Cultivation of mushroom (*Pleurotus ostreatus*) and the microorganisms associated with the substrate used

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ABSTRACT

This study was conducted to investigate the microorganisms associated with substrate used in the cultivation of mushroom (*Pleurotus ostreatus*) in a mushroom farm. The substrate which is a mixture of sawdust, rice bran, lime, and water was composted for 30 days and pasteurized for use in the cultivation. Samples of pasteurized substrates were randomly collected for the analyses using standard microbiological techniques. Microorganisms isolated and characterized and their frequency of isolation from the substrate include species of fungal genera *Aspergillus* (40.9%), *Fusarium* (22.7%), *Mucor* (5.6%) *Penicillium* (17.0%), *Rhizopus* (11.6%) and *Trichoderma* (2.3%). The species of bacteria genera isolated and frequency of isolation was *Bacillus* (36.67%) *Clostridium* (16.67%), *Enterobacter* (18.33%), *Escherichia* (15%), and *Pseudomonas* (13.33%). Aspergillus and Bacillus species had the highest frequency of occurrence for fungi and bacteria respectively. Proper composting promotes the development of a number of saprophytic soil microorganisms that helps in the degradation of the substrate. The presence of cellulolytic fungi such as *Aspergillus, Penicillium* and *Trichoderma* are associated with the composting process and do accelerate composting for efficient recycling. Generally, the presence of fungi and bacteria in the pasteurized substrate is attributed to the ineffectiveness of the method adopted for pasteurization and the possession of spores that were heat tolerant by most of the isolates. The presence of *E. coli* which is an indicator of faecal contamination is attributed to the feacal contamination of the streams and river banks around the Sawmill where logs are retained before being sawn with the sawdust as a by-product. The presence of potential pathogens such as *Bacillus, Clostridium, E. coli* and *Enterobacter* can lead to bacterial disease of edible mushrooms and economic loss. The presence of these microorganisms also has serious implications for human health.

Keywords: Mushroom, *Pleurotus ostreatus*, sawdust, substrate, fungi, bacteria, *E. coli*

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Introduction

Mushrooms are the fruiting bodies of different kinds of fungi. Beneath the mushroom in the soil or substrate, is the fungus itself, consisting of a mat of intertwined hyphae, sometimes several feet in diameter. The mushroom first appear as white tiny balls consisting of short stem (stipe) and a cap (pileus) which begin to open up like an umbrella.

Unlike higher plants, mushrooms lack the ability to use solar energy. Mushrooms extract their carbohydrates and proteins from a rich medium of decaying organic matter vegetation. About 100,000 species out of the numerous other species are known to be edible and occurring in various parts of the world under different climatic and environmental conditions. Mushroom has continued to attract human

attention because of its enigmatic nature, its long established nutritional and health qualities, beliefs in its spiritual potency and curative qualities and its very curious nature which defies classification both from the point of view of edibility as well as proper classification as plant or animal.

Mushrooms provide a rich addition to the diet in form of protein, carbohydrates, minerals, vitamins, fat and fiber. In terms of nutrition, mushroom falls between the best vegetable and animal protein source. They have a high percentage of all the nine essential amino acids that promote good health; they are low in calories (less than 35k cal per 100g) with trace of sugar and without cholesterol. Mushrooms are richer in vitamins (B1, B2 Niacin, B12 Pantotemic acid and vitamin C) Most mushroom practically oyster mushroom has a very high content of vitamins A, C, D, and K as well as water (Mile and Chang, 2004). The fats content in mushroom is very low, less than 8% dry weight which consists mostly of unsaturated fatty acids which is less harmful to health than saturated fatty acids found in animal fats. Mushroom contains very little of carbohydrates (little sugar and no starch at all), which makes it ideal for diabetic patients and people prone to obesity. Mushroom contains more minerals than the amount of minerals found in vegetables. The high fiber content of mushroom is an invaluable advantage over other food items for easier and better digestion and assimilation in the alimentary canal.

