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JOURNAL OF PURE AND APPLIED SCIENCES

ISOLATION, SCREENING AND IDENTIFICATION OF BEST FUNGAL ISOLATE FROM SPOILT ORANGE THAT HAS POTENTIAL FOR *POLYGALACTURANASE* PRODUCTION

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ABSTRACT
Polygalacturonase (PG) is one of the most important enzyme in fruits juice industries. In this study PG was produced by fungi isolated from spoilt orange. Five pure fungal isolates were obtained. The isolates 1-5 were morphological and microscopically identified as *Aspergillus niger, Aspergillus fumigatus, Saccharomyces, Aspergillus flavus, Penicillium digitatum* respectively. The result of the decay test shows that the isolates: *Aspergillus niger, Aspergillus fumigatus, Saccharomyces, Aspergillus flavus, penicillium digitatum* produce rot diameter of 25.00 ±0.04, 37.00±0.02, 20.00 ±0.02, 30.00 ±0.01, 28.00 ±0.11 (mm) respectively after 14 days of incubation, which confirmed that all the five isolates were responsible for the spoilage of the orange. On screening of the best isolate that has high potential for extracellular polygalacturonase production using plate assay method, the result shows that *Aspergillus niger* produce zone of clear diameter of 21±0.12(mm) and total enzyme activity of 7.19(umol/min/ml), *Aspergillus fumigatus* produce zone of clear diameter of 32±0.02(mm) and total enzyme activity of 12.79(umol/min/ml), *Saccharomyces* produce zone of not clear diameter of 08±0.15(mm) and total enzyme activity of 2.24(umol/min/ml), *Aspergillus flavus* produce zone of clear diameter of 26 ±0.03(mm) and total enzyme activity of 8.35(umol/min/ml), *Penicillium digitatum* produce zone of not clear diameter of 14±0.16(mm) and total enzyme activity of 4.69(umol/min/ml) after 48h of incubation. Hence *Aspergillus fumigatus* was found to be the isolate with the highest potential for polygacturonase production. The result of molecular identification of the isolated *Aspergillus fumigatus* shows that it is 93.2% similar to *Aspergillus fumigatus* strains based on 18S rRNA analysis gene sequencing, so probably is a new strain of the fungi.

Keywords: Fungi, Polygalacturonase, Fungal isolates, Molecular, Sequencing

INTRODUCTION
Fruits are liable to numerous of disease condition and this leads to damage of the fruits which cause enormous economic disaster(Sagarika *et al*., 2020). Different fungal species were isolated and identified from orange and banana with the percentage of occurrence (Mohammed, 2018). According to Agrios, 2005 and Barth *et al,* 2009 about 20% to 30% of vegetables and fruits were lost every year after harvest due to spoilage. Rashad, 2011 also reported that *Aspergillus* specie was found to cause spoilage of orange, apricot, tomatoes, apple, kiwi and mango. These microorganisms can cause the spoilage of fruits, the seed itself, during the growth of the fruits; it can be during the harvesting period and postharvest handling;it can even be during storage and distribution of these fruits (Barth *et al*., 2009). Pectinaseenzymes that break down pectin into simplerforms are produce by fungi. These filamentous fungi have the ability to secrete this hydrolyzing enzyme into their culture media, which led to the extraction of this useful enzyme (Berry and Paterson, 1990; Chinedu *et al*., 2008). Enzymes that are used in industries are expensive, especially in developing countries like Nigeria, because they are gotten from purified substrates and usually apparent organisms. It is therefore important to look for cheaper substrates from local sources, for enzymeproduction and also to study

fungi with high enzyme producing potential isolated locally. There is a scanty report in this area of research in Nigeria, mainly due to over dependence on the crude oil reserve (Adebare, 2012). The aim of this study is to isolate and morphologically identify the fungi that have potential for polygalacturonase production and molecularly identify the fungus that has highest potential for producing polygalacturonase.

