

# Lignocellulolytic Activity of Soil Fungi Isolated from Different Organic Farming Sites

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## ABSTRACT

The lignocellulolytic activity of soil fungi isolated from different organic farming site at Ibere, Ikwuano local government area of Abia state, south east Nigeria was studied. Samples of soil numbering 100 were collected from four different farming sites and analyzed using the pour plate technique and tannic acid/carboxyl methyl cellulose test for lignocellulolytic enzyme production. While the enzymes activity assay was carried out in submerged fermentation using mixed chopped Maize husk and Yam peel as substrates. From the soil sampling, Twenty eight (28) Fungal species belonging to the Genera; penicillium *Aspergillus* *Rhizopus* *Mucor* *Paraconiothyrium* *Trichoderma* *Fusarium* and *Curvularia* were isolated. Out of the 28 isolates, 4 isolates (*Penicillium*, *Aspergillus*, *Rhizopus* and *Paraconiothyrium* species) were positive for lignocellulolytic enzyme activity based on their ability to degrade lignocellulose substrates. The effect of various conditions on enzyme activity showed that the highest activity was recorded at the 5<sup>th</sup> day of incubation, at temperature ranges of 25°C to 45°C and pH ranges of between 5 to 9. The molecular identification of the isolates were carried out using PCR and 16S rRNA. Sequencing and the isolates were identified as *Aspergillus niger*, *Penicillium rolfsii*, *Rhizopus oryzae* and *Paraconiothyrium brasiliense*. Therefore, the lignocellulolytic fungi isolated, could serve as useful source of commercial production of lignocellulolytic enzyme and also be applied to the soil to degrade organic residue, thereby improving the soil fertility and agricultural waste management in the environment.

**Keywords:** Lignocellulolytic activity, Organic farming, Fungi, Enzymes, substrates.

## INTRODUCTION

Conventional agriculture or farming involves the use of chemical fertilizers and other synthetic products to increase the yield of crops. It involves farming practices such as tilling/ploughing the soil, removal of organic matter and monocropping [1]. These practices generally require the use of a substantial amount of chemical energy and input of energy which damages and distorts the ecological balance of the land. It alters or destroys the natural environment, deteriorates soil quality and eliminates biodiversity, depletes soil fertility, pollutes ground water, soil structural degradation, soil compacting, surface sealing and crusting [2]. Based on these harmful effects on the soil environment, there is therefore need for alternative farming method that maintains soil structure and productivity such as conservation agriculture, organic farming or sustainable agriculture.

Organic farming is a system which avoids or largely excludes the use of synthetic inputs (such as fertilizers, pesticides, hormones, feed additives etc) but rely upon crop rotations, crop residues, animal manures, off-farm organic waste, mineral grade rock additives and biological system of nutrient mobilization and plant protection[3]. This farming system promotes better use of agricultural inputs through the combined management of available soil, water and biological resources so as to minimize the use of external inputs [4,5]. Its primary feature, is the maintenance of a permanent or semi-permanent soil cover, which could be either a live crop or dead mulch, which serves to protect the soil from sun, rain and wind, and feed soil biota with little soil disturbance characterized with minimum or no till. However, the microbial community is essential as it provides a 'biological tillage' that serves to replace the functions of conventional tillage [6]. The retention of

adequate levels of crop residue on soil surface is a key principle of Conservation Agriculture/ organic farming. This residues/waste contains a high proportion of lignocellulose. Lignocellulose is the major structural component of plants and represents a major source of renewable organic matter. Decomposition of crop residues depends on autochthonous soil microbes, length of decomposition period, and soil and environmental conditions. Fungi are the main cellulase-producing microorganisms [7]. Fungi are an important component of soil micro-biota, constituting more of the soil biomass [8] than bacteria, depending on depth and nutrient conditions of soil. Fungi being filamentous in nature have an advantage in the decomposition of lignocellulosic waste as they possess ability to produce prolific spores that can quickly invade substrates and supported by a broad variety of enzymes with complementary catalytic activities [9]. Fungi, such as white-rot fungi, brown-rot fungi, and soft-rot fungi, can degrade lignocellulose biomass effectively [10,11]. Other lignocellulose-degrading fungi include brown-rot and softrot fungi, which can also effectively depolymerize cellulose biopolymers into simple sugars; however, lignin can only be partially degraded [12,13].

