Characterization of the microrganisms associated with *Pleurotus ostractus* and *Pleurotus tuber-regium* spent mushroom substrate

*S. E. Okere[1] and C. G. Ikechi-Nwogu[2]

[1]*Department of Crop Science and Technology, Federal University of Technology P.M.B 1526 Owerri, Imo State, Nigeria. 08037565046*

[2]*Department of Plant Science and Biotechnology, University of Port Harcourt Choba Rivers State, Nigeria.08032325098*

**Corresponding author's email: chinyerum.nwogu@yahoo.com*

Abstract

The microbial characterization of two spent mushroom substrate (SMS) from *Pleurotus osteratus* (Jacq. ex Fr.) P. Kumm and *Pleurotus tuber-regium* (Rumph. ex Fr.) Singer were investigated. The microbiological properties of the spent mushroom substrate were determined using standard methods. Quantitative microbiological analysis revealed that *P. osteratus* spent mushroom substrate (POSMS) contained 7.9×10^5 and 1.2×10^3 cfu g^{-1} of total heterotrophic bacteria and total fungi count, respectively, while *P. tuber-regium* spent mushroom substrate (PTSMS) contained 1.38×10^6 and 9.0×10^2 cfu g^{-1} of total heterotrophic bacteria count and total fungi count respectively. The fungal species from PTSMS included *Aspergillus* and *Cladosporum* species, while *Aspergillus* and *Penicillium* species were found in POSMS. The bacterial species encountered from PTSMS included *Bacillus, Acinetobacter, Alcaligenes, Actinobacter* and *Pseudomonas* species while *Bacillus, Actinobacteria, Aeromonas, Lactobacillus* and *Aerococcus* species were encountered from POSMS. Therefore, based on the findings from this study it can be concluded that spent mushroom substrates contain microorganisms that can be utilized both in bioremediation of oil polluted soils as they contain important hydrocarbon utilizing microorganisms such as *Penicillium*, *Aspergillus* and *Bacillus* species and also as sources of plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas* and *Bacillus* species which can induce resistance on plants. However, further studies are recommended to confirm these findings further.

Keywords: Characterization, Microorganisms, *Pleurotus ostractus*, *Pleurotus tuber-regium,* Spent mushroom substrate

Introduction

Edible mushrooms are a group of fungi (Basidiomycetes) that grow naturally on tree trunks, leaves, roots of trees as well as on dead and decaying woody materials (Stamet, 2000; Lindequist *et al.*, 2005). They are achlorophylous organisms and thus can be grown in jars and bottles devoid of sunlight. Nutrients such as vitamins, minerals and micro and macro nutrients have been detected in most oyster mushrooms which are called rot fungi because of their ability to degrade lignocellulosic materails and are able to colonize different agricultural wastes as substrates (Jonathan *et al.,* 2012).

Several reports have emphasized that the microorganisms associated with mushroom compost after flushing /harvesting are quite lignolytic and posses xenobiotic degrading genes (Fragoeiro, 2005; Chukwura, 2012). Spent mushroom substrates are rich sources of xenobiotic degrading bacteria, actinomycetes and lignolytic fungi. These microorganisms degrade pollutants to innocuous compounds such as carbon dioxide and water (Semple *et al.,* 2001). These microorganisms can biotransform pollutants into less toxic substances and or lockup pollutants with the organic matrixes thereby reducing pollutants bioavailability (Semple *et al.,* 2001).

According to Ibiene *et al.* (2015), identities of bacteria and fungi that colonize the spent mushroom substrate are not well documented. The SMS are rich in plant growth promoting rhizobacteria (PGPR) that colonize the roots of plants to enhance plant growth and reduce diseases, nematodes or insect damage (Reddy *et al.*, 2013). There has been much research interest in PGPR and with an increasing number of PGPR now being commercialized for crops. Organic growers may have been promoting these bacteria without knowing it. The addition of compost and compost teas promote existing PGPR and may introduce additional helpful bacteria to the fields. However, PGPR such as *Pseudomonas* and *Bacillus* species have attracted much attention for their role in reducing plant diseases. Some PGPR use scarce resourses and thereby prevent or limit the growth of pathogenic microorganisms. Even if nutrition are not limiting, the establishment of benign or beneficial organisms on the roots limits the chance of a pathogenic organism that arrives later will find space to become established. Numerous rhizosphere organisms are capable of producing compounds like HCN that are toxic to pathogens (Reddy, 2013). Thereafore, the objective of this study was to provide basic information regarding those microorganisms

especially bacteria and fungi that are associated with *P. ostractus* and *P. tuber-regium* spent mushroom substrates and suggest how they can be used to enhance crop production as soil amendent and to induce disease resistance.