MATERIALS AND METHOD

Spoilt Fruit Materials

Twenty spoilt oranges were collected from Yanlemo Market Naibawa, Kano state and were taken to Microbiology laboratory, Biological Sciences Department, Bayero University Kano, Kano state for the analysis.

Isolation of fungi

Fungi were isolated according to method describedby Chadha *et al*., 2005. Spoilt orange were selected randomly and examined. The oranges were chopped into small pieces about 3mm width with disinfected blade. The surface of the orange which was compared with the initial isolates (Tafinta *et al*., 2013).

Screening of fungal isolate for galacturonase activity

Screening of best isolates for extracellular was disinfected in 1% hypochlorite for 2 min. Samples were inoculated on potato dextrose agar media (PDA) under sterilized condition andthen incubated at 28^{o}C for 5 days. The different colonies that appear on the plates were purified by sub-culturing the individual colonies several times until pure culture was obtained.

Decay Test

Decay test was carried out to see if the fungi isolated were responsible for the spoilage of the oranges. Healthy oranges were disinfected with 3:1 alcohol. Tissues were cut out from the fruits using disinfected cork borer. One week old fungal culture were placed in these holes under disinfected condition, then covered and sealed off. The same protocol was carryout for each of the fungal isolates. The oranges that wereinoculated with the isolates and the control wereplaced in disinfected nylon bags and incubated at 28 ± 3^{o}C for 14 days. The diameter of the orange spoilage was measured. Isolation of the fungi from the inoculated orange was carried out polygalacturonase was carried out by using plate assay method (Sapna *et al*., 1996). A medium containing (pectin 10.0g, diammonium orthophosphate 3.0g, K_2HO_4 2.0g, KH_2PO_4 2.0g, $MgSO_4$ 0.1g, Agar 20.0g, Distilled water 1000ml, pH 4.5) was inoculated with the pure isolates and incubated at 37^{o}C for 48h. Iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330mL H_2O) was poured on the plates to detect clear zones.

Production of Polygalacturonase in Solid State Fermentation

Inoculum was prepared by suspending the fungi in sterile distilled water; haemocytometer was used to prepare a suspension containing 10^6spores/ml. The fermentation medium contained 10g of processed orange peels mixed with minerals salt, carbon and nitrogen source solution inside a 500ml Erlenmeyer flask and autoclaved at 121^{o}C for 30 minutes. The flask was inoculated with 2ml of the inoculum and the total moisture was made to 75%. Then, the flasks were incubated at 35^{o}C for 48hours in a shaker at 150rpm. The enzyme was extracted by adding 100ml of 0.05M sodium acetate buffer shaken for 20mins, filtered through muslinclothe. The culture filtrate was centrifuge at 4^{o}C,10,000 rpm for 10mins. The crude extract was made to undergo polygalacturonase assay (Ahmad *et al*., 2011).

Assay of Polygalacturonase Activity (PG)

Polygalacturonase activity was determined by measuring the concentration of galacturonic acid released from citrus pectin using the 3, 5, dinitrosalicyclic acid reagent (DNSA) assay(Miller, 1959). The Mixture containing 0.8ml1% citric pectin (Sigma) in 0.2M acetate buffer,pH 5.0 and 0.2ml of crude enzyme solution was incubated at 50^{o}C for 10min (Silva *et al*., 2002).One unit (U) of enzyme activity was defined asthe amount of enzyme which releases 1µmol ofgalacturonic acid per minute.

Morphological Identification

Pure cultures of the fungal isolates were morphologically identified on the basis of characteristics such as colony color, shape, size, and color of the back of the Plate. Placing small portions of the fungal growth on a glass slide and staining it

with a drop of lactophenol in cotton blue solution which was then observed under X10 and X40 objective lens of a light microscope. The presence of septa, the shape of spores, and other microscopic features were observed (Samson *et al*., 2004). The characteristics and appearance of the fungal isolates were compared with Atlas of mycology Robert and Ellen (1988).

