly reduce the viscosity while rhamno-PG catalyze pectin split at the non-reducing rhamnogalacturonan chain [5].

Pectinolytic enzymes are used in food, textile, and wastepaper industries. They are non-polluting biochemical catalysts available in dry powdered and liquid forms. Enzymatic liquid goes into process medium instantly and disperse evenly through the application process as opposed to powdered products [10].In the food industry, they are used for fruit juice extraction and clarification, coffee and tea fermentation, oil extraction, improvement of chromaticity and stability of red wines [6]. Pectin lyase is a major component of commercial preparation in fruit juice and wine industries because it decreases viscosity without damaging the volatile ester content responsible for specific aroma of various fruits [7]. Apple, grape, and passion fruit juice have been effectively clarified by pectin lyase [11].

Pectinase production is expensive due to several factors, including substrate cost. There is ongoing research into finding cheap and locally available substrates suitable for pectinase production. Peels of banana, mango, citrus, and oranges are substrates used for pectinase production [5]. However, new substrates would offer additional options to pectinase-producing industries. The aim of this study was to produce polygalacturonase and pectin lyase from fruit wastes readily available in Nigeria as substrates. Fungal isolates were used because of their ability to degrade pectin-containing wastes producing pectinolytic enzymes in the process.

## 2. MATERIAL AND METHODS

### 2.1 Microorganism

The fungi species used in this study were isolated and identified as reported by Amande and Adebayo-Tayo [4]. They were maintained on Potato Dextrose Agar (PDA) slants, stored at 4$^{o}$C and sub-cultured fortnightly.

### 2.2 Substrates

Fresh plantain and mangoes were purchased from Bodija Market in Ibadan, Oyo state, South West Nigeria and were conveyed in clean plastic containers to the Postgraduate Laboratory of the Department of Microbiology, University of Ibadan. They were washed thoroughly with tap water to remove adhering substances. Using a clean knife, the fruits were peeled into clean trays labeled accordingly. The peels were minced into pieces and hot air oven dried at 55$^{o}$C until a constant weight was achieved [12]. They were ground into powder (particle size 300µm) and sealed in polyethylene bags ready for analysis. The Nigeria Beans Testa was collected from beans cake (akara) sellers in Bodija market.

### 2.3 Preparation of Inoculums

The inoculum was prepared as described by Amande et al. [13]; 1mL of the inoculum was used to inoculate the fermentation medium.

## 2.4 Fermentation

Solid-state fermentation (SSF) was carried out in 250 mL Erlenmeyer flask containing 15 g of substrate and 10mL of distilled water. The substrate was sterilized at 120$^{o}$C for 40 minutes [14]. One mL of the inoculum was used to inoculate the fermentation medium. The flasks were incubated at room temperature (25±2$^{o}$C) for 12 days. To estimate the Polygalacturonase, Pectin Lyase and Total Protein, 30mL of sterile distilled water was added and mixed properly. The mixture obtained was shaken gently and filtered using nylon cloth. The filtrate was centrifuged at 15,000 rpm for 10 minutes using Hitachi high speed refrigerated centrifuge (HimacCR21GII, Max. rpm 21000, -20 – 40$^{o}$C) and the resulting supernatant was used for conducting the assays. Estimations and pH of the fermenting substrates were carried on day 3, 6, 9 and 12. At each time point, the pH of the fermenting medium was measured using a standard benchtop pH meter before the addition of sterile distilled water for subsequent analyses.

## 2.5 Enzyme Assay

### 2.5.1 Polygalacturonase(PG) Assay

Polygalacturonase (PG) activity was determined by measuring the release of reducing groups from citrus pectin using the 3,5-dinitrosalicyclic acid (DNSA) reagent assay [15]. The reaction mixture containing 0.8mL of 1% Citric Pectin in 0.2M acetate buffer, pH 5.0 and 0.2mL of crude enzyme solution, was incubated at 40$^{o}$C for 10 minutes using a modified method of Soares *et al.* [16]. One unit of enzyme activity (U) was defined as the amount of enzyme which released one µmole of galacturonic acid per minute.