Materials and Methods

Source of spent mushroom substrate

POSMS and PTSMS used for this study were obtained from Dilomat Farms and Services located at the Faculty of Agriculture, Rivers State University of Science and Technology, Port Harcourt, Rivers State Nigeria, after 4 months of fruitbody production and was used immediately for microbiological analysis.

Media preparation

The medium used for isolation and heterotrophic bacterial count was agar at the concentration of 28 g L^{-1}. This was prepared by adding 28 g of nutrient agar powder to 1 L of distilled water in an Erlenmeyer flask and then plugged with cotton wrapped with aluminum foil. Mineral salts medium was used in screening bacteria and fungi as well as counting bacteria and fungi in samples. The mineral salts medium contains the following per litre: 10 g NaCl; 0.42 g $MgSO_4.7H_2O$; 0.29 g KCl; 1.25 g KH_2PO_4; 0.42 g NH_4NO_3; 0.83 g K_2HPO_4 and 15 g agar. The pH adjusted to 7.2 and 3.7 for bacterial and fungal growth, respectively. Lactic acid was incorporated into the fungal medium at a concentration of 1% to inhibit bacterial growth. Appropriate volumes of these media were dispensed into Erlenmeyer flasks, plugged with cotton wool wrapped with aluminum foil. The media was sterilized and allowed to cool. Upon cooling, the agar was poured into sterile glass Petri dishes and allowed to solidify. All solidified media were inoculated and incubated at room temperature (28-30 ºC) for 24 hours (nutrient agar) and 72 hours (mineral salts agar) to check for sterility.

The medium for fungal count and isolation was sabouraud dextrose agar (SDA) amended with 1% lactic acid. This was prepared by adding 65 g SDA to 1 L of distilled water and sterilized. This was allowed to cool to 47 ºC before the lactic acid was added and plated out in sterile Petri dishes.

Isolation of microorganisms

Viable aerobic heterotrophic bacteria present in the samples were isolated on nutrient agar plates. The samples were diluted using sterile physiological saline. The spread plate technique was used. 1 g sample was aseptically added to 9 mL of sterile physiological saline in a test tube to give 10^{-1} dilution. Subsequent tenfold serial dilution was made from these initial dilutions up to 10^{-6}. Each dilution was inoculated onto sterile nutrient agar plates by aseptically pipetting 0.1 mL of the dilution and thoroughly spreading it on the agar surface with sterile bent glass rod. The inoculated plates were incubated at 37 ºC for 48 h. The diiferent colonies that grew on the medium were picked and subcultured by steak plate method. The fungi present in the samples were isolated using the same proceedure but with sabouraued dextrose agar plates. A portion of each fungal colony which developed was picked using a sterile inoculating needle and aseptically sub-cultured onto fresh sabouraud dextrose agar plates. The plates were kept as stock cultures for subsequent use.

Enumeration of heterotrophic microorganisms

Heterotrophic bacteria and fungal populations in the sample were enumerated. Ten-fold serial dilutions of the samples were prepared A volume of 0.1 mL of different dilutions was spread-plated in duplicate plates of nutrient agar and incubated at 37 ºC for 48 h. Colonies numbering between 30–300 on plates were counted. Fungal population was enumerated in the same manner using sabouraud dextrose agar amended with 1% lactic acid. The inoculated sabouraud dextrose agar was incubated at 37 ºC for 72 h. The means of duplicate colony counts were used to calculate and compute the number of heterotrophic microorganisms in the original samples.

Identification of fungal isolates

Fungal isolates were examined macroscopically and microscopically using the needle mounts method. A wet mount slide was made by tranferring a small amounts of the culture with a dissecting needle to a drop of lactophenol cotton blue stain on a grease-free clean glass slide, covered with a cover glass and examined under low power objectives. The isolate were identified according to Malloch (1981).

Identification of bacterial isolates

Cultural and biochemical characterization of the isolates were observed after culturing on nutrient agar. The following test were carried out: gram reaction, cell morphology, test for spores, catalase, oxidase, citrate utilization, motility, indole, and starch hydrolysis as well as their ability to ferment some sugars- glucose, sucrose and lactose. The tests were performed following the procedure of Cruickshank *et al*. (1980) and the identification was done using Bergey (1993) manual of determinative bacteriology.