Molecular Identification

Isolate 2 which have highest potential for producing polygalacturonase was selected and was identified at molecular level through; DNA extraction, Amplification of 18S rRNA genes, Gel electrophoresis and sequencing.

DNA Extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from the isolate using CTAB method (Zhang *et al*., 2010). Amplification of the 18S region of isolate 2 was performed using polymerase chain reaction, and 18S universal primers were used. The primers amplify the whole region of the 18S Rrna gene Zymo Taq premix (Zymo Research) supplied by Inqaba Biotech supply the PCR master mixture add full stop TheZymo Taq premix contain Taq DNA Polymerase, PCR Buffer, $MgCl_2$ (1.75uM), and ultrapure dNTPs. Agarose Gel preparation; 1 grams of agarose powder was suspended in 100ml of X1 TAE buffer and boiled in Microwave. About 2ul of ethidium bromides was added and mix thoroughly. About 25 µL of PCR reaction mixture was added to a 0 .2mL thin-walled PCR tube. The PCR mixture contain 12.5 µL master mix, 8.5 µL sterile distilled water, 2 µL DNA template, 1 µL forward primer, 1 µL reverse primer. The primer used were ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) at concentration of 10Mm. Afterwards, the samples were placed in the thermal cycler forthe process of amplification. Initial denaturation 95°C for 5 minutes, followed by 30 cycles of denaturation 95 °C for 30 seconds, annealingtemp 52.6°C for 30 seconds, extension 72°C for 30 seconds, followed by final extension 72 °C for 10 minutes. Purification of unpurified PCR Products was carried out by inqaba Biotec West Africa.

Sequencing Analysis

Sequencing was Sanger method which was carried out by Inqaba Biotec West Africa. Sequence obtained from the sequencing was compared to known 18s rRNA gene sequences in National Center for Biotechnology Information database using BLAST.

Phylogenetic Analysis

The 18S rRNA sequence obtained was pasted into BLAST in NCBI website and compared with the available nucleotide sequence database and the closet homologs of the sequence were identified. Twenty five closely related 18S Rrna gene sequences were recovered from GenBank in National Center for Biotechnology Information database, and compared with isolate 2 (*Aspergillus fumigatus*). The analysis involved 26 nucleotides sequences with *Penecillium christogenum* as an out group. Mega 11 was used to trim and aligned the sequence (Tamura *et al*., 2021).

RESULT AND DISCUSSION

Five fungal strains were isolated from Spoilt orange (Figure 1); the morphological characteristics of the fungi isolated from the spoilt orange were shown in fig 1.Table 1 shows the microscopic characteristics and the fungal specie identified. The fungal species identified *were Aspergillus niger, Aspergillus fumigatus, Sacchromyces, Aspergillus flavus, and Penecillium digitatum*. Tafinta *et al.*, 2013, reported that *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus* and *R. stolonifer* and some yeasts were found in the spoilt sweet orange fruits sold in Sokoto State, Nigeria. Mailafia *et al*., 2017 also reported the occurrence of the fungi and their frequencies in spoilt fruits in gwagwalada market Abuja Nigeria.

Isolate	Colonial appearance	Microscopic characteristics	Fungal specieidentified

1	Colonies are initially white, quickly becoming darkbrown with conidial production. Reverse was thesame	Smooth color conidiophores and conidia. The conidiophores are protrusion from septate and hyaline hyphae. Biseriated phialidea that radiate round the entire vesicle	*Aspergillus niger*
2	Colonies are dark green withwhite edges. Reverse appearcreamy	Rod septate, uniseriate phialide, no metula, rough terminating conidiophore in boom-like whorl of branches	*Aspergillus fumigatus*
3	Colonies are creamy white. Reverse appearthe same color	Cells appear circular/ovoid, apiculate or elongated	*Sacchromyces*
4	Colonies are yellow-green, reverse appear creamy	Dome-shaped vesicle, septate, cornidiophore are rough. Phialides loosely radiate on most of vesicle, phialides are both uniseriate and biseriate	*Aspergillus flavus*
5	Colonies are greenish-blue, reverse appear cream color.	Narrow septate hyphae. Separated brush-like conidiophore.	*Peniillium digitatum*