Fungi play a central role in the degradation of plant biomass by producing an extensive array of carbohydrate-active enzymes responsible for polysaccharide degradation. Jorgensen and Olsson [14] reported that several species of *Penicillium* were shown to have the ability to produce cellulolytic enzyme systems. Krogh *et al.*, [15] investigated 12 filamentous fungi from genus *Penicillium* for the production of cellulolytic and xylanolytic enzymes and found some *Penicillium* spp. as good producers of enzymes. Many species of *Penicillium* have been reported as good producer of xylanase [16] like *Penicillium janthinellum* [17], *Penicillium janczewskii*, *Penicillium oxalicum* [18]. Sohail *et al.*, [19] reported that *Alternaria* sp. MS28 has the ability to produce cellulases in presence of various substrates. The endophytic fungi *Alternaria alternata* also have been reported to produce xylanase [20]. Many Species of Aspergilli produces cellulases and exhibits strong hydrolytic activity towards cellulose [21]. Some species of Aspergilli like *A. flavus* [22], *A. niger* [23 Chinedu *et al.*, 2008] and *A. terreus* [24 Jahromi *et al.*, 2011] were found effective in the biodegradation of different lignocellulosic materials by producing oxidative and hydrolytic enzymes. *A. niger* is known globally for its ability to produce broad range of extracellular glucohydrolases, including xylanases, pectinases, and b-glucosidase [25].

However, in recent years using fungi as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity. Filamentous fungi are attracting greater attention as potential sources of plant cell wall hydrolyzing enzymes, because they secrete high levels of the enzymes [26]. Although, residue degrading fungi are already present in soil, their population varies greatly from place to place and field to field depending on the management, edaphic and environmental conditions. Our hypothesis is that isolation and augmentation of fast degrading autochthonous fungi from fields will enhance their population density upon field inoculation. Therefore, the aim of this study is to isolate and identify lignocellulolytic fungi from soil, determine the lignocellulolytic enzyme activity by their ability to degrade organic plant residues and to study the effects of various growth conditions on the enzyme activity.

## MATERIALS AND METHODS

### Soil sample collection

The soil samples were collected from 5 different farming sites practicing organic farming in Ibere (Itunta, Ngwugwo, Obuohia, Inyila, Elemaga) in Ikwuano Local Government Area, Abia State. Each site was divided into four grids. Soil was dug out at the depth of 15cm using soil auger of diameter 5mm and samples collected into sterile polythene bags and taken to the laboratory.

### Isolation of Fungi

The soil suspension method was used in the isolation of the fungi. One gram soil sample was transferred aseptically into 9 mL sterile distilled water, mixed thoroughly and serially diluted up to  $10^{-5}$ . From this, 0.1 mL was inoculated onto Potato Dextrose Agar (PDA) in Petri plates and incubated at 28°C for 7 days. After the growth, pure cultures were obtained by repeatedly subculturing on PDA plates. These pure cultures were stored on PDA slants at 4°C.

## Identification of Fungal Isolates

Macroscopic examination was done by characterization of the mycelia structure and colour. Microscopic characterization was carried out by observing the morphological structure according to Ainsworth and Bisby [8].