Results and Discussion

Cultural description and microscopy of fungi Isolates

The fungi species isolated from the two spent mushroom substrate namely *P. ostratus* and *P. tuber-regium* is presented in Tables 1. A total of 6

fungal species comprising 2 species from *P. tuber-regium* and 4 species from *P. ostreratus* were isolated. They were coded as isolate PT1 - PT2 for *P. tuber-regium* and PO1 - PO4 from *P. Osteractus,* respectively. These microorganisms were characterized as *Aspergillus* and *Cladosporum* species from *Pleurotus tuber-regium* and *Aspergillus* and *Penicillium* species from *P. osteractus* (Table 1). These identifications were based on cultural description and basic microscopy as described by Samson *et al*. (1995).

Isolates PT1, PO1, PO2 and PO3 had black or brown colouration in both front and reverse view of culture plate with or without cracked lines with aseptate hyphae with branched conidiophores having vesicles that produce characteristic of conidia with or without rough head of pigmented oval shaped spores which were identified as *Aspergillus* species (Samson *et al.,* 1995). PT2 isolates had dark brown surface with slightly raised center and few green spots with septate hyphae that produce oval spores with double or single celled was identified as cladosporum species while isolate PO4 with light green (sky blue) colouration in the front view but green in reverse view of culture plate with single branched septate hyphae and characteristics of conidia was identified as penicillium. The fungi species encountered in this study were consistent with the findings of Obodia *et al.* (2010) which have been found to degrade cellulose, hemicellulose, starch and to some extent lignin (Ryckeboer *et al.,* 2003).

Biochemical characterization of bacteria isolate

The bacteria species isolated from *Pleurotus ostractus* and *Pleurotus tuber-regium* is presented in Tables 2. A total of 11 species comprising 5 species from PO and 6 species from PT were isolated in this study and they were coded as isolates PO1- PO5 from PO and PT1-PT6 from PT. These micro organisms were characterized as Bacillus, actinobacter, aeromonas, lactobacillus, aerococcus, acinetobacter, alcaligenes, actinobacter and pseudomonas species. These identifications were based on a series of morphological and biochemical test. Isolates PO1, PT1 and PT3 were gram positive, motile and spore bearing rods with negative indole test, citrate, slant, non gas formation and starch hydrolysis and ferment most sugars were identified as bacillus species which is in aggreement with the findings of Prescott *et al.* (1999) and Jonathan (2006).

PO2 isolates were gram negative, non motile rods with negative indole, citrate, gas formation, H_2S production, slant, butt and starch hydrolysis but produces acid or gas on glucose, sucrose and lactose but negative on maltose. They were identified as actinobacteria species (Table 2). Isolates PO3 were gram negative, non motile, non- spore bearing rods with negative indole, gas formation H_2O production, slant but positive oxidase and citrate test. they produce acid on glucose snd sucrose fermentation but non gas and acid production with lactose and maltose. They were identified as aeromonas species. Also based on Bergey (1986) manual of determinative bacteriology. PO4 isolates are gram positive, non motile and spore producing rods. They had negative reactions with indole, oxidase test and do not produce gas and H_2S and do not hydrolyse starch but positive citrate test. They produce acid and or gas on glucose, sucrose, lactose and maltose and identified as lactobacillus species.

Isolates PO5 are gram positive coccus, motile, non spore producing with negative indole, oxidase, gas formation, H_2S production, slant but positive citrate test and starch hydrolysis. They produce acid on glucose, sucrose, lactose and maltose fermentation. They are thereafor identified as aerococcus specie. PT2 isolates are gram negative, non motile, non spore producing rods but negative indole, oxidase gas formation, H_2S production. They react positively with citrate and produce acid or glucose, sucrose and maltose fermentation but negative acid or gas production on lactose fermentation and identified as acinetobacter specie. PT4 isolates are gram negative, motile, non spore bearing rods with negative indole, gas formation, H_2S production, slant and non starch hydrolysis. They react positively with oxidase, citrate and butt with none acid and gas production on the fermentation of glucose, sucrose, lactose and maltose and identified as alcaligenes species.

Isolates PT5 are gram negative, non-motile, non- spore bearing rods with negative indole, oxidase, gas formation, H_2S production, but positive citrate test and acid production on glucose, sucrose, lactose and maltose fermentation and they are identified as actinobacter species. PT 6 isolates are gram negative, motile, non-spore bearing rods with negative indole, gas formation, H_2S production. They produce acid and or gas on the fermentation of glucose, sucrose, lactose and maltose and identified as pseudomonas species. The bacteria ecology observed in this study differs significantly from the observation of made by Jonathan (2006) who worked on the bacteria ecology of mushroom compost. This result revealed that a new set of microorganisms colonize the SMS immediately after the end of fruitbody production. This however could be the first account of this observation.

