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Full Length Research Paper

Bacteria associated with compost used for cultivation of Nigerian edible mushrooms *Pleurotus tuber-regium* (Fr.) Singer, and *Lentinus squarrosulus* (Berk.)

JONATHAN SEGUN GBOLAGADE

Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria. E-mail: gbolyjoe@yahoo.com.

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The bacteria involved in an outdoor single phase composting using sawdust and wheat bran as substrates for cultivation of *Pleurotus tuber-regium* (Fr.) Singer, and *Lentinus squarrosulus* (Berk.), two Nigerian edible mushrooms were identified. Composting was carried out for 2 weeks. The highest core and peripheral temperatures were 68 and 48ºC, respectively, while the lowest temperature was 32ºC. The highest number of bacteria in the core and peripheral compost were 1.46 x 10^6 and 6.90 x 10^5 cfu/ml, respectively. Bacteria isolated and characterized from the fermenting agricultural substrates include *Bacillus polymyxa, Enterobacter aerogenes, Micrococcus roseus, Citrobacter freundii, Bacillus subtilis, Clostridium perfringens, Bacillus licheniformis, Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli*. The implication of the presence of these bacteria is discussed.

Key words: *Lentinus squarrosulus, Pleurotus tuber-regium,* bacteria mushrooms, compost, fermentation.

INTRODUCTION

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. By the end of 18th century, composting using agricultural wastes as substrates for mushroom growing was recognized as essential tool for mushroom growers (Bahl, 1988; Quimio et al., 1990).

Composting is a fertilizing mixture of partially decomposed organic matter from plant and animal origin (Piet et al., 1990). Composting is a solid-waste fermentation process, which exploits the phenomenon of microbial degradation and mineralization (Mckinley and Vestal, 1984). The main purpose of composting to a mushroom grower is to prepare a substrate in which the growth of mushroom is promoted to 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). Up till now, the identities of bacteria species involved in composts used for cultivation of *P. tuber-regium* and *L. squarrosulus* from Nigeria have not been well documented. Therefore, the objective of this present study is to provide information regarding those bacteria and proffer suggestions on how they could be used in a control fermentation process, which could further improve mushroom cultivation in Nigeria.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

The compost used in this study was prepared by using sawdust of *Terminalia superba* locally known as 'afara' among the Yoruba people of South Western Nigeria. This was collected from Bodija Saw Mill Ibadan, Nigeria and wheat bran (used as nutrient supplement) was obtained from the International Institute of Tropical Agriculture (IITA Ibadan, Nigeria). The fruit bodies of two mushroom samples (*Pleurotus tuber-regium* and *Lentinus squarrosulus)* were collected from decaying wood at the reserved forest of University of Ibadan Botanical Gardens. These mushrooms were tissue cultures according to the method of Jonathan and Fasidi (2001). Likewise, the spawn of Pleurotus *tuber-regium* and *Lentinus squarrosulus* were prepared adopting the procedure of Fasidi and Ekuere (1993).

Table 1. Colonial morphology of the bacteria isolated from fermenting compost.

	A	B	C	D	E	F	G	H	I	J
Shape	Circular	Irregular	Circular	Circular	Irregular	Circular	Irregular	Irregular	Rhizoid	Circular
Elevation	Convex	Flat	Raised	Raised	Raised	Raised	Raised	Raised	Convex	Convex
Size	0.4cm	1.5cm	0.2cm	0.7cm	1.0cm	0.7cm	0.4cm	0.4cm	0.5cm	0.1cm
Edge	Entire	Lobate	Entire	Erose	Erose	Entire	Serrated	Undulate	Lobate	Entire
Texture	Smooth/ Glistering	Dry/ Smooth	Smooth/ Dry	Smooth/ Glistening	Rough/ Dry	Smooth/ Glistening	Smooth/ Glistening	Rough/ Dry	Rough/ Dry	Smoot h/ Dry
Consistey	Butryous	Rubbery	Butryous	Butryous	Butryos	Mucoid	Butryous	Rubbery	Rubbery	Butryous
Emulsification	Easy	Easy	Easy	Easy	Easy	Easy	Easy	Difficult	Difficult	Easy
Opacity	Opaque	Transparent	Opaque	Opaque	Opaque	Translucent	Translucent	Opaque	Opaque	Opaque
Chromogenicy	Yellow	Cream	White	Cream	White	Cream	Green	White	Yellow	Yellow

COMPOSTING

The compost was prepared by outdoor single-phase solid-waste fermentation (Nair and Price, 1991). About 60.0 kg of fresh sawdust were mixed thoroughly with 6.0 kg (10%, w/w) of wheat bran. Water was added until moisture content was between 40-60%. This is usually being determined by the 'rule of thumb' method (Buswell, 1984). The substrates were then stacked into heap of about 1.5 m wide, 1.5 m high and 1.5 m long. This was covered with black polyethylene bags and left for 2 weeks with turning and re stacking every 3-4 days to produce homogenous compost. Temperatures of the core and peripheral compost were measured twice daily (12.00 noon and 6.00 p.m.) during the period of the experiment. Likewise, fermenting compost samples were taken daily for bacteriological analysis.

