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Screening of Fungi Isolates from Soil, Pulp Waste Water and Rotten Wood for Cellulase Producing Potentials

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ABSTRACT: Pulp waste water, soil from agricultural waste dump site and rotten wood were investigated for the presence of fungi with cellulolysic ability. Fungi Isolates obtained from the samples were identified based on cultural and morphological characteristics. Seven species of fungi namely, *Aspergillus niger, Rhizopus oryzea* and Fusarium from soil, *Penicillium notatum, Mucor resmosus* and *Aureobasidium sp* from rotten wood, *Trichoderma citrinoviride, Fusarium salani, A niger and P notatum* from pulp waste water were isolated. Among the seven fungi species , *A. niger, F. salani*, and *P. notatum* recorded the highest frequency of occurrence, (2), while *T. citrinoviride*, *R. oryzea*, *Aureobassidium sp* recorded frequency occurrence of one (1)each and the cellulytic activity was determined by the ratio of zone of clearing and colony diameter. The difference in zone of clearing produced by the fungi isolates (*A. niger, T. citrinoviride, F. salani*), on CMC containing plate were not significantly different at ($p< 0.05$). Highest cellulase activity ratio was exhibited by *T. citrinoviride* (1.39), followed by *A. niger* from soil (1.30).This result highlights the potential of *T citrinoviride* as strain for industrial production of cellulolysic enzyme.

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The demand for energy from is increasing due to growing population and industrialization (Bakare *et al.,* 2019), and Agricultural and industrial wastes are among the causes of environmental pollution (Milala *et al.,* 2005). These wastes which includes cereal straws, leaves, corn cobs, rice bran, wheat bran sugarcane baggers are highly underutilized in Africa and Nigeria in particular (Milala *al. et*, 2005). Cellulose is the most abundant components of plant biomass which is found in nature almost exclusively in plant cell wall and also produced by some bacteria species (lynd *et al.,* 2002). Perpetual renewal of plant biomass through the process of photosynthesis ensures inexhaustible supply cellulose (Sadaf *et al*., 2005). Cellulose is a linear unbranched homopolysaccharide consisting of glucose sub-unit joined together via β 1 - 4 glycosidic linkages. Individual cellulose molecules vary widely in length and are usually arranged in bundles or fibers (Walsh, 2002) Cellulose is commonly degraded by an enzyme called cellulase. Cellulotic enzymes are synthensized by a number of microorganisms. Fungi and bacteria are the main natural agent of cellulose degradation (Lederberg 1992). The specific cellulolytic activity shown by bacterial species is found to be depending on the sources of occurrence (Saxena *et al*., 1993). Cellulase is a group of hydrolytic enzymes that are capable of degradation all types of lignocellulosic materials (Muhammed *et al.,* 2013). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo (1,4) –β-D- glucanase (endoglucanase cellulase, CMCase (E C3.2.1.4), which cleaves β- linkage at random, commonly in the amorphous part of cellulose, exo-(1,4)-β-d- glucanase (cellobiohydrolase, from non-reducing or reducing end generally from the crystalline parts of cellulose and β-glucosidae (cellobiase [(EC 3.2.1.21)] which releases glucose from cellobiose and short- chain cellooligosaccharides (Bhat and Bhat 1997). Cellulose and other lignocellulytic enzymes have significant applications and biotechnological potentials for various industries including chemicals, fuel, food animal feed, textile and laundry, pulp and paper and agriculture (Ramesh *et al*., 2011. The aim of this research is to screen fungi isolates from three environmental sources for cellulolysic activity.

MATERIALS AND METHOD

Sources of Fungi: Fungi species were isolated from different environmental sources in Kaduna North West Nigeria. These include soil from agricultural waste dump where corn wastes have been left to decompose into soil. Pulp waste water was obtained from nice top paper mill industries, Goningora and rotten wood obtained from timber market in Panteka, Tudun wada, Kaduna State, Nigeria.