Most mushroom have exceptional medicinal potentials and properties, especially in diseases such as high blood pressure, asthma, respiratory tracts infections, anaemia, rheumatism, diabetes, hepatitis, tumor, cancer etc. (Wasser, 2011) and because of their resistance to other diseases which meat protein consumers are prone to have. Mushrooms in particular also improve the eyesight and are therefore recommended by many medicinal experts for pregnant women and practicing vegetarians and under nourished children. Research has also shown that some medicinal mushroom isolated has cardiovascular, antiviral antibacterial, antiparasitic, anti-inflammatory, and antidiabetic properties, currently several extracts have widespread used in Japan, Korea and china, as adjustments to radiation treatment and chemotherapy.

Physical and environmental factors such as temperature affect mushrooms. Insects, mice, rodents, snails, termites, cockroaches, spiders, earthworms, lizards, millipedes, toads, saprophytic, fungi, bacteria, viruses, ants, nematodes, etc also affect mushrooms. Fluorescent pseudomonas species cause the majority of mushroom bacteria disease (Fermor, 1987; Gill, 1995). However unidentified soft rot pathogen have appeared sporadically in the mushroom industry for many year (Fermor and Lincoln, 2000). The loss of mushrooms to soft rot disease may be restricted in the absence of any additional chemical controls. Often rot bacteria, if undetected, can cause potentially grave problem in mushroom marketing if serious lapses in post harvest cold chain handling occur.

Mushrooms derive all their energy and growth material from their growth media through biochemical decomposition processes. The medium used to cultivate mushroom is known as substrate. Substrate which is known as compost is a solid waste fermentation process, which exploits the phenomenon of microbial degradation and mineralization (Mckinley and Vestal, 1984).

Cultivation of mushroom in the farm is basically turning waste to wealth. Man tries to mimic nature to be able to produce mushroom. Mushrooms grow well at relative humidity levels of around 95-100%, and substrate moisture levels of 50 -70%. By the end of 18th century, compositing using agricultural wastes as substrate for mushroom growing was recognized as essential tool for mushroom growers (Bahl, 1988). The first detailed record of mushroom cultivation occurred in A.D. 600 during

the reign of Lious XIV when Tournefort described a successful method of growing mushroom *Agaricus bisporus* on stable manure. Compost is a fertilizing mixture of partially decomposed organic matter from plant and animal origin (Piet *et al*., 1990). The main purpose of compositing to a mushroom grower is to prepare a substrate in which the growth of mushroom is promoted in the practical exclusion of other microorganisms. Fermor *et al*., (1985) reported that a composted substrate improved mushroom fruit body yield but, reduced infestation by insects, fungi and bacteria pathogens. Microorganisms colonizing mushroom compost during composting process are regarded as active agents which determine the chemical composition and mineralization thereby making it possible for mushroom growth (Fermor *et al*., 1985). There is practically no substance existing in nature that is not used by one microorganism or another (Iranzo *et al*., 2004). Mushrooms extract their carbohydrates and proteins from a rich medium of decaying organic matter vegetation. This rich organic matter first must be prepared into a nutrient rich substrate that mushrooms can consume. When correctly made this nutrient may become available exclusively to the mushroom and should not support the growth of too many other organisms. The sequence to produce this specific substrate for the mushroom is called composting or compost substrate preparation and it has distinct goals or objectives. It is the grower's responsibility to provide the necessary ingredients and environmental conditions for these chemical and biological processes required to complete these goals. It is managing these ingredients and conditions that makes composting for growing mushrooms so demanding.

The objective of this study is to carry out the composting and processing of sawdust, lime, and rice bran into a substrate for the cultivation of edible oyster mushroom (*Pleurotus ostreatus*) and to isolate and identify some microorganisms associated with the substrate used in the cultivation of *Pleurotus ostreatus*. The results obtained should generate information regarding the organisms that survived the composting and processing of the substrate and how they can either improve the cultivation of edible mushroom or cause economic loss.