### 2.5.2 Pectin Lyase Assay

Pectin Lyase (PL) activity was determined by measuring the increase in absorbance at 235nm (Lambda 25 UV/Vis Spectrometer, range 190 – 1100 nm, accuracy ± 0.1 nm) of substrate solution (0.8mL of 1% citrus pectin in 0.2M tris – HCl buffer, pH 8.5), hydrolyzed by 0.2mL enzyme solution at 40$^{o}$C [14]. One unit of enzymatic activity (U) was defined as the amount of enzyme which released 1 µmol of unsaturated uronide per minute, based on the molar extinction coefficient (5500) of the unsaturated product [17].

### 2.5.3 Total Protein Content Assay

Total protein was determined by the burette method, as described by Amande and Adebayo-Tayo [4] using 5mg of albumin/ml as the protein standard. Briefly, 3mL of burette reagent was added to 2mL of test protein solution in a sterile test tube and the mixture was properly mixed. The tubes were then warmed at 37$^{0}$C for 10 minutes with shaking and finally the tubes were cooled, and absorbance was noted at 540nm using Lambda 25 UV/Vis Spectrophotometer (range 190 – 1100 nm, accuracy ± 0.1 nm).

## 2.6 Statistical Analysis

Results obtained in this study were subjected to analysis of variance using one way ANOVA and differences between means were separated by Duncan Multiple Range Test [18; 19].

## 3. RESULTS

### 3.1 Fungal Polygalacturonase Production

Fungal PG was produced on all four substrates, but mango peels was the best substrate identified (Fig 1). The PG produced on beans testa ranged from 0.0377 – 21.8850U/g (Fig. 1) with *Aspergillus piperis* producing the highest on day 3 (Fig. 1a). On mango peels, the PG produced ranged from 6.6612 – 141.0095 U/g with *Aspergillus tamarii*, *Aspergillus piperis*, *Aspergillus terreus* producing the highest on day 3 (Fig. 1a). *Aspergillus tamariii* produced the same quantity of the enzyme on day 3, 6 and 9 (Fig 1a-b). PG production on plantain peels ranged from 0.0919 – 25.5506 U/g, with *Aspergillus tamari* producing the highest on day3. The PG produced on BT: MP: PP ranged from 0.2707 – 44.4448 U/g with *Aspergillus parasiticus* producing the highest on day 3. The amount of PG produced by fungal strains varied significantly between days (*P* =.05).