Isolation of Fungi: Fungi isolation was carried out according to Fawole and Oso (1988). About 5g of the soil sample was weighed into a 250ml conical flask

containing 100ml distilled water. The suspension was mixed vigorously to obtain uniform mixture. It was serially diluted to 10^{-10} and then dilution 10^{-4} and 10^{-5} were spread and plated on Potatoe Dextrose Agar (PDA) which was supplemented with 1% chloramphenicol for selective isolation of the fungi. The inoculated media was incubated at 30°C for 5days. Colonies obtained after incubation were sub cultured to obtain a pure cultures. The isolated pure cultures were stored at 4°C for identification and further use (Dilli, 2018). Fungi were also isolated from pulp waste water through serial dilution method (Jamila *et al.,* 2018). Isolation of fungi from Rotten wood was made by direct spreading of the Rotten wood on PDA and incubated at 30°C for 5days (Sadaf *et al.,* 2005).The colonies obtained were sub cultured to obtain a pure culture of the isolates . The fungi isolates were maintained on Agar slant at 4°C and sub cultured for active mycelia at regular intervals.

Test for Fungi Ability to utilize Cellulose: Screening of isolates for cellulytic activity was performed by planting pure culture on agar plate containing Carboxymethyl cellulose supplemented with streptomycin (100mg/ml) (Acharya *et al*., 2008). The plates were incubated at 30^0C for 5 days, to visualize the zone of hydrolysis. The plates were stained with aqueous solution of Congo Red Dye (CRD) (0.1%) and allowed for 15minutes and washed in 1M NaCl (Apun *et al*., 2008). Colonies with clear zones are indication of cellulase activity and the ratio of diameter was determined. The ratio was determined by dividing the clearing zone diameter by the colony diameter.

Morphological Identification of Fungi: The isolated fungal pure culture were aseptically scraped from the plate and placed on clean slides. The slide were stained with Lactophenol in cotton blue and covered with cover slips. The stained slides were then observed under a microscope (Dilli, 2018). Identification was based on cultural characteristics (colour, shape of colony, surface of the colony as well as microscopic structures (septate or non-septate hyphae, structure of conidia and hyphae) using current universal identification key (Hawkword *et al.,* 1995).

*Statistical Analysis***:** Data obtained from test of fungi ability to hydrolyse cellulose was analysed using one way analysis of variance (ANOVA) to verify the differences between the fungi isolates.

RESULTS AND DISCUSSION

Fungal isolates obtained from soil, rotten wood and paper mill effluents. A total of 10 fungal isolates were obtained from the three environmental sources (Soil, rotten wood, paper mill effluent) as shown in Table 1. All the isolates were of seven species namely *Aspergillus niger, Rhizopus oryzea, Mucor resormosus, Fusarium sp. Penicillium sp, Aureobasidium sp.* and *Trichoderma sp.*

Table 1 Fungal isolates obtained from soil, rotten wood and paper mill effluents.

SOURCE	CODE	DESCRIPTION OF FUNGAL ISOLATES	SUSPECTED ORGANISM
Paper effluent, Soil		Colony with dense layer of dark brown to black with age. Conidiophores stripes, smooth walled hyaline but often brown in colour. Vesicle globose to sub-globose. Phialides borne on brown septate nuclei. The often septate conidia globose to sub- globose, 3.5-5mm in diameter, brown, ornamented with irregular spines and ridges.	*Aspergillus niger*
Soil		Colony whitish becoming brownish gray with age. Stolid smooth or slightly rough colourless to yellowish brown. Sporangiophore globose, ovoid or	*Rhizopous oryzea*
Wood		Irregularly shaped, often polygonal. Colony whitish, becoming brownish-gray with age. Sporangia hyaline, brownish to gray. Tall and short sporangiophore with branches. Collumella ovoid ellipsoidal, cylindrical- ellipsoidal and slightly pyriform usually with truncate base and light brown in colour. Chlamydiosphore were barrel- shaped when young subglobose and yellowish in colour in old culture	*Mucor resmosus*
Soil paper effluent		Arial mycellium sparse, dense and floccus, agar surface is green to bluish- brown or leathery, greyish- white, cream to buff micro-conidiophores which from sporodochia 4-7days.	*Fusarium salani*
Paper effluent wood		A raised floccose aerial mycellium changing to yellowish or greenish gray. Conidiophores stripe rough- walled mostly bi to terverticulate, multi- branched conidiophore	*Penicilluim Notatum*
Wood		Colonies flat creamy, shiny when young, turning dark with age. Appears to be granular in PDA with green conidia distributed throughout. An irregular yellow zone without conidia. White	*Aureobasidium spp*
Paper effluent		pustules around. The inoculums grown in the green mats of conidia. Conidia are globose; philiades are flask shape and short. Conidiophores system appears single.	*Trichoderma spp*