## Molecular characterization

Fungal isolates were identified by sequence analysis of rDNA gene. The DNA extraction was done using a modified CTAB (Cetyltrimethyl ammonium bromide) method [27]. The rDNA region consisting of internal transcribed region (ITS) 1, 5.8S subunit and ITS 2 region, will be amplified using TTS 1 and ITS 4 primers [28]. The PCR reaction was carried out using 1.0mm MgCl<sub>2</sub>, 5p moles each of forward and reverse primer and 1U of Taq DNA polymerase. The PCR thermal profile consisting of initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30sec., annealing at 54°C for 1min, extension at 72 °C for 1min, followed by final extension at 72°C for 7min. PCR products, was purified using gel extraction kit (Applied Biosystems Ltd.) and checked with agarose gel electrophoresis (1.5%) for its purity and integrity. The purified PCR products were then used directly for DNA sequencing (3130xl genetic analyzer from applied Biosystems).

## Enzyme production

### Cellulose enzyme production

The cellulose enzyme production was determined by inoculating the organisms on carboxymethyl cellulose (CMC) agar media and the plates incubated at 32°C for 7 days. The plates were stained with Gram's iodine for 3–5 min [29] and zone of clearing was observed.

### Lignase enzyme production

The presence of polyphenol oxidase which indicates Lignase production was determined using Tannic Acid media proposed by Cruz-Hernandez *et al.*, [30] with some modifications. Tannic acid and the other constituents were separately autoclaved and mixed after cooling. A fungal inoculum disc of 5-mm was cut from the hyphal edge of 5-day-old PDA culture and placed at the centre of the media plate and incubated at 32°C for 7 days. The appearance of a dark brown pigment will be observed.

## Enzyme Activity Assay

Lignocellulolytic enzymes activity assay was carried out on mixed chopped Maize husk and Yam peel medium according to the method of Saparrat *et al.* [31]. The medium was prepared by mixing 100g each of blended dry maize husk and dry yam peel together. Then, 20g of the mix was collected and put in a flask, 5g of yeast extract added to it and suspended in 1Litre of distilled water. The suspension was thoroughly shaken and 50ml dispensed in 250ml flask each and autoclaved at 121°C for 15min. After cooling, the straw was inoculated with five discs (5 mm) of freshly grown 5-day old culture and incubated at 25° C, for 9 days. The fermented substrate was extracted with 100 ml of citrate buffer (0.05 M, pH 4.8) by shaking the mixture at 120 rpm for 1 h. The slurry was filtered through muslin cloth, followed by filtration through Whatman filter paper No. 1. Final, the filtrate was centrifuged at 9000 rpm for 10min at 4°C. The supernatant was used for the assay of various lignocellulolytic enzyme activities. The isolates showing highest levels of these enzymes was finally selected and maintained on PDA slants at 4°C in a refrigerator for further identification studies.

### Effect of incubation time on the lignocellulolytic activity

Flasks containing 5% of maize husk and yam peel medium each was inoculated with the test organism and incubated at 25°C for 24, 48, 72, 96, and 120 hrs for 5 days. These were subsequently filtered and the lignocellulase activities of the filtrates were determined [32].

## Effect of temperature on the lignocellulolytic activity

Flasks containing 5% of maize husk and yam peel medium each was inoculated with the test organism and incubated at 25, 30, 45 and 50°C respectively for 5 days. These were subsequently filtered and the lignocellulase activity of the filtrates determined. [32].

## Effect of pH on the lignocellulolytic activity

Flask containing 5% of maize husk and yam peel medium each was inoculated with the test organism. The pH was adjusted to 4, 5, 6, 7, 8 and 9 respectively using buffer solutions and incubated at 25°C for 5 days. The flasks were subsequently filtered and the lignocellulase activity of the filtrates determined. [32].

## RESULTS

### Isolation and identification of Fungi

From the result of the isolation process, a total of twenty eight (28) fungal isolates were recorded and were identified based on their colonial morphology as members of the Genus; *Aspergillus*, *Mucor*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Curvularia*, *Paraconyterium*, and *Penicillium species*.