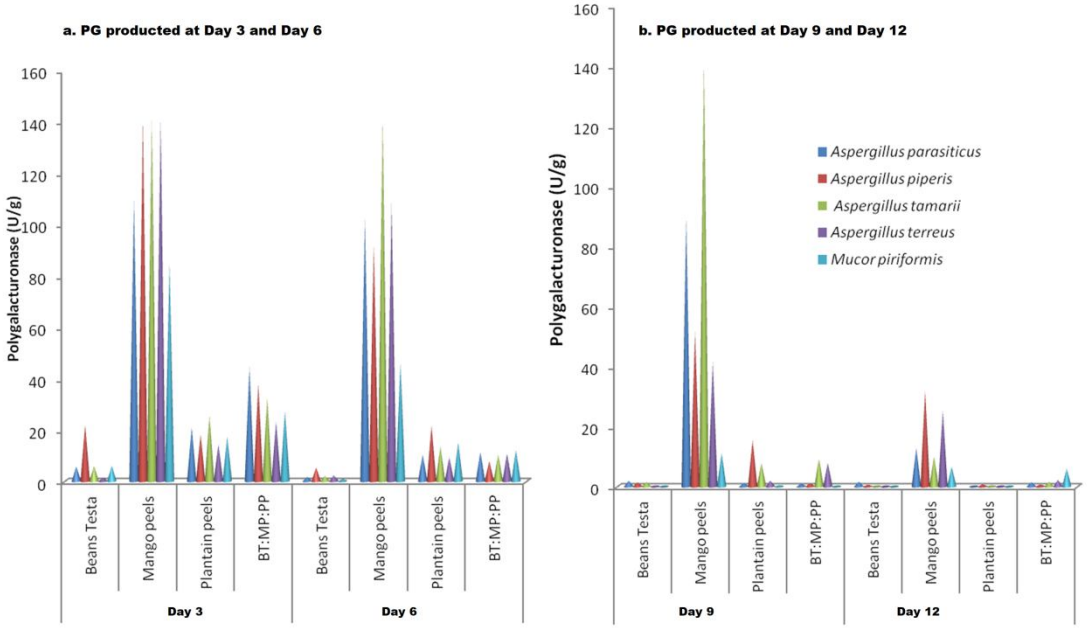


**Figure 1: Fungal polygalacturonase (PG) from fruit substrates, bean testa (BT), mango peels (MP), and plantain peels (PP) in solid-state fermentation. a) Fungal PG (U/G) produced at day 3 and 6.b)Fungal PG produced at day 9 and 12. The substrate, BT: MP: PP is a 1:1:1 combination of bean testa (BT), mango peels (MP), and plantain peels (PP).**

All fruit substrates favored fungal pectin lyase production (Fig 2). The PL produced on bean testa, ranged from 50.50 – 1626.55U/g with *Mucor piriformis* producing the highest on day 9 (Fig 2b). On mango peels, PL produced ranged from 164.50 – 6845.50U/g with *Aspergillus parasiticus* producing the highest on day 3 (Fig. 2a). On plantain peels, the PL produced ranged from 104.50 – 10,852.50 U/g with *Mucor piriformis* producing the highest on day 6 (Fig 2a). On the composite substrate, BT: MP: PP, PL produced ranged from 117.50 – 9600.50 U/g with *Aspergillus parasiticus* producing the highest on day 12 (Fig. 2b).PL production by fungal strains varied between days (*P*=.05).