Materials and Methods

Collection of Sawdust, composting and sampling of substrate for analysis

Sawdust was collected in large polypropylene sacks from a Sawmill on a river bank in Mile 3 Area of Port Harcourt and immediately transported to the mushroom farm for processing into substrate. The substrate for the mushroom cultivation was prepared by composting.

The compost was prepared by outdoor single phase solid waste fermentation (Nair and Price, 1991). The combination of sawdust (75%), rice bran (25%) and lime (5%) was thoroughly mixed and a little water and left for 30 days with turning every 2 weeks to produce homogenous compost with the necessary conditions for the growth of mycelium. The moisture content of the compost was between 40-60%. This is usually determined by the rule of thumb method (Buswell, 1984). The purpose is to produce the proper medium for the growth of mushroom mycelium by allowing the composted materials go through some physical and chemical changes.

Bagging

The treated sawdust is introduced into heat-resistant polypropylene bags. Bagging is a very important aspect of the mushroom production process. The humidity of the sawdust, its level of compactness and the thoroughness of the mixture are all

important considerations in the ability of the spawn to penetrate or colonize the bags. The substrate must pass the "moisture squeeze test" for good results, otherwise the inoculation would have been a waste and the goal of achieving maximum level of colonized bags defeated. Some of the processes in mushroom Production are shown in Plate 1. Plate 1a shows the bagging and compacting of the substrate.

Pasteurization

The compost substrate is pasteurized to reduce or eliminate undesirable microbes like insects, nematodes, fungi, and bacteria. This is not a complete sterilization process but a selective killing of pests that will compete for nutrients or directly attack the mushroom, yet minimize the loss of desirable microbes.

Pasteurization of the substrate was carried out by subjecting the bagged substrate to thermogenic composting process to eliminate bacteria and other microbes not conducive for the growth of mycelium for mushroom production. Pasteurization in its general usage aims at the elimination or destruction of harmful microorganisms. The heat applied should be sustained long enough to achieve this objective and leave only bacteria with beneficial effects on mycelial growth. The duration of the heat depends on the nature of power utilized and its intensity, however about three hours of sustained heat is considered adequate. Plate 1b shows the introduction of the bagged substrates into the pasteurization room. At the end of the pasteurization process, samples of the substrate was aseptically collected at random and bulked together in sterile container to produce a composite sample which was transported to the laboratory within 10 minutes for microbiological analysis.

Inoculation

The seed of mushroom is inoculated into the bags of the pasteurized substrate. Inoculation can be referred to as the "planting" of mushroom. Inoculation of the bags was not delayed beyond 24 hours after pasteurization. However, the bags were allowed to cool down before the inoculation process. Absolute care and hygiene are very essential and were observed during the inoculation process. The inoculated bags of substrate were immediately transferred to the incubation room. This process can be referred to as spawning. Plate 1c shows the inoculation of pasteurized substrate.

Incubation and Cropping

The inoculated substrates were finally incubated in a dark, but well ventilated atmosphere ideal for the development and spread of the mycelium. *Pleurotus ostreatus* can tolerate temperature of 25^{o}C without any harmful effect but this need not be excessive or continuous. The cropping process was the exposing of the developing mushroom (mushroom initials or embryonic mushroom) so as to give them the opportunity to sprout and blossom into full mushroom. The cropping house is the final testing ground for all mushroom operations. The final requirement for the growth or flushing of mushrooms is water. A very important slogan to bear in mind at all times is "**no water no mushroom**". This is particularly important during the harmattan or dry season when a single day's negligence can lead to an almost irreparable loss or damage. Plate 1d shows the harvesting of mushroom.