Table 1: Morphological Characterization of the Fungal Isolates

Isolate	Morphological features	suspected Organism
I	Dark brown in colour, occurs in patches and gritty	<i>Aspergillus</i> sp
II	Pinkish-White cotton-like in culture, fluffy	<i>Fusarium</i> sp
III	Brown in appearance, smooth dense	<i>Curvularia</i> sp
IV	Greenish in appearance, occurs in patches	<i>Trichoderma</i> sp
V	Grey fluffy appearance with numerous dark spores	<i>Mucor</i> sp
VI	Grey, fluffy appearance with numerous dark spores round the colony	<i>Rhizopus</i> sp
VII	Blue or green, fuzzy texture	<i>Penicillium</i> sp
VIII	Dark brown or white fluffy in colour	<i>Paraconiothyrium</i> sp

### Lignocellulolytic Enzyme production

Out of the 28 fungal isolates, 2 isolates belonging to the Genera *Aspergillus* and *Penicillium* produced a clear zone around the colonies growing on the Carboxyl Methyl Cellulose (CMC) medium. This shows they are cellulolytic organisms (Fig 1-2). While 2 other isolates belonging to the genus *Rhizopus* and *Paraconiothyrium* species respectively produced dark-brown colouration/zone around colonies growing on Tannic acid medium. This thus indicates that they are Lignolytic organisms (Fig 3-4).

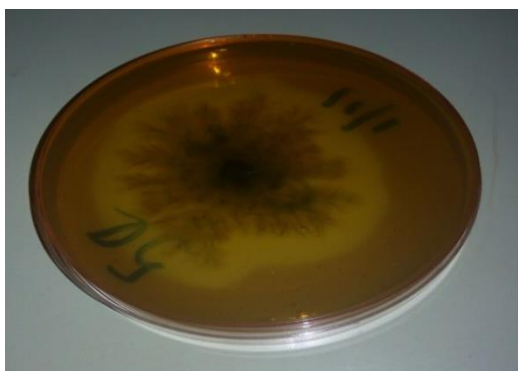


Fig 1: *Aspergillus* sp showing clear Zone around the colony on CMC medium

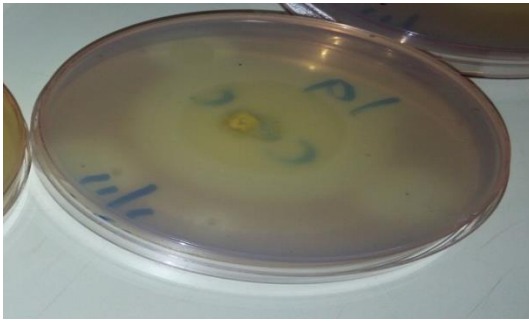


Fig 2: *Penicillium sp* showing clear Zone around the colony on CMC medium



Fig 3: *Rhizopus sp* showing dark-brown colouration on Tannic acid medium



Fig 4: *Paraconiothyrium sp* showing dark-brown colouration on Tannic acid medium

### Lignocellulolytic enzyme activity in submerged fermentation

The four fungi isolates that showed Lignocellulolytic enzyme production in the primary screening was used for the enzyme activity studies in submerged fermentation. From the result of the study, all four isolates demonstrated increased enzyme activity from the first day to the fifth day and a decrease in the sixth day. The fungal isolate *Paraconiothyrium sp* showed the highest enzyme activity (12.1 IU/ml) followed by *Aspergillus sp* (11.2 IU/ml), *Rhizopus sp* (8.2 IU/ml) and *Penicillium sp* (7.8 IU/ml) showing the lowest activity.