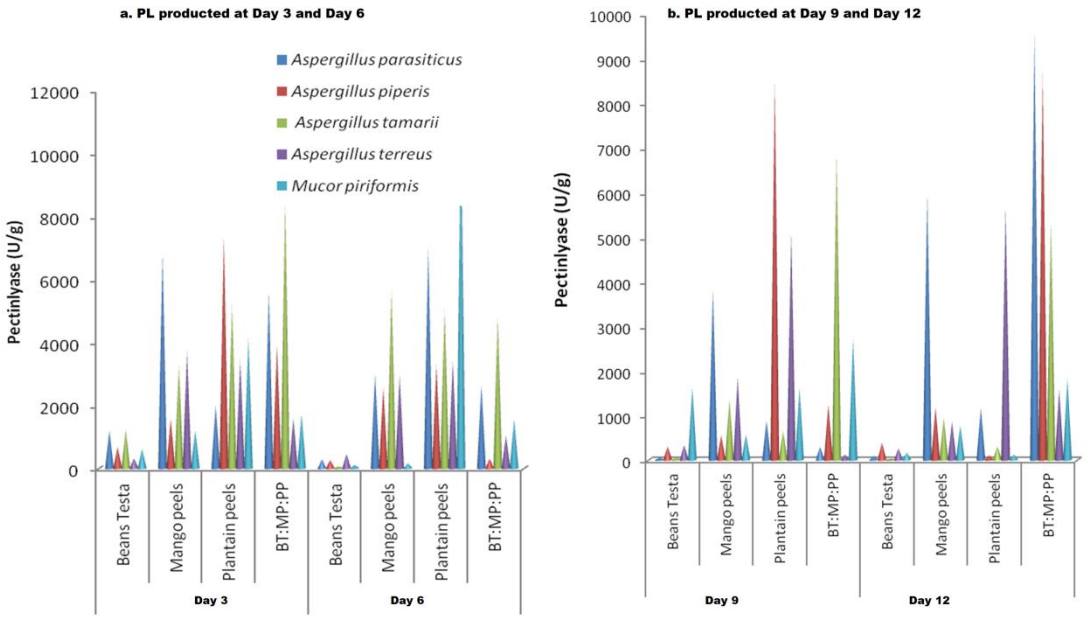


**Figure 2: Fungal Pectin lyase (PL) from fruit substrates, bean testa (BT), mango peels (MP), and plantain peels (PP) in solid-state fermentation. a) Fungal PL(U/g) produced at Day 3 and 6. b) Fungal PL produced at Day 9 and 12. The substrate, BT: MP: PP is a 1:1:1 combination of bean testa (BT), mango peels (MP), and plantain peels (PP).**

### 3.2 Total protein (TP) content

Concentration of the total protein for the fermenting substrates changed from day 3 to 12 (Table 1a-b). On beans testa, TP ranged from 1.9033 – 44.7109 mg/mL with *Aspergillus piperis* producing the highest on day 6 (Table 1a). On mango peels, TP ranged from 11.9894 – 65.2474 mg/mL with *Aspergillus terreus* producing the highest on day 3 (Table 1a).

**Table 1a Total Protein (mg/mL) Produced by Fungi Isolates using Beans Testa (BT), Mango Peels (MP), Plantain Peels (PP) and BT:MP:PP(1:1:1) as substrate inSolid state fermentation.**

| ISOLATE | Day 3 | | | | Day 6 | | | |
|---|---|---|---|---|---|---|---|---|
| | Substrate/ Protein Concentration (mg/mL) | | | | | | | |
| | Beans Testa | Mango Peels | Plantain Peels | BT:MP:PP | Beans Testa | Mango Peels | Plantain Peels | BT:MP:PP |
| *Aspergillus parasiticus* | 17.8894$^{c}$ | 49.1334$^{c}$ | 30.2646$^{b}$ | 48.1915$^{b}$ | 9.0215$^{b}$ | **41.3865$^{a}$** | 27.0367$^{c}$ | **65.2474$^{a}$** |
| *Aspergillus piperis* | 10.0087$^{e}$ | 52.3376$^{b}$ | 24.6715$^{c}$ | 18.6979$^{e}$ | **44.7109$^{a}$** | 29.6678$^{d}$ | 20.1725$^{d}$ | 24.2957$^{d}$ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Aspergillus tamari* | 13.0285$^{d}$ | 27.5259$^{d}$ | 13.6763$^{e}$ | 25.5865$^{d}$ | 2.8925$^{e}$ | 30.5592$^{c}$ | 35.1979$^{b}$ | 11.3602$^{e}$ |
| *Aspergillus terreus* | 19.0418$^{b}$ | **65.2474$^{a}$** | 20.5871$^{d}$ | 32.7194$^{c}$ | 5.8285$^{d}$ | 36.2129$^{b}$ | 19.8152$^{e}$ | 26.7958$^{c}$ |
| *Mucor piriformis* | **44.6743$^{a}$** | 16.8742$^{e}$ | **34.0075$^{a}$** | **53.5023$^{a}$** | 6.1614$^{c}$ | 21.7894$^{e}$ | **40.8725$^{a}$** | 47.0725$^{b}$ |

**Values with same superscript are not significantly different (*P*=.05)using Duncan's multiple range test. Values in bold font are relatively higher than other values in the same column.**

On Plantain peels, TPranged from 13.6763 – 65.2474 mg/mL with *Aspergillus piperis* and *Aspergillus terreus* producing the highest on day 9 and 12 respectively (Table 1b). On BT: MP: PP, TP ranged from 7.2152 – 65.2474 mg/mL with *Aspergillus parasitucus* and *Mucor piriformis* producing the highest on day 6 and 9 respectively. The TP produced by fungal strains varied significantly on different substrates and between days of incubation (*P*= .05).