Plate 1a: Bagging of substrate

Plate 1b: Introduction of bagged substrate into oven for pasteurization

Plate 1c: Inoculation of pasteurized substrate

Plate 1d: Cropping / Harvesting Process

Plate 1: Some of the processes in mushroom production

Microbiological Analysis of Substrate used for cultivation of Pleurotus ostreatus:Fungal Analysis

The procedure used involved placing of 1g of the substrate samples with 9ml of sterile normal saline inside a test tube. This was shaken vigorously to form uniform solution of 10^{-1} concentration. The stock was subjected to decimal dilution using sterile pipettes to form 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. These homogenate were used to determine different types of fungi that were present in the compost and their spore forming unit per gram (sfu/g). A sterile pipette was used to transfer 0.01ml of the 10^{-3} dilution onto sterile plates of Sabouraud dextrose agar (SDA) (which was prepared according to the manufacturer's instruction. About 32.5g of powered SDA medium was dissolved in 500ml of distilled water and pasteurized by autoclaving at 121^{o}C for 15 minutes and allowed to cool to about 45^{o}C before pouring carefully into sterile Petri dishes). A glass spreader was used to spread it round the plate and incubated at room temperature of 37^{o}C for 4 - 7 days. Cultures were prepared in triplicates. Colonies that appeared at the end of incubation were counted and the unit expressed in term of spore forming unit per gram (sfu/g). The distinct viable colonies were subcultured to get a pure culture of a single organism.

Characterization and Identification of Fungi in the Substrate

Pure fungal cultures were observed while still on plates and after wet mount in lacto-phenol on slides under the compound microscope. The following standard characterization tests were performed in duplicate: Macroscopic examination of fungus was carried out by observing the colony morphology, diameter, colour (pigmentation), texture and surface appearance.

Microscopic examination was done by needle mount or wet mount method Harrigan and McCance (1990) and by observing sexual and asexual reproductive structures like sporangia, conidial heads, arthrospores and vegetative mycelium.

A wet mount was done for each fungal isolate. A drop of lacto-phenol was dropped on a clean slide aseptically, a piece of fungal hyphae under test was teased into it using 2 (two) sterile needles. The teasing was done carefully and slowly so as to make good spread of the fungal hyphae. The slides were then gently covered with a cover slip to avoid air bubbles. The slides were observed under low and high power objective for the cultural characteristics, pigmentation, sporangia, conidia, arthrospores, vegetative mycelium, septate and non-septate hyphae. Observed characteristics were recorded and compared with the established identification key of Barnett and Hunter (1972) and Malloch (1997).

Bacteriological Analysis

The media used is nutrient agar. It was prepared according to the manufactures instruction. 14g of the powered Nutrient Agar medium was dissolved in 500mls of distilled water and pasteurized by autoclaving at 121^{o}C for 15 minutes and allowed to cool a little before pouring carefully into sterile Petri dishes and allowed to solidify.

One gram (1g) of the substrate sample was measured out using weighing balance and was mixed with 9ml of sterile normal saline inside a test tube. This was shaken vigorously to form uniform solution of 10^{-1} concentration. The stock was subjected to decimal dilution using sterile pipettes to form 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions. These homogenate were used to determine different types of bacteria that were present in the substrate and their colony forming unit per gram (cfu/g). A sterile pipette was used to transfer 0.01ml of the 10^{-4} dilution onto freshly prepared nutrient agar in sterile plates. A sterile glass spreader was used to spread the liquid round the plates and incubated at 37^{o}C for 24 hours. Cultures were prepared in triplicates. Colonies that appeared at the end of incubation were counted and the unit expressed in term of colony forming unit per gram (cfu/g). Discrete colonies were subculture on fresh plates to get a pure culture of a single organism.

The distinct viable colonies of isolates were Gram stained and examined under microscope. These were further subjected to standard biochemical tests. The identification of the isolates was based on series of morphological test and biochemical test as well as physiological characteristics using the standard characterization definitions of Buchanan and Gibbons (1986). Bacterial identification was by comparison of the characteristics of the isolates with those of known Taxa (Buchanan and Gibbons, 1986).