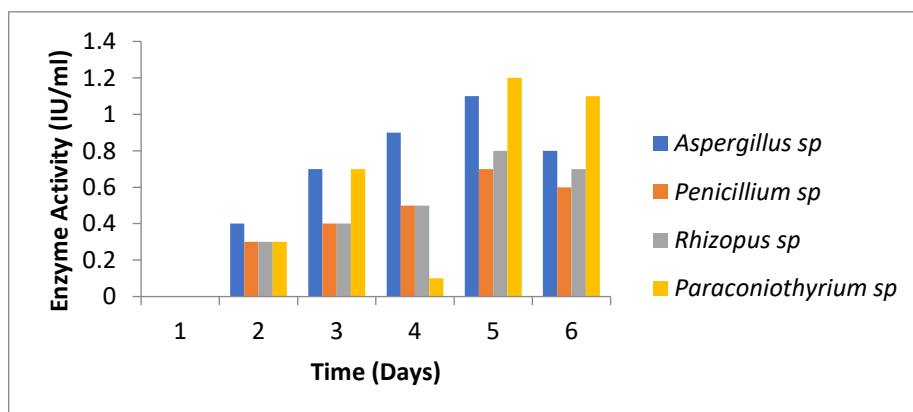


Fig 5: Lignocellulolytic enzyme activity of Fungal isolates in submerged fermentation



### Effect of temperature and pH on enzyme activity

The effect of temperature and pH on enzyme activity was analyzed and presented in Tables 6 and 7 respectively. From the result of the study, the enzyme activity increased with increase in temperature with the highest activity recorded for most organisms at 25 to 30°C except for *Paraconiothyrium sp* with maximum enzyme activity at 45°C. Beyond these temperatures, the enzyme activity decreased with increasing temperature (Fig 6).

For the effect of pH on enzyme activity, the result shows that the highest enzyme activity was recorded at pH range of 5 and 6 for the organisms except for *Aspergillus sp* with maximum enzyme activity at pH 9. Beyond these pH ranges, the activity decreased with increase in pH (Fig 7).