Table 1: Microscopic Identification of the fungalisolates using lactophenol blue

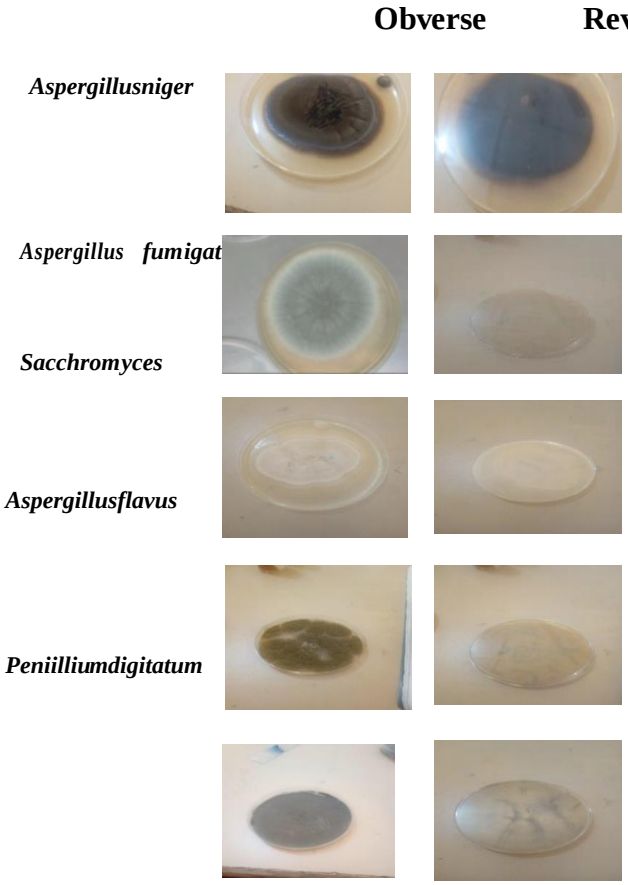


Figure 1: Plates of pure fungi (isolate1-5), isolated from spoilt orange: *Aspergillus niger* (isolate 1), *Aspergillus fumigatus* (isolate 2), *Sacchromyces* (isolate 3), *Aspergillus flavus* (isolate 4), *Penicillium digitatum* (isolate 5).

Table 2 shows the result of spoilage of fresh oranges after 14 days of incubation with the isolate. The result obtained agrees with the study conducted by Tafinta *et al*., 2013, Baiyewu *et al*., 2007 and Chukwuka *et al*., 2010, also reported that all the fungi isolated in this research were responsible for the orange spoilage.

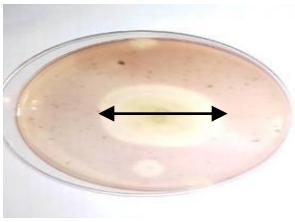
The isolates were screened for extracellular polygalacturonase using plate assay method, and checked for activity in fermentation medium. Based on the screening *Aspergillus fumigatus* and *Aspergillus flavus* were found to be active polygacturonase producers (Fig. 2), produces clearzone of diameter 32±0.07 mm and 26 ±0.03 mm respectively after 48h of incubation (Table 2). Table2 represents the total enzyme activities of the five isolates, *Aspergillus fumigatus* produce the highest enzyme activity of 12.79umol/min/ml. Hence*Aspergillus fumigatus* was found to have highest potential of producing extracellular polygalacturonase. Phutela *et al.*, 2005, Fawole and Odunfa (2003), reported that *Aspergillus fumigatus*, *Aspergillus niger* were active producers of extracellular polygalacturonase. Kumar *et al.*, 2012 reported that both polygalacturonase and pectin lyase were also produced by *Aspergillus foetidus* using mango peel assubstrate.