Results

A total of 11 genera of fungi and bacteria were isolated from the pasteurized substrate (sawdust and rice bran) used for the cultivation of *Pleurotus ostreatus*. The fungal genera included *Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus* and *Trichoderma* while those of bacteria included *Bacillius, Clostridium, Enterobacter, Escherichia* and *Pseudomonas*. Cultures of some of the fungi isolated from the substrate used for the cultivation of *Pleurotus ostreatus* are as shown in Plate 2 below.

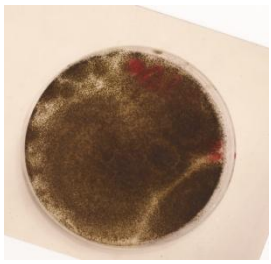

Plate 2a: *Aspergillus niger*

Plate 2b: *Fusarium* sp

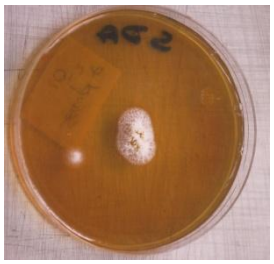

Plate 2c: *Mucor* sp

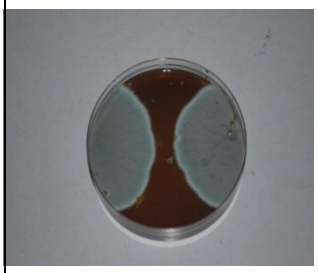

Plate 2d: *Penicillium* sp

Plate 2e: *Penicillium italicum*

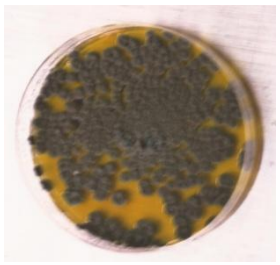

Plate 2f: *Trichoderma harzianum*

Plate 2: Some of the fungi isolated from the substrate used in the cultivation of *Pleurotus ostreatus*

The frequency of isolation of fungi and bacteria and their error bars with 5% value is as shown in Figure 1 and 2 respectively. *Aspergillus* (40.9%) and *Bacillus* (36.67%) species had the highest frequency of occurrence for fungi and bacteria respectively. While *Trichoderma* (2.3%) and *Pseudomonas* (13.33%) species had the least frequency of occurrence for fungi and bacteria respectively.