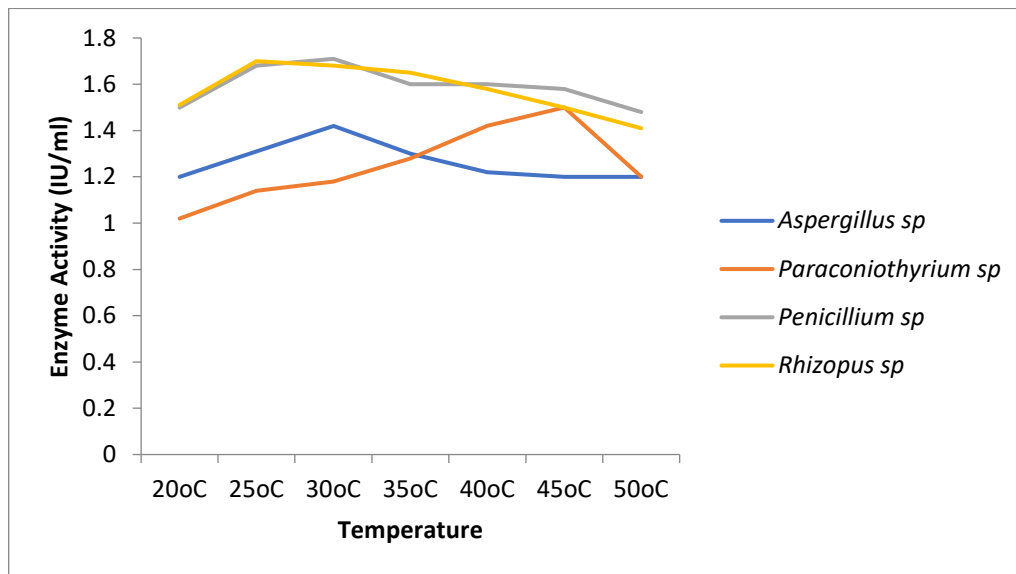


Fig 6: The effect of temperature on enzyme activity

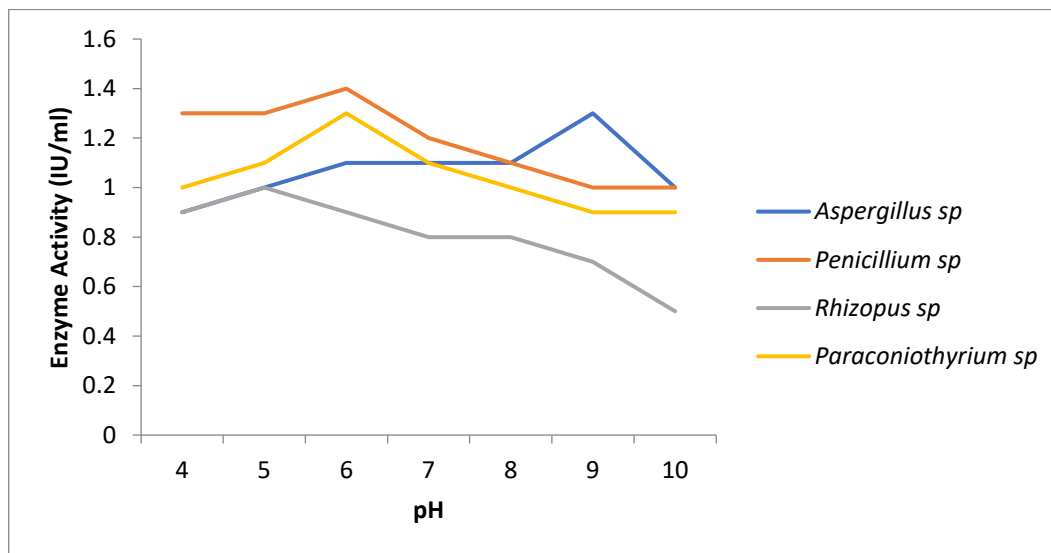


Fig 7: The effect of pH on enzyme activity

### Molecular identification of Isolates

The molecular identification of the four fungal isolates was carried out and the Agarose gel electrophoresis presented in Fig 8 and the BLAST sequence shown in Table 1. The sequences were submitted to NCBI GenBank with accession numbers **MG669190**, **MH856397**, **MH865589**, **KJ767103**. The BLAST search showed 99% similarity with *Aspergillus niger*, *Penicillium rolsfii*, *Rhizopus oryzae* *Paraconiothyrium brasiliense* respectively.

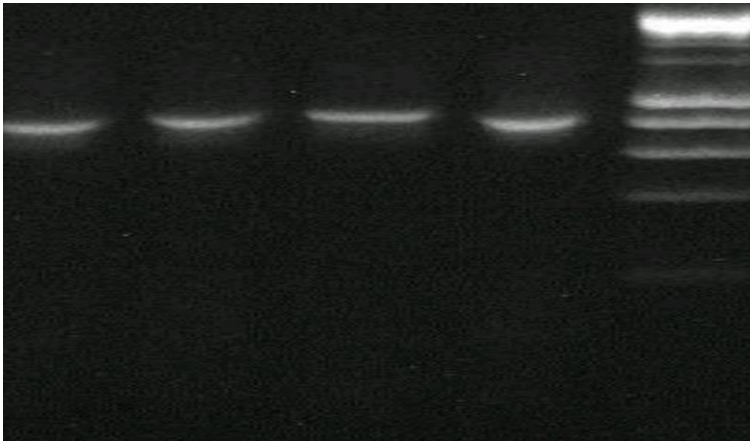


Fig 8: Agarose gel electrophoresis of the isolates showing PCR products of Fungal isolates.