The frequency of occurrence of fungi isolates obtained from soil, rotten wood and pulp waste paper: Penicillum notamum, Aspergillus niger and Fusarium salani had frequency occurrence of two each. While *Mucor resmosus*, *Rhyzopus oryzea,Aureobasidium sp* and *Trichoderma citrinoviride* recorded frequency occurrence of one each as shown in figure 1

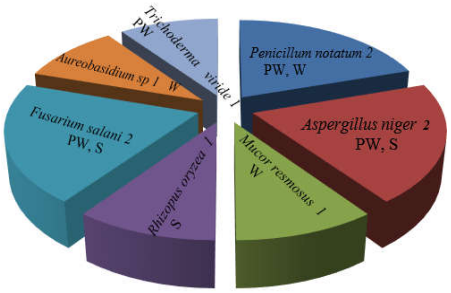

Fig 1 the frequency of occurrence of fungi isolates obtained from soil, rotten wood and pulp waste water. **KEY:** PW= Pulp waste water**,** S= Soil, W= Rotten wood

The screening of fungal isolates for cellulase production: Trichoderma citrinoviride exhibited highest clearing zone to mycelium diameter ratio of (1.39mm) followed by *Aspergillus niger* from soil (1.30mm.) as shown in Table 2., while *Penicilium notatum* isolated from pulp waste water had the least ratio of (0.09mm).The zone of clearing produced by *Trichoderma spp, Aspergillus spp, Fusarium spp* did not differed significantly at P> 0.05 from each other but differed significantly at P < 0.05 from *penicillium spp*, *Rhizopus spp and Fusarium spp.*

Zone of clearing to mycelium diameter ratio of the isolates: Trichoderma sp exhibited highest clearing zone to mycelia diameter ratio (1.39mm) followed by *A. niger* from soil (1.30mm) while *Rhizopus sp* recorded least (0.09mm) as shown in Figure 2

Table 2: Zone of clearing to mycelium diameter ratio of fungi isolates

Name of isolates	Mean Colony Diameter	Mean Clearing Zone(Mm)	Clearing Zone to Colony Diameter Ratio	Sources
Aspergillus sp	24.7	32.3	1.3.	S
Aspergillus sp	25.3	31.0	1.2	PE
Fusarium sp	26.3	3.10	1.1	S
Mucor sp	*	*	*	W
Rhizopus sp	21.0	2.3	0.10	S
Penicillium sp	33	4	0.12	W
Penicillum sp	35	3	0.09	PE
Trichoderma sp	25.3	35.3	1.39	PE
Fusarium sp	25.8	30.9	1.19	PE
Aureobasidium sp.	-----	------	------	W

Measurement of clearing zone was not possible due to overgrowth of mycelia throughout the plates: No growth observed. **KEY: S=Soil, PE=Paper effluent, W=Rotten wood*

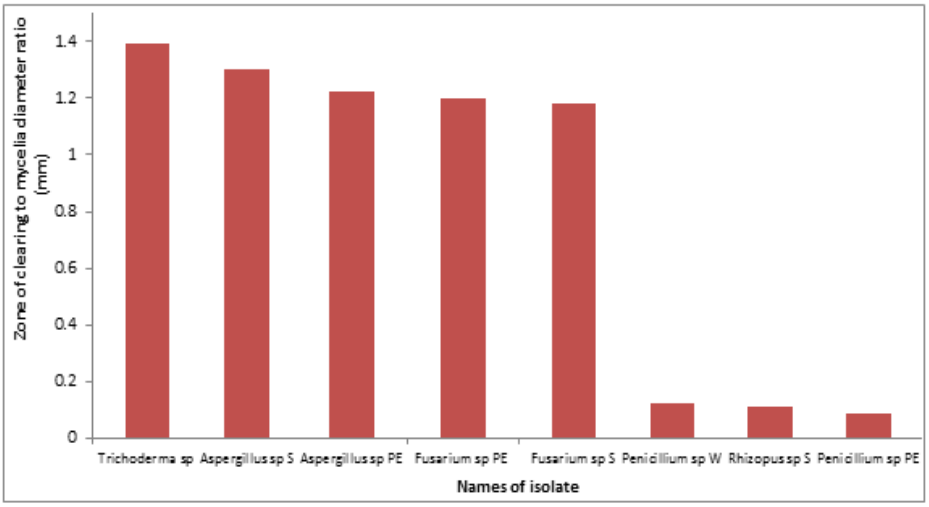

Fig 2: Zone of clearing to Mycelium diameter Ratio (mm) of fungi isolates
KEY: S=Soil, PE=Paper effluent, W=Rotten wood