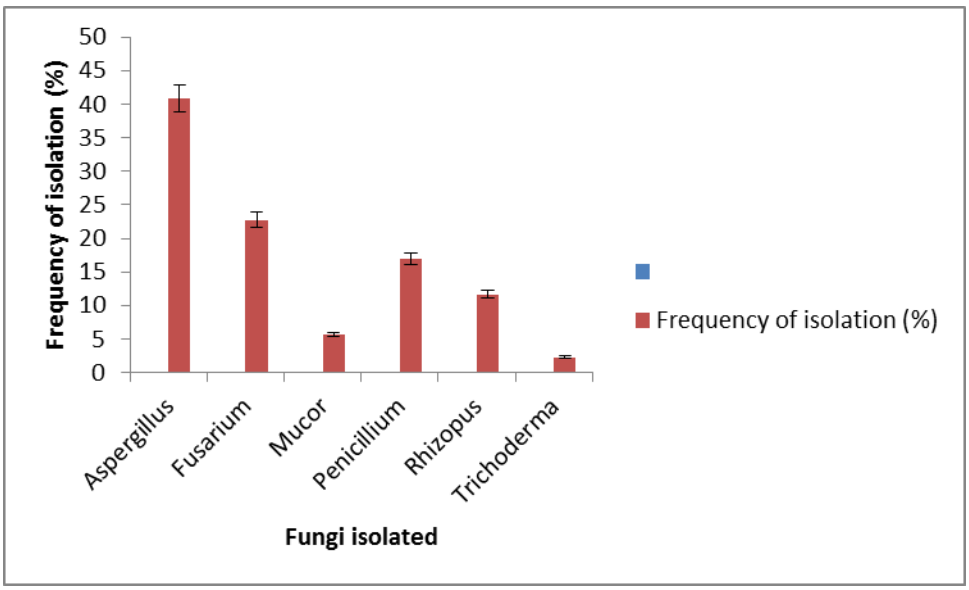

Fig. I: Frequency of isolation of fungi from the substrate

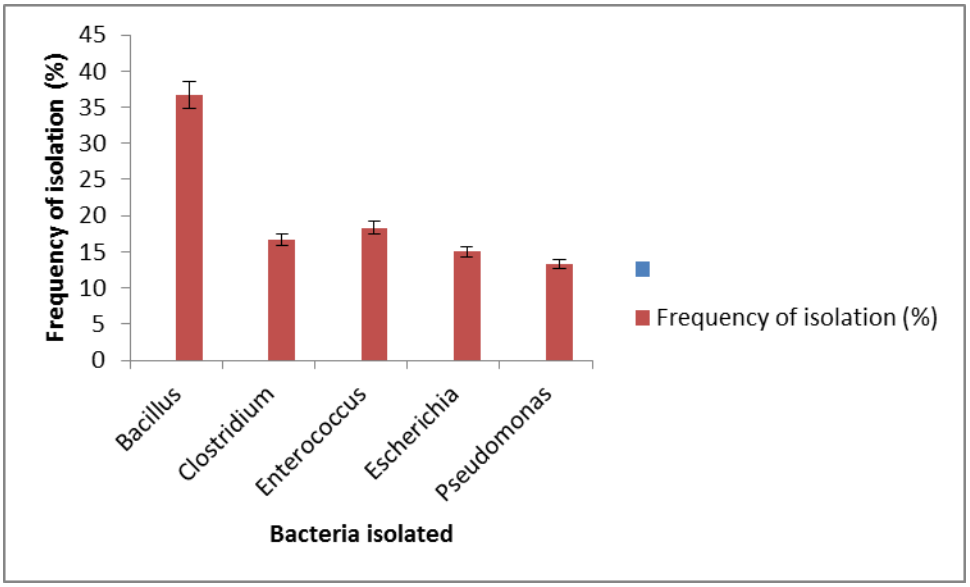

Fig. 2: Frequency of isolation of bacteria from the substrate

Discussion

The present study has revealed the types of fungi and bacteria that are associated with the substrate used in the cultivation of edible oyster mushroom *Pleurotus ostreatus*. Microorganisms isolated from the substrate include species of *Aspergillus, Fusarium, Mucor*, *Penicillium, Rhizopus* and *Trichoderma*. The species of bacteria genera isolated was *Bacillus, Clostridium, Enterobacter, Escherichia*, and *Pseudomonas*. Aspergillus (40.9%) and Bacillus (36.67%) species had the highest frequency of occurrence for fungi and bacteria respectively. Rabia et al (2007) also reported Aspergillus, Mucor, Penicillium, and Trichoderma as some of the fungi and Bacillus, Clostridium. Pseudomonas among the bacteria isolated from compost. They also reported that members of the genus Aspergillus were most prevalent (38%) followed by Bacillus comprising of 20% of the total microbial isolates.

The isolation of anaerobic bacteria such as *Bacillus species* and *Clostridium perfringen* agrees with reports of Mckinley and Vestal (1984) that composting is not a complete aerobic process. Precott *et al*., (1999) suggested that some *Bacillus* and *Clostridium species* inhabit high temperature habitats. Jones (1993) reported that these bacteria produce spores, which are heat resistant thus making them to survive in an extremely high temperature of compost. In this present study, these bacteria also survived the pasteurization process. *Escherichia coli* and *Enterobacter aerogenes* were also isolated from the substrate in this study. They were also reported by Brock *et al.*, (1986). The isolation of coliform group *E. coli* and *E. aerogenes* may be due to contamination from the river (water source) associated with the logs before they were sawn from which the sawdust was a by-product. The presence of *E. coli* which is an indicator of faecal contamination is attributed to the faecal pollution of the river water and banks by humans and animals around the saw mill. The presence of *Pseudomonas aeruginosa* may be related to its ability to survive in vast number of habitats (Brock *et al*., 1986).