Table 1: Sequence Blast Analysis of Isolates

Isolate No.	Sequence BLAST	Name of Organism
I	TATCTTCTTAATCTGAGGTCACCTGGAAGAATGGTTGGAAAACGTAC GGACAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATA CGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTC CCCCCGAGAGGGGGACGGCGACCCAACACACAAGCCGGGCTTGA GGGCAGCAATGACGCTCGGACAGGCATGCCCCCGGAATACCAGGG GGCGCAATGTGCGTTCAAAGACTCGATGATTCATGAATTCTGCAAT TCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACC AAGAGATCCATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTC AGACTGCACGCTTTCAGACAGTGTTTCGTGTTGGGGTCTCCGGCGGG CACGGGGCCCCGGGGGGCAGAGGCGCCCCCGGCGGCCGACAAGC GGCGGGCCCCGCCGAAGCAACAGGGTACAATAGACACGGATGGGGA GGTTG	<i>Aspergillus niger</i> strain APBSDFS58 with NCBI accession number MG669190
II	CTTTTCTTAGTCTGAGGTCACCTGGATAGATTGATTGGGGTCCCG GACGGGCGCCGGCCGGGCCTACAGAGCGGGTGACGAAGCCCCATA CGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGTCCGCC CCCCCGAAACCGGGGGGGCGGGGCCCAACACACAAGCCGTGCTTGAG GGCAGCAATGACGCTCGGACAGGCATGCCCCCGGAATACCAGGGG GCGCAATGTGCGTTCAAAGACTCGATGATTCATGAATTCTGCAATT CACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCA AGAGATCCGTTGTTGAAAGTTTTAACTGATTAGTTAATCACTCAGA CTGCAATCTTCAGGCAGAGTTCAATGGTGTCTTCGGCGAGCGCGGG CCCGGGGGGCGGATGCCCCCGGCGGCCGTGAGGCGGGCTCGCCGA AGCAACAAGGTACGATAAACACGGGTGGGAGGTTGGACCCAGAGG GCCCTCACTCGGTAATGATCCTTCCGCAGGTTACCTACGGAAACCT TGTTACGCTTTTTTTACTTCCC	<i>Penicillium rolszii</i> strain CBS 368.48 with NCBI accession number MH856397
III	CAGTATGCTACATGAGTTCAGTAGCATAGTTTGCAAGTGTGCATGG ATTATACTCTTAGTACTTTACTTCCTGGGCGAACCAAAGAAAAAGA TCCTGAGACCAGCGTAATATTCCTGCCTAGCAAGCCAGACAGAAA ATCACACACATTTTAGGTGCTCACTGTAATAAAACAGCGATGCGAC CCATTACCACATAAAACAATGTTATGTGTGGGTTTGTGATGATACT GAAGCAGGCGTACTCTATAGAAAACCATAGAGTGCAAGCTGCGT TCAAAGACTCGATGATTCATGAATATGCAATTCACACTAGTTATC GCACTTTGCTACGTTCTTCATCGATGCGGAGAACCAAGAGATCCATT GTTAAAAGTTGTTTTTTATTAACTTTATAATACTGAATTTCTAGGT	<i>Rhizopus oryzae</i> strain CBS 130156 with NCBI accession number MH865589

	TTATTATGAAGGGTGCTCCTGAAACCAGGAGTGGCATCGATCAAAC CCCAGATAGGTCTACCCATGACCAGTCTGAGTCTCTCAGCCAAATT TTCACAGTGTAGAAGCAATCACTTACCCCAGAGGAAACCCTAAGA GGTAAGGCGCTTTAACATAATTAATGATCCTTCCGCAGGTTACCT ACAGAAAACATTGTTACGTTTTTTTCACCTTCCA	
IV	GCCGGTCACCCGTCCGAGTGTCTAAGACGGTAATGTTGCTTCGTGG ACACGCACCCACGCCCTCGAGAAGCGCAATGTGCTGCGCGAGAG GAGGCAAGGACCGCTGCCAATGATTTTGAGGCGAGTCCACGCGCAG AGGCGGGACAGACGCCCAACACCAAGCAGAGCTTGAGGGTGTAGA TGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATG TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTAC TTATCGCATTTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATC CATTGTTGAAAGTTGTAACGATTGTTTGTATCAGAACAGGTAATGCT AGATGCAAAAAAAGGTTTTGTATGGTTCCAACGGCAGGTTGCCCTG CCGAGGGAGAACGAAGGTACTCGTAAAAAAGGGTGCAGACGTGC GGCGCGTACCGCCACGGGTAAGCGTGGGGCCGCGACCGCAGCTGG TTTTGGATGGATAATGATCCTTCCGCAGGTTACCTACGGAAACCTT GTTACGCTTTTTTAACCTTCCA	<b><i>Paraconiothyrium brasiliense</i> isolate A2S4-11 with NCBI accession number KJ767103</b>