**Table 1b Total Protein (mg/mL) produced by Fungi isolates using Beans Testa (BT), Mango Peels (MP), Plantain Peels (PP) and BT: MP: PP (1:1:1) as substrate in solid state fermentation**

| **ISOLATE** | **Day 9** | | | | **Day 12** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Substrate/ Protein Concentration (mg/mL)** | | | | | | | |
| | Beans Testa | Mango Peels | Plantain Peels | BT:MP:PP | Beans Testa | Mango Peels | Plantain Peels | BT:MP:PP |
| *Aspergillus parasiticus* | 4.3979$^{d}$ | 28.5066$^{c}$ | 17.7743$^{d}$ | 44.7409$^{b}$ | 8.1398$^{d}$ | **31.6043$^{a}$** | 30.4055$^{b}$ | 33.9302$^{b}$ |
| *Aspergillus piperis* | 4.8496$^{c}$ | 16.8926$^{e}$ | **65.2474$^{a}$** | 11.0023$^{e}$ | 10.9145$^{b}$ | 31.5575$^{b}$ | 17.8496$^{e}$ | 28.3145$^{d}$ |
| *Aspergillus tamariii* | **7.4302$^{a}$** | **31.9915$^{a}$** | 46.9269$^{b}$ | 35.4743$^{c}$ | 3.5915$^{e}$ | 30.4474$^{c}$ | 28.4635$^{c}$ | 30.9743$^{c}$ |
| *Aspergillus terreus* | 5.9247$^{b}$ | 29.9109$^{b}$ | 14.2582$^{e}$ | 28.2129$^{d}$ | 10.0539$^{c}$ | 25.9614$^{d}$ | **65.2474$^{a}$** | **46.8872$^{a}$** |
| *Mucor piriformis* | 1.9033$^{e}$ | 25.3926$^{d}$ | 21.7011$^{c}$ | **65.2474$^{a}$** | **11.6129$^{a}$** | 11.9894$^{e}$ | 23.1828$^{d}$ | 7.2152$^{e}$ |

**Values with same superscript are not significantly different (*P* =.05) using Duncan's multiple range test. Values in bold font are relatively higher than other values in the same column.**

### 3.3 Changes in pH during substrate utilization and pectinase production by fungi isolates

Fungal strain fermenting a substrate was the key driver of pH change (Fig 3). The initial pH of bean testa substrate at Day 0 was 4.11. During the growth and utilization of beans testa by the fungi isolates there were significant changes in pH. *A. parasiticus, A. piperis, and A. tamari* changed the pH of the medium to alkalinity with their peaks at 7.74, 7.49, and 7.04 respectively while *Mucor piriformis* recorded steady change of pH to acidity giving the lowest pH of 3.07 at day 12(Fig. 3a).