BACTERIOLOGICAL ANALYSIS

The procedure used involved placing 10.0 g of the compost sample with 90.0 ml of sterile de-ionized water inside 250 ml conical flask. 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}, and 10^{-6} and 10^{-7} concentrations using the method of Ejifor and Okafor (1985). These homogenate were used to determine different types of bacteria that were present in the compost and their colony-forming unit per milliliters (cfu/ml).

A Pasteur pipette was used to transfer 0.25 ml of the last three dilutions on to a sterile plate of nutrient agar (NA) (oxoid) and Eosine methylene blue agar (EMB) (Difco). The plates were agitated for even spread of the inoculum and incubated at 37ºC for 24 h. Colonies that appeared at the end of incubation were counted and the unit expressed in term of colony forming unit per milliliter (cfu/ml). The distinct viable colonies were Gram stained and examined under oil immersion objective (x100). The colonies were then streaked on to appropriate agar to obtain pure cultures. Preliminary colonial morphology and microscopic examination were carried out on bacterial isolates. They were further subjected to standard biochemical tests shown on Table 2. Bacteria identification was carried out on the isolates by comparing the results obtained with the standard characterization definitions of Skermann (1967) and that of Bergey's manual of determinative systematic bacteriology (1986).

RESULTS AND DISCUSSION

Ten bacteria species were isolated from saw dust/wheat bran based compost used in this study. They were coded isolate A, B, C, D, E, F, G, H, J and I (Tables 1 and 2). These microorganisms were characterized as *Enterobacter, aerogenes, Bacillus polymyxa, Micrococcus roseus, Citrobacter freundii, Bacillus subtilis, Clostridium perfringens, Pseudomonas aeruginosa, Bacillus cereus, Bacillus licheniformis* and *Escherichia coli* (Table 2). These identifications were based on series of morphological and biochemical tests as well as physiological characteristics using the standard characterization definitions of Skermann (1967) and that of Bergey's manual of determinative bacteriology (1986).

The endospore forming rods, Gram positive, catalase positive that readily form chains and ferment most sugars (Isolates B, E, H, and I) were identified as *Bacillus* species (Prescott et al., 1999). A simplified classification based on morphology, endospore position, starch and gelatin hydrolysis, motility, nitrate reduction and oxidative fermentation were used to classify Isolates B, E, H. and I as *B. polymyxa, B. subtilis, B. cereus* and *B. licheniformis* respectively (Bergey's manual, 1986). The isolation of these bacteria from composting agricultural substrates suggests that a form of fermentation had taken place during the composting process. This is because Kolawole and Okonkwo (1985), Popoola and Ekueshi (1985), Okafor (1977) and Akinrele (1970) linked these organisms with fermentation of various agricultural substrates.

The bacterial cells that formed endospores and were oxidase negative and have anaerobic fermentative ability were identified as *C. perfringens* (Table 2). These bacteria had circular raised colony with 0.7 cm diameter. They were non-motile, Gram-negative short rods. The isolation of *C. perfringens* agrees with reports of McKinley and Vestal (1984) that composting is not a complete aerobic process. Therefore, anaerobic micro-environment cannot be completely eliminated and the presence of non-oxidative bacteria cannot be avoided. The isolation of *Bacillus* and *Clostridium* species from fermenting compost is not a surprise; this is because similar organisms were also isolated from fermenting cocoa beans (Rambouts, 1952; Ojey, 1981). Precott et al. (1999) also suggested that some *Bacillus*

Table 2. Biochemical properties of the isolates from the mushroom compost.