Table 2: Rot Diameter (14 days), Zone Diameter and Polygalacturonase Total Activity Produce by the Fungi Isolated from Spoilt Orange Fruits.

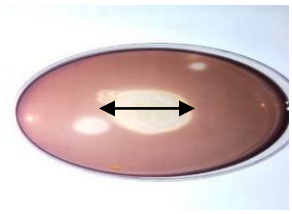
Fungal	Diameterof rot (mm)	Zone(mm)	PG

Isolates			Total Activity (µmol/min)
Aspergillus Niger	25.00±0.04[a]	21±0.12[a] (not clear)	7.19[a]
Aspergillus fumigatus	37.00±0.02[b]	32±0.07[b] (clear)	12.79[b]
Sacchromyces	20.00±0.02[c]	08±0.15[c] (not clear)	2.24[c]
Aspergillus flavus	30.00±0.01[d]	26 ±0.03[d] (clear)	8.35[d]
Penicillium digitatum	28.00±0.11[e]	14±0.16[e] (not clear)	4.69[e]

Results are presented as mean ± standarddeviation. Values bearing different superscript down the column are statistically different($p<0.05$). PG: Polygalacturonase



***Aspergillus fumigatus (*Isolate 2)**



***Aspergillus flavus* (Isolate 4**)

Figure 2: Plates of ***Aspergillus fumigatus*** **(Isolate 2)** and ***Aspergillus flavus*** **(Isolate 4**) showing the clear zone of 32±0.07mm and 26±0.03 mm respectively.

M S N

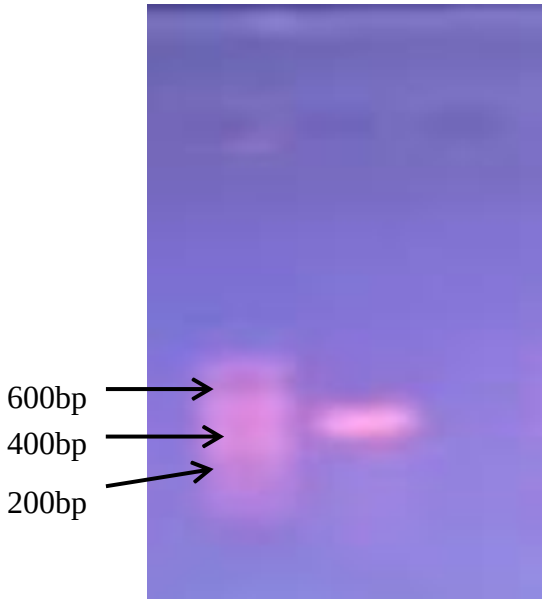


Figure 3: Amplification of 18S rRNA gene from isolate 2, M: Molecular marker (DNA ladder, Invitrogen, USA), S: Sample *Aspergillus fumigatus* (Isolate 2), N: Negative control without DNA. Primers used were ITS1 and ITS4

Below is the result of 18S rRNA sequence of *Aspergillus fumigatus* (Figure 4), the fungi has amplified DNA sequence of approximately 465 base pairs. Figure 5 is the result of region of local similarity between sequences after BLAST of *Aspergillus fumigatus* (isolate 2) in National Centre for Biotechnology information NCBI of 18S Rrna sequences Database. *Aspergillus fumigatus* (isolate 2) was 93.2% similar to *Aspergillus fumigatus* strains.