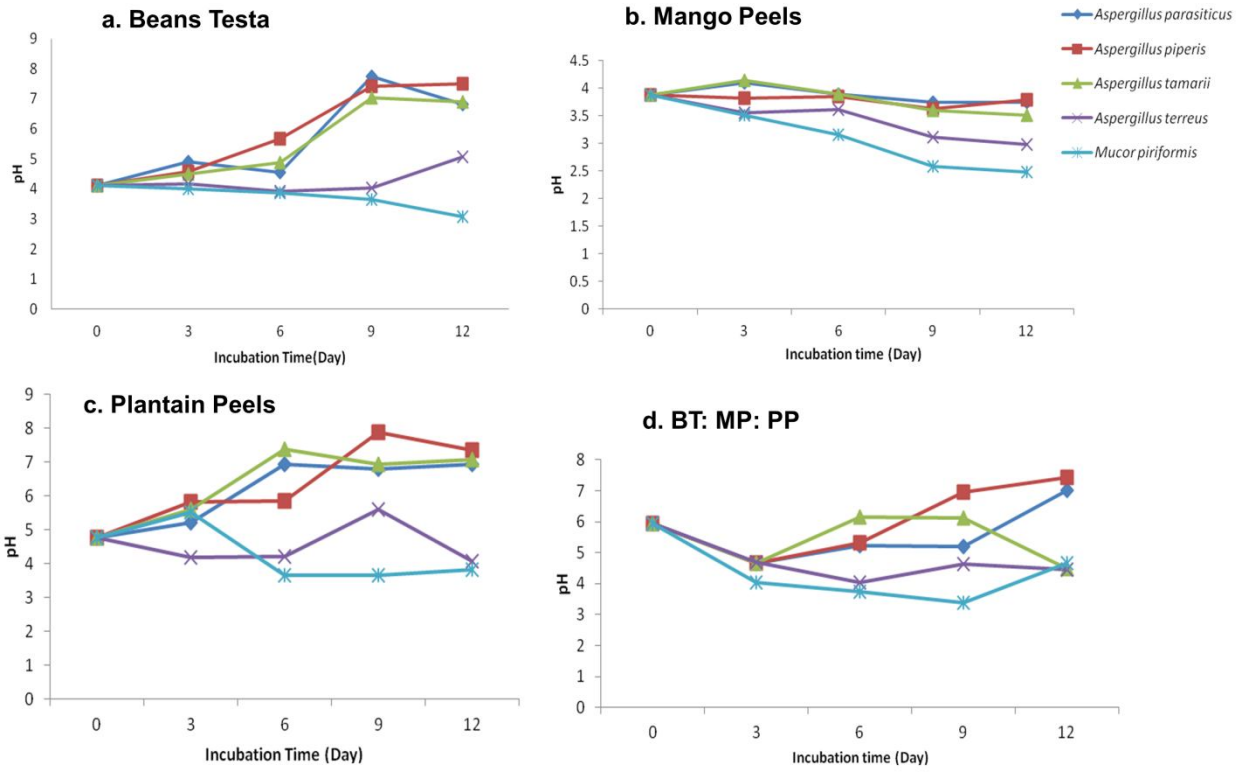


**Fig. 3: pH change of fermenting substrates, a) beans testa, b) mango peels, c) plantain peel and, d) BT:MP:PP during pectinase production. Each media was seeded with one fungi strain. The substrate, BT: MP: PP is a 1:1:1 combination of bean testa (BT), mango peels (MP), and plantain peels (PP).**

The initial pH of the ground mango peel substrate at Day 0 was 3.88.The isolates tend to reduce further the pH of the medium thus making it more acidic. *A. terreus* and *Mucor piriformis* caused the greatest reduction in the pH,i.e. 2.97 and 2.48 respectively, at the end of fermentation (Fig. 3b). *A. piperis*, *A. tamarii*, and *A. terreus* produced the highest quantity of PG at pH 3.82, 4.14 and 3.55 respectively (Fig 1).

The initial pH of the ground plantain peel substrate at Day 0 was 4.76. *A. parasiticus*, *A. piperis* and *A. tamarii* changed the pH of the medium towards alkalinity with their peaks at pH 6.96, 7.88 and 7.38 respectively .*A. terreus* and *Mucor piriformis* changed the pH of the medium towards acidity, i.e. 4.09 and 3.65 respectively (Fig. 3c). *Mucor piriformis* produced the highest PL at pH 3.65 (Fig 2).

The initial pH of the composite substrate (BT: MP: PP) was 5.93.*A parasiticus* and *A. piperis* caused an increase of the pH with their peak at 7.00 and 7.43 respectively. However, *A .tamarii, A. terreus* and *M.piriformis* lowered the pH of the medium to 4.49, 4.05 and 3.39 respectively (Fig. 3d).

## 4. DISCUSSION

Several states in the Federal Republic of Nigeria produce record number of fruits. Benue State for instance generates tons of fruit waste that can be processed and sold to industries producing depolymerizing pectinases (hydrolases and lyases).