Total Microbial Count in the SMS

The total microbial count present in the two spent mushroom substrates is presented in Table 3. The result of the total microbial count of the SMS revealed that the total heterotrophic bacteria present in the *Pleurotus ostreatus* spent mushroom substrate 7.0×10^5 cfu g^{-1} was lower than *P. tuber-regium* spent mushroom substrate which had 1.38×10^6 cfu g^{-1} though cultivated on the same substrate (sawdust).

The result obtained from the microbiological count could be attributed to the differences in the moisture content of the substrate as very wet substrates favours microbial colonization as against dry substrates. This result did not also agree with the result obtained by Obodai *et al.* (2010) who reported THBC in *Pleurotus* SMS within the range of 1.85×10^4 and 6.0×10^6 cfu g^{-1}. The total fungi count obtained from *P. ostreatus* was 1.2×10^3 cfu g^{-1} which was higher than 9.0×10^2 cfu g^{-1} obtained from *P. tuber-regium* spent mushroom substrate which is in agreement with the result obtained by Ibiene *et al.* (2015). A closer look at the result also revealed that the SMS with higher bacterial count recorded lower total fungi count which could be attributed to the fact that the SMS with higher bacterial count may have released certain secondary metabolites such as antibiotics which may have inhibited the growth of fungi. This observation is not in agreement with Ibiene *et al*. (2015) who observed lower bacteria counts and higher fungi count in all the SMS studied but the result is consistent with the result obtained by Obodai *et al*. (2010) in which they reported that bacterial counts were at all points higher than the fungi count. The total microbial composition of the two spent mushroom substrate revealed that *P. ostreatus* SMS recorded total bioload of 7.91×10^5 cfu g^{-1} significantly lower than *P. tuber-regium* SMS which had 1.38×10^6 cfu g^{-1} total bioload.

Conclusion

The result obtained from this study revealed that PTSMS and POSMS contained hydro carbon utilizing micro organisms required for bioremediation of oil polluted soil as well as PGPR that releases elicitors that could induce resistance for plant diseases. Thereafore SMS could be effectively utilized in bioremediation of oil poluted soils and as sources of PGPR in the management of plant diseases.

Table 1: Identification of fungal isolates.

Isolate no.	Cultural description	Microscopy	Probable genera
PT1	Black/brown colouration in both front and reverse view of culture plate	Aseptate hyphae with rough head of pigmented oval shaped spores	*Aspergillus* spp
PT2	Dark brown surface with slightly raised centre and few green spots	Septate hyphea that produce oval spores double or single celled	*Cladosporum* spp.
PO1	Greyish-dark surface , light yellow reverse with cracked lines	Aseptate hyphea with branched conidiaphores bearing vessicles producing chains of conidia	*Aspergillus* spp.
PO2	Light green surface with few cracked lines at the reverse	Aseptate hyphea showing large spherical visicle giving rise to melulae with or without conidia	*Aspergillus* spp
PO3	Black surface and brown reverse with cracked lines	Aseptate hyphea with branched conidiophores having vesicles that produce characteristics of conidia with the presence of foot cells.	*Aspergillus* spp
PO4	Light green (sky blue) colouration in the front view but green in front in reverse view of culture plate	Single branched septate hyphea and characteristics of conidia	*Penicilliun* spp

Key
PO –*Pleurotus ostreatus*
PT-*Pleurotus tuber-regium*

Table 2: Biochemical Characterization of Bacteria Isolate.

I solate no	Gram reaction	Cell morphology	Test for spores	Indole test	Oxidase test	Citrate test	Gas formation	H_2S production	Slant	butt	Starch hydrolysis	Moti-lity test	Sugar fermentation				Probable genera
													G	S	L	M	
PO1	+	Rods	+	-	+	-	-	-	-	+	+	+	A/G	A	A	A	*Bacillus* spp
PO2	-	Rods	-	-	+	-	-	-	-	-	-	-	A/G	A	A	-	*Actino bacteria* spp
PO3	-	Rods	-	-	+	+	-	-	-	+	+	-	A	A	-	-	*Aeromonas* spp
PO4	+	Rods	-	-	-	+	-	-	-	-	-	-	A/G	A/G	A/G	A	*Lacto bacillus* spp
PO5	+	Cocci	-	-	-	+	-	-	-	+	+	+	A	A	A	A	*Aerococcus* spp
PT1	+	Rods	+	-	-	+	-	-	-	+	-	-	A/G	A/G	A/G	A	*Bacillus* spp
PT2	-	Rods	-	-	-	+	-	-	-	-	+	-	A	A	-	A	*Acinetobacter* spp
PT3	+	Rods	+	-	-	+	-	-	-	+	-	+	A/G	A	A	A/G	*Bacillus* spp
PT4	-	Rods	-	-	+	+	-	-	-	+	-	+	-	-	-	-	*Alcaligenes* spp
PT5	-	Rods	-	-	-	+	-	-	-	+	-	-	A	A	A	A	*Actinobacter* spp
PT6	-	Rods	-	-	+	+	-	-	-	-	-	+	A/G	A/G	A	A	*Pseudomonas* spp