TGGGTGTCGGYTGGCGCCGGCCGGSCCT ACAGASCAGGTGACAAARCCCC
ATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCC
CCCGGGAKAGGGGGACGGGGGCCCAAC ACACAAGCCGTGCTTGAGGGCA
CMATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATG
TGCGTYCAAARACTCSATGATTCACTGA ATTCTGCAATTCACATTACTTATC
GSATTTCGCTGCGTTCTTCATCGATGCCG GAACCAASASATCCSCTGYTGAA
AGTTTTAACTGATTACGATAATCAACTCAGACTGCMTACTTTCAGAACAGS
GTTCATGTTGGGGTCTTCGGCGGGCGCSGGCCCGGGGGCGCAAGGCCTCCC
CGGCSGCCSTCKAAASGGCCGGCCCGCCSAACCAACAACGYACYATARACW
CSGGTGGGAGGTTGG

Figure 4: The 18S rRNA sequence of the *Aspergillus fumigatus* (isolate 2). DNA sequencing results shown an amplified productof 465 bp.

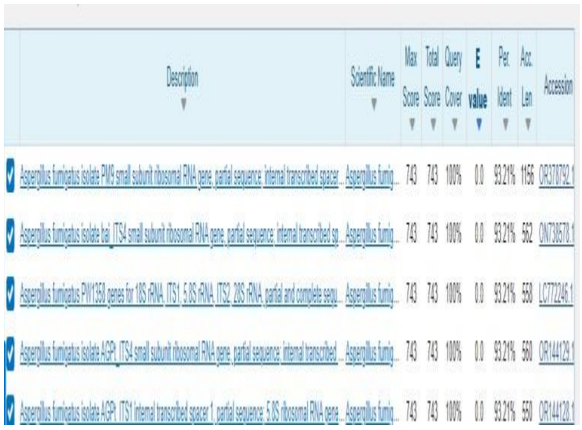


Figure 5: Blast result of *Aspergillus fumigatus* (isolate 2) against NCBI 18S Rrna sequences Database.

The phylogenetic evolutionary tree relationship was constructed using MEGA11 (Figure 6). Isolate 2 wasidentified as *Aspergillus fumigatus* sp., with 93.2% similarity to the strains compared, probably is a new strain.

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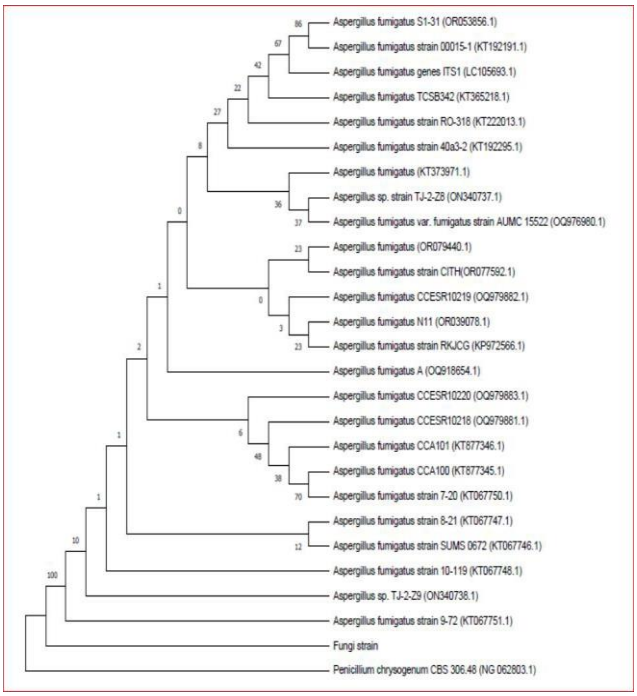

Figure 6: The 18S rRNA genetic relatioship between 25 other related references microorganisms.

Penicillium chrysogenum was used as an outgroup

CONCLUSION

Five isolates were isolated from spoilt orange fruit. All the isolates were found to be responsible for the orange spoilage. These isolates were morphologically identified as *Aspergillus niger, Aspergillus fumigatus, Sacchromyces, Aspergillus flavus and Penecillium digitatum* respectively. Among the fungi isolated, *Aspergillus fumigatus* (isolate 2)

was found to have highest potential of producing extracellular polygalacturonase. The isolate was 93.2% similar to *Aspergillus fumigatus* strains based on 18S rRNA analysis gene sequencing, probably is a new strain of the fungi.

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