ISOLATE CODE	GRAM REACTION	CATALASE TEST	OXIDISE TEST	INDOLE TEST	METHYL RED TEST	VOGES-PROSKAUER TEST	MOTILITY	STARCH HYDROLYSIS	GELATIN HYDROLYSIS	CITRATE UTILIZATION	NITRATE REDUCTION	UREA HYDROLYSIS	HYDROGEN SUPHIDE	SPORE STAINING	GLUCOSE	GALACTOSE	SUCROSE	LACTOSE	MANNITOL	REFFINOSE	MALTOSE	ARABINOSE	FRUCTORSE	CELLULAR MORPHOLOGY	PROPABLE ORGANISM
A	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+	Rods	*Enterobacter aerogenes*
B	+	+	+	-	-	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+	Rods	*Bacillus polymyxa*
C	+	+	+	-	-	-	+	-	-	-	+			-	+	-	-	-	+	-	+	-	+	Cocci	*Micrococcus roseus*
D	-	+	-	-	+	-	+	-	-	+	+	+	+	-	+	-	+	+	+	-	-	+	+	Short rods	*Citrobacter freundii*
E	+	+	+	-	-	+	+	+	+	+	+			+	+	+	+	+	+	-	-	+	+	Rods	*Bacillus substilis*
F	+	-	-	-	+	-	-	+	-	+	-			+	+	-	+	-	-	-	-	+	+	Rods	*Clostridium perfringens*
G	-	+	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	-	+	+	+	-	+	Rods	*Pseudomonas aeruginosa*
H	+	+	+	-	-	+	-	+	+	+	+			+	+	-	-	-	-	-	-	-	+	Rods	*Bacillus cereus*
I	+	+	+	-	-	+	-	+	+	+	+			+	+	+	+	-	+	-	-	+	+	Rods	*Bacillus licheniformis*
J	-	+	-	+	+	-	+	+	-	-	+	-	-	-	+	+	+	-	+	-	-	+	+	Short rods	*Escherichia coli*

Represents Positive result; - represents Negative result while blank represents Indeterminate result.

Table 3. Compost temperature during the period of fermentation.

Day	12 Noon (ºC)		6.00 p.m. (ºC)	
	Core	Peripheral	Core	Peripheral
1	32	32	32	32
2	46	44	47	43
3	54	45	55	44
4	65	46	65	46
5	67	47	68	48
6	64	45	63	44
7	57	43	57	41
8	53	38	55	38
9	49	36	48	37
10	45	36	46	35
11	43	35	44	35
12	42	34	43	33
13	39	33	38	32
14	34	32	33	31

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 the compost. Similar reasons could be adduced for the presence of *Citrobacter freundii* and *Micrococcus roseus* since they are facultative anaerobes (Table 2).

Isolate J was indole positive, Gram-negative short rods that fermented lactose, maltose and fructose. Its growth on Eosine methylene blue agar to form Green metallic sheen was used to classify it as *E. coli* (Brock et al., 1986). *Enterobacter aerogenes* (Isolate A) was Gram-negative short rod. It has circular, convex and glistening colonies (Table 1). This organism, which ferments lactose and fructose (Table 2), was catalase positive and oxidase negative. The isolation of coliform group (*E. aerogenes* and *E. coli*) may be due to water source used in the mixing of the compost. The water might have been contaminated with these enterobacteriaceae. The bacteria were predominantly present at the early stage of composting process and died off when the temperature increased. The presence of *P. aeruginosa* (Isolate G) in the fermenting compost may be related to its ability to survive in vast number of habitats (Brook et al., 1986).

Table 3 shows that daily temperature of the core compost increased steadily from the 1st day (32ºC) and attained it highest value (68ºC) on the 5th day of composting process. A gradual temperature decrease was observed from the 6th day until the end of composting process (14th day). 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. Generally, the temperature of the core compost was higher than the peripheral (Table 3). This is because the core area of the compost is in a close state thereby restricting heat exchange and aeration (Jones, 1993).

The bacteria count of the core and peripheral compost is shown on Table 4. For the peripheral, there was an increase in bacteria count for the first 2 days. On the 3rd day, the value dropped significantly. This may be due to the confluent growth of *B. lichenformis* and *B. polymyxa*, which produce bactracin and polymyxin antibiotics, which could have inhibited the growth of other bacteria. There was an irregular trend of bacteria count between core area of the compost and the peripheral area probably because of the different bacterial succession due to the temperature changes in the fermenting compost (Table 4). Only few bacteria could survive high temperature observed in the core area. The available nutrients will be used for the growth of these few bacteria with less competition.

From the results obtained, it can be concluded that various bacteria genera were involved in the decomposition of compost making it suitable for the growth of *P. tuber-regium* and *L. squarrosulus*. The pure cultures of these bacteria could be incorporated into agricultural wastes in a controlled fermentation unit. The

next report will compare fruit body yield of *P. tuber-regium* and *L. squarrosulus* using composted and non-composted agricultural substrates.

Table 4. Viable bacteria count of peripheral and core area of the compost for 2 weeks.

Date	Colony Forming Unit Per Millilitre (cfu/ml)	
	Peripheral	Core
1.	6.0×10^6	6.0×10^4
2.	6.20×10^5	1.21×10^6
3.	6.90×10^5	1.46×10^6
4.	6.0×10^5	1.37×10^6
5.	5.8×10^5	9.3×10^5
6.	5.3×10^5	3.6×10^5
7.	4.9×10^5	3.2×10^5
8.	4.3×10^5	6.4×10^5
9.	1.68×10^5	1.6×10^5
10.	1.52×10^5	1.2×10^5
11.	1.60×10^5	1.04×10^5
12.	2.0×10^5	1.00×10^5
13.	1.85×10^4	0.96×10^4

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