Key
- A- Acid production
- A/G-acid and gas productions
- \+ Positive
- \- Negative
- PO –*Pleurotus osteratus*
- PT- *Pleurotus tuber-regium*

Table 3: Microbial count in the spent mushroom substrate.

Sample	Total Heterotrophic Bacteria Count (cfu g^{-1})	Total fungi count (cfu g^{-1})	Total bioload (cfu g^{-1})
Pleurotus ostreatus SMS	7.9×10^5	1.2×10^3	7.91×10^5
Pleurotus tuber-regium SMS	1.38×10^6	9.0×10^2	1.38×10^6

References

Chukwura EI, 2012. Microbial transformation of Biospheric wastes for economic growth, 21st Inaugural lecture, Nnamdi Azikiwe University, Awka Nigeria.

Fragoeiro SIS, 2005. Use of fungi in bioremediation of pesticides, A Ph.D thesis submitted to the Applied Mycology Research Group, Institute of Biosciences and Technology, Cranefield University.

Ibiene AA, Okereutugba PO, Orji FA, Dike EN, 2015. Physico-chemical and culture-dependent micrbiological characterization of spent Pleurotus composts from three different agro-based wastes. *J. Adv. Biol. Biotechnol.,* **3**: 173-183.

John GH, Noel RK, Peter HAS, James TS, Stanley TW, 1994. Bergey's manual of determinative bacteriology 9th. Ed.

Jonathan SG, 2006. Bacteria associated with compost used for cultivation of Nigerian edible mushroom *Pleurotus tuber-regium* (Fr) Singer and *Lentinus squarrosulus* (Berk). *Afr. J. Biotechnol.,* **5**: 338-342.

Jonathan SG, Oyetunji OJ, Asemolaye MA, 2012. Influence of spent mushroom compost (SMC) of *P. ostractus* on the yield and nutrient compositions of *Telfaria occidentalis* HOOK. FA. (Pumpkin), a Nigerian leafy vegetable. *Nat. Sci.,* **10**: 149-156.

Linderquist U, Niedermeyer THT, Julich W, 2005. The pharmacological potentials of mushroom. *Ecam,* **2**: 285-299.

Malloch D, 1981. Moulds, their isolation, cultivation and identification. University of Toronto Press, Toronto. pp. 97.

Obodia M. Amua-Awua W, Odam-Hen GT, 2010. Physical, chemical and fungal phenology associated with the composting of wawa sawdust (*Triplocliton scleroxylon*) used in the cultivation of oyster mushroom in Ghana. *Int. Food Res. J.*, **17**: 229-237.

Okere SE, 2017. Studies on the microbial metabolites in spent mushroom substrate and the management of cassava mosaic disease. A Ph.D thesis submitted to the Post Graduate School University of Port Harcourt, Rivers state, Nigeria.

Prescott LM, Hawley JP, Klein AO, 1999. Microbiology (4th. Ed) Megraw Hill, USA.

Ryckeboer J, Mergaert J, Vacs K, Klammer S, Chercq DE, CoosemansD, Insam J, Swings J, 2003. A survey of bacteria and fungi occuring during composting and self heating process. *Ann. Microbiol.,* **53**: 349-410.

Reddy PP, 2013. Recent advances in crop protection, Springer India 2013. pp. 1-11.

Semple K, Reid B, Fermor T, 2001. Impact of composting strategies on the treatment of soil contaminated with organic pollutants. *Environ. Poll.,* **112**: 269-283.

Stamets P, 2000. Growing goumet and medicinal mushrooms. Berkely Ten Speed Press.

Samson AR, Hockstra ES, Frisvad JC, 1995. Introduction of Food – Borne fungi. 4th ed. Netherlands: Pensen and Loogen.