Ten fungi isolates were obtained from soil, pulp waste water and rotten wood. This is not surprising as fungi are saprophytes and thrive on decomposing plant materials. Sadaf *et al*., (2005), isolated fungi from soil, air and infected plants, while Lakshmi and Narasimha (2012) isolated fungi from forest litter soil. The seven fungi

species isolated belong to the *Aspergillus niger, Fusaruim salani, Rhizopus oryzea*, *Trichoderma sp, Fusarum sp, Penicillium notatum*, *Mucor sp, Penicillium notatum* and *Aureobasidium sp.* (Sadaf *et al*., 2005), earlier reported presence of fungi species from soil, air and infected plant. Pulp waste water had the highest fungal load of 4.This could be related to the fact that the conditions of pulp waste water is optimum for growth of different fungi species. This could also be attributed to availability of nutrients for different fungal growth Ekundayo and Anotupin, (2015). Soil from agricultural waste dumps recorded 3 fungal bio- loads. This subscribes to the report of Sanchez (2009) that lignocelluloses are good substrates for fungi and are available in the waste dump soils. The variation in the fungal load form different habitat depends on the ability of the microorganism to colonise, metabolize and obtain necessary nutrients from the environment as well as prevailing environmental condition and physicochemical properties of the sources. This is in line with the report that chemical constituent and physicochemical properties of ligonocellulosic biomass make them suitable receptacles for cellulytic fungi (Al-Qahtani *et al*., 2013).

Among the ten fungi species isolated*, Aspergillus niger, Fusarium salani*, and *Penicillium notatum* had the highest frequency occurrence, while only one strain of *Trichoderma sp.* was isolated. The low frequency of isolation of Trichoderma could be due to the slow growth rate of the fungi and therefore slow growth competition with the rapidly growing fungi like Aspergillus spp and Rhizopus spp. The fungi species obtained from different habitat (Soil, pulp waste water and rotten wood) unveiled different cellulase activity based on carboxyl methyl cellulose agar plate assay. Plate screening based on clear zone formation of cellulase produced by microorganisms have been reported for *Penicilluim funiculosum, Aspergillus repens, Mucor resmosus, penicillium simplissimum, Epicoccum nigrum, Penicillium. Fellutanum* (Dele, 2007) and *A. niger* (Damisa, 2013). For any organism to successfully utilize cellulose in a medium as a carbon source for growth, it must first hydrolyse the cellulose to glucose. This cellulysic ability is possible if the organism can produce cellulase and exude them from its cell into the cellulose medium (Apun *et al*., 2000). Among the fungi isolates, *Trichoderma sp* from pulp waste water produced the highest cellulase activity (1.39) based on the zone of clearing to colony diameter ratio, followed by *A. niger* from soil (1:3S). Ekundayo and Arotupin,(2015) recorded *A. niger* from saw mill soils having highest cellulase activity ratio of 1.9.This is in contrast with the result of this study which recorded *T. citrinoviride* from pulp waste water as having cellulase activity of 1.39 while *A. niger* from Agricultural waste dump sites recorded cellulase activity of 1.3. This difference could be as a result of differences in habitat of isolations of the fungi. This result is in agreement with findings of Sadaf *et al*., (2005), which reported *Aspergillus sp, Fusarium sp., Rhizopus sp., Trichoderma sp.* and *Pennicillum sp* as cellulytic fungi. Wood and Care, (1972) equally reported *Trichoderma sp.* and *Aspergillus sp.* as important cellulysic fungi Zakpa *et al.,* (2009), reported *Aspergillus niger* to have high cellulolstic ability on Mendel's agar medium based on clearing zone to colony diameter ratios. The differences in the diameter of zone of clearing formed by the isolates could be as a result of the ability of the cellulase produced to diffuse through the medium and a function of their genetic make up to secrete cellulase with high speed (Gautam *et al.,* 2010).

Conclusion: The investigations for effective microorganisms that possess the capacity to hydrolyse lignocellulosic wastes are a continuous research. Pulp waste water and soils from Agricultural waste dump site is found to be ideal environment for cellulolytic fungi. *T citrinoviride* and *A. niger* isolated in this studies could be used for industrial production of callulase after further research.

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