## DISCUSSION

The lignocellulolytic enzyme activity of fungi organisms isolated from farms practicing organic farming techniques were carried out in this study. The organisms isolated from the sites are; *Aspergillus*, *Mucor*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Curvularia*, *Paraconiothyrium*, *Penicillium* and *crystosporium species*. These are predominantly fungi genera found in most agricultural soil and are widely involved in decomposition of organic matter, nutrient cycling, enhancing nutrient uptake and biodegradation activities [33]. From the results, out of the 28 fungi organisms isolated, only 4 (*Aspergillus*, *Rhizopus*, *Paraconiothyrium* and *Penicillium species*) were lignocellulolytic-enzymes producers. These observations reflect the reports of other previous studies which also stated that these organisms produce Lignocellulose enzymes [34,35]. Silva *et al.*, [36] also reported that enzymes from soil fungi such as ligninolytic filamentous *Aspergillus niger*, *Penicillium glabrum* and *Cladosporium cladosporioides* are capable of removing petroleum hydrocarbons from polluted soils. These organisms are capable of degrading lignin and cellulose in plants because of their ability to produce these enzymes [37].

The enzyme activity of the fungal isolates was studied and the results showed that there was an increase in activity progressively from the day 1 to day 5 of growth while declining from day 6. This could be as a result of substrate depletion, inactivation of enzymes or cell death [38]. *Paraconiothyrium sp* showed the highest enzyme activity (12.1 IU/ml) while *Penicillium sp* (7.8 IU/ml) showing the lowest activity. This shows that the organism *Paraconiothyrium sp* has high degradation ability and has been reported to degrade plant cell wall. Their Lignocellulose-degrading ability has been reported to be associated with the possession of genes encoding lytic polysaccharide monooxygenase (LPMOs) which are involved in the depolymerization of lignin [39, 35]. However, a study of the effect of different environmental factors such as temperature and pH on the enzyme activity of the organisms showed that the optimum enzyme activity was between the ranges of 25oC to 30oC for most of the organisms and 45°C for *Paraconiothyrium sp*. While the pH ranges for the highest enzyme activity was between 5 and 6 for most of the organism except for *Aspergillus* which was 9. This is true of most enzyme activity studies which have consistently stated that Very high enzyme activities are recorded at optimal temperature conditions of between 30°C to 45°C and pH of 5.5 to 6.0 [40,41,42]. These results are good and encouraging for the practical application in our study area because it is tropical weather and the temperature and pH ranges support the optimum enzyme activity.

## CONCLUSION

From our studies, it has been shown that some fungal species isolated from the farming sites are able to



produce lignocelluloses enzymes capable of degrading organic residues which can enhance soil fertility and plant growth. Therefore, by exploiting the enzymes produced by these fungi isolates *Aspergillus niger*, *Penicillium rolfsii*, *Rhizopus oryzae* and *Paraconiothyrium brasiliense*, a consortium can be formulated and applied to the field for effective and controlled decomposition/degradation of lignocellulolytic organic matter. This will in turn, reduce the use of other unhealthy, environmentally unfriendly farming practices used by most communities in the area.

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## Conflict of interest

The authors have no conflict of interest and states that this work is an original work and is not under review at any other publication.

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