*Aspergillus* sp. was the main PG producer and performed favorably on all substrates tested. The maximum PG was produced by *A. tamarii, A. terreus* and *A. piperis.* Sohail *et al*. [20] reported that members of the genus *Aspergillus* had greatest diversity in terms of high levels of hydrolases (pectinase) production. Our findings agree with an earlier observation by Rogaia Al-Gashgari [21] who reported the highest pectinase production by *A*. *terreus*, *A*. *flavus*, *A*. *fumigatus*, *A. niger*, and *A. parasiticus*. The 141.0095 U/g of PG obtained in solid state fermentation is higher than that earlier reported by Niture and Pant [22].

Pectinase lyase was produced in relatively large amount on all substrates. The highest PL production was obtained on the 6$^{th}$ day of fermentation by *Mucor piriformis* on plantain peels substrate. A similar report observed maximum pectin lyase production by *Moniliella* sp. on the 6$^{th}$ day of fermentation [14].Lotfi *et al*. [23] screened some *Zygomycetes* strains for pectinase activity and observed that strains of *Mucor* showed higher pectinase activity than *Rizopus*. They concluded that *Mucor* strains should be considered as a resource for pectinlytic enzymes.

Mango peels was the best substrate for PG production while plantain peels and BT: MP: PP gave higher yields of pectin lyase. Of all the substrates investigated, beans testa (BT) gave a generally poor yield of pectinase. Pectin acts as an inducer to produce pectinolytic enzymes by microbial systems. Mango has been reported to contain substantial amounts of pectin having a high gelling grade [2]. However, the pectin content of beans ranges from 0.27 – 1.11% [24]. The low yield of pectinase on BT could be because it has the least amount of pectin as compared to the other substrates investigated. A study maintained that mango peels could be used as a valuable, economic, and abundant media source for the commercial production of natural enzymes such as pectinase [25].

Almost all the organism produced a greater yield of the PG on the 3$^{rd}$ and 6$^{th}$ day of fermentation, but PG activity almost disappeared on the 9$^{th}$ and 12$^{th}$ day. However, the pectin lyase activity of most of the organism became more pronounced from day 6 and peaked at day 12. The different peaks of enzymatic activity observed indicate the sequential production of isoenzymes by fungi as described by several authors [26;27;28]. Another author also reported decline in the PG activity upon prolonged incubation using *Aspergillus fumigatus* isolated from decomposing orange peels [29]. Maximum production of pectic enzymes from different molds varies from 1 to 6 days [30]. Sarvamangala and Dayanand [31] observed a gradual increase in the production of pectinase from deseed sunflower head

by *A. niger* after 72 hours of fermentation period in submerged fermentation and up to 96hours in solid state condition.

The pH of the cultivation medium is an important factor in the production of pectinases for it influences the sort and content of those enzymes produced by fungus [32]. In this study, the maximum PG production on mango peels by *A. piperis, A. tamari* and *A. terreus* were at pH 3.82, 4.12, and 3.54 respectively while the maximum PL production on BT:MP:PP by *Mucor piriformis* was at pH 3.65 . The optimum production of pectic enzymes has been shown to be within the acidic pH range [33; 34].Silva *et al*. [27] found that *P. viridicatum* showed maximum production of polygalacturonase and pectinlyase at a pH of 4.5 and 5.0, respectively. However, the mechanism by which pH acts on the production of pectic enzymes is not known [35].

## 5. CONCLUSION

The need for a cheap and readily available substrate for pectinase production will continue to grow. This study has demonstrated fruit wastes like mango peels, plantain peels, and beans testa are useful substrates. Where a substrate has little pectin content like beans testa, a composite medium of beans testa: mango peels: plantain peels will increase pectinase yield. Generally, mango and plantain peels are a better substrate for pectinase production while beans testa is not. The optimum pH for pectinase production falls within the acidic pH range.

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