Ivors *et al*., (2000) observed a similar rise in temperature, while working on fermented agricultural substrates used for the cultivation of *Agaricus bisporus*. Carlile and Watkinson (1996) suggested that temperature had significant effect on the succession of microorganisms involved in fermentation process. The presence of the

two substrates also increased the variety of microorganism. Fungal species were found to be numerous during both mesophilic and thermophilic phase of composting. Their presence also helps to degrade the compost. The study of Nakasiki and Ohtaki (2002) explains that when a microorganism is incubated in the presence of two or more substrate, the substrate will be degraded in the order of the variety of microorganisms.

Proper composting promotes the development of a number of saprophytic soil microorganisms. Dubey and Maneshwan (2005) have stated that the cellulolytic fungi such as *Aspergillus, Penicillium* and *Trichoderma* accelerate composting for efficient recycling of dry crop waste with high rate and reduce the composting period for about 1 month.

The fungal genus of *Aspergillus* had the highest frequency of isolation from the substrate for cultivation of *Pleurotus ostreatus* in this study. The species isolated included *A. niger, A. flavus,* and *A. terreus*. Their association with different compost has been reported by previous researchers (Ryckeboer *et al*., 2003; Iranzo *et al*., 2004; Taiwo and Oso 2004; Anastasi *et al*., 2005; Wouters *et al.,* 2005;).

Proper composting promotes the development of a number of saprophytic soil microorganisms that helps in the degradation of the substrate. The presence of cellulolytic fungi such as Aspergillus, Penicillium and Trichoderma as reported in this present investigation are associated with the composting process and do accelerate composting for efficient recycling.

Generally, the presence of fungi and bacteria in the pasteurized substrate is attributed to the possession of spores that were heat tolerant by most of the isolates. The presence of E. coli which is an indicator of faecal contamination is attributed to the feacal contamination of the streams and river banks around the Sawmill where logs are retained before being sawn with the sawdust as a by-product. The presence of potential pathogens such as Bacillus, Clostridium, E. coli, Enterobacter and Pseudomonas can lead to bacterial disease of edible mushrooms and economic loss. The presence of these microorganisms also has serious implications for human health. It is therefore suggested that a thorough and complete pasteurization of the compost is important to prevent the spread of disease. In fact, this practice may be quite dangerous with crops heavily infested with virus or Trichoderma. Fermor and Lincoln (2000) reported that mushroom soft rot outbreaks are by no means commonplace but, when they do occur, their often dramatic symptoms can cause consternation on the farm. It is also a potential threat to mushroom industries in tropical and sub-tropical regions. Soft rot bacteria, if undetected, can cause potentially grave problems in mushroom marketing if serious lapses in post-harvest cold chain handling occur.

Conclusion and Recommendations

The process of composting sawdust is not only ecologically sound since it utilizes waste materials as the substrate on which cultivation of the mushroom is to occur but also it shortens the period of fruit body formation to approximately two months. This makes this important source of protein and nutrient readily available and assures food security. Since most families in Nigeria cannot afford the expensive poultry, beef, fish and sea foods as sources of protein, the cultivation and consumption of mushroom is advocated.

From the results obtained, it was observed that various bacteria genera and fungi were involved in the decomposition of sawdust making it suitable for the growth of mushroom. The present study supports the idea that knowledge regarding species composition of the microorganisms of different composts can help to optimize compost quality. The pure culture of these bacteria and fungi could be incorporated into sawdust, agricultural and domestic wastes in a controlled fermentation or composting

unit to enhance and accelerate the composting process. However, since these bacteria and fungi survived the pasteurization process, and are potential pathogens, they should be completely eliminated during the pasteurization process as to prevent the infection of sprouted mushrooms that would lead to economic loss and transmission of diseases to humans.

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