VIRULENCE FACTORS PRODUCING POTENTIAL AND ANTIBIOGRAM OF BACTERIA ISOLATED FROM BIOSOLID FOR LAND APPLICATION

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ABSTRACT

Sewage sludge samples from a water treatment plant in Nigeria were subjected to an in-vessel composting to improve its quality for agricultural applications. Treated samples were analyzed formicrobiological properties, assayed for virulence factors and antibiotic susceptibility pattern using standard analytical and aerobic culture protocols. Bacteriological analysis of the initial mixture (sewage sludge/ sawdust) revealed counts of 5.0$\log_{10}$CFU/g, 4.59, 2.434.74 and 4.92 $\log_{10}$CFU/g for heterotrophic bacteria, coliform, Vibrio and salmonellae-shigellae in fresh sludge compost respectively. These values were significantly ($p \leq 0.05$) reduced in the bio-solid produced after 40 days of composting. Pathogens such as *Salmonella* and *Shigella* sp and *Vibrio* were not detected in the biosolid after 40-day composting however some bacteria; *Bacillus polymyxa, Vibrio* sp *and Pseudomonas aeruginosa,Pseudomonas flourescens and Citrobacter diversus* survived the 30 - 40 days treatment process and may be risky in the final product meant for land application. These isolates assayed for virulence factors producing potential and the results have shown that the most prevalent virulence factors produced by the lipase, protease and lecithinase 18 (60.0%), while the least was gelatinase 8 (26.7%). *Bacillus polymyxa, Vibrio* spp*and Pseudomonas aeruginosa* produced all the factors (100%) while *Pseudomonas flourescens* and *Citrobacter diversus* exhibited only 1 (16.7%) of the factors assayed. The antibiogram of the virulence factors elaborating Gram positive isolates revealed that 5 (100.0%) were sensitive to Levofloxacin, while 1 (20.0%) was sensitive to Chloramphenicol, Norfloxacin, Amoxicillin and Ampiclox. This study shows that although the treated sludge (biosolids) met the United States Environmental Protection Agency (USEPA) permissible limits for microbiological standards for land application of compost, routine monitoring of the stored biosolid is necessary to avoid the proliferation of potential pathogens in the organic fertilizer.

INTRODUCTION

The problem of waste generation, control and management has become a well-known phenomenon in most Nigerian cities. It has been estimated that municipal solid waste generated in a typical Nigerian city could be as high as 4 million tonnes, including about half a million of untreated industrial wastes (Kofoworola, 2007). The average waste generation rate in Abuja is estimated to be between 0.55-0.58 kg per person per day (Imam *et al*. 2008). The use of waste to generate other useful by-products is recently common due tothe advent of new technologies for the conversion of wastes of any form into valuable products. However, sludge waste is another kind of waste which applications are rarely exploited, especially in developing countries.

Sewage sludge is the solid, semi-solid, or liquid residue generated from the treatment of domestic sewage. It is very rich in nutrients (nitrogen and phosphorus), organic matter and some trace elements needed for plant growth (Fatunla *et al.,* 2017). Recently, focus has been on

the composting of good-quality sludge for agricultural and horticultural use. When sewage sludge and woodchips are mixed and composted, stabilized product results from the action of aerobic-thermophilic microorganisms, which utilize a part of the organic material for their growth and activity. During this decomposition, the composting biomass heats to temperatures in the pasteurization range of 55ºC to 70ºC, with resulting destruction of enteric pathogenic microorganisms. The principal pathogens of concern in sewage sludge include bacteria such as *Salmonella* sp responsible for typhoid fever and food poisoning, *Shigella* sp associated with bacillary dysentery, *Yersinia* sp implicated with acute gastroenteritis) and *Vibrio cholerae* that causes cholera. *Campylobacter jejuni* and and the pathogenic strains of *Escherichia coli* associated with gastroentiritis are readily found in sewage sludge (Fatunla *et al.,* 2017). The end result of sewage sludge composting is a humus-like material called biosolid which is ordinarily devoid of pathogens and is useful as soil conditioner and a source of plant nutrients (Hornick *et al*., 1984; Nilsson and Dahlstrom, 2002; Andreolli *et al.,*2007; Fatunla *et al*., 2017).It has been reported that proper land utilization of stabilized sewage sludge can make a positive contribution to agriculture, forestry, horticulture and city development as well as land reclamation (Wang, 1997and Forste, 1997). However, it should be done with good knowledge of microbial properties and disease causing potential of bacteria commonly associated with sewage sludge and biosolid. In this study, bacteria isolates that survived the composting process were evaluated for presence of virulence markers and their antibiotic susceptibility pattern in order to evaluate biosolid's suitability for agricultural applications.

MATERIALS AND METHODS

Collection and Composting of Sewage Sludge

Sludge samples were obtained from Lower Usuma Dam Water Treatment Plant (LUDWTP) located in Abuja Nigeria between latitude 9º 01' 12" N and longitude 7º 25' 16" E. The samples were subjected to composting using the In-Vessel Composting method described by Fatunla *et al.,* (2017). The composting cycle lasted for 40 days and substrate temperature was measured daily at a depth of 50 cm at different positions inside the vessel. The compost was air-dried and bagged.

Bacteriological Analysis of Samples

Bacteriological samples were taken before composting (B_0) and after composting or before biosolid generated was bagged (B_s) respectively for analysis. The number of heterotrophic bacteria was determined on simple nutritive agar after incubation at 37°C for 48hrs. *Salmonella* sp. was determined on Selenite F medium and Salmonella Shigella agar after 18-24 hours incubation at 36°C. The total coliform and fecal coliform were determined with MacConkey agar and Eosin-Methylene Blue agar respectively after incubation at 37°C for 24 hours (Cappuccino and Sherman, 2002).

Virulence and Antibiotic Susceptibilty Assay of Bacterial Survivors of the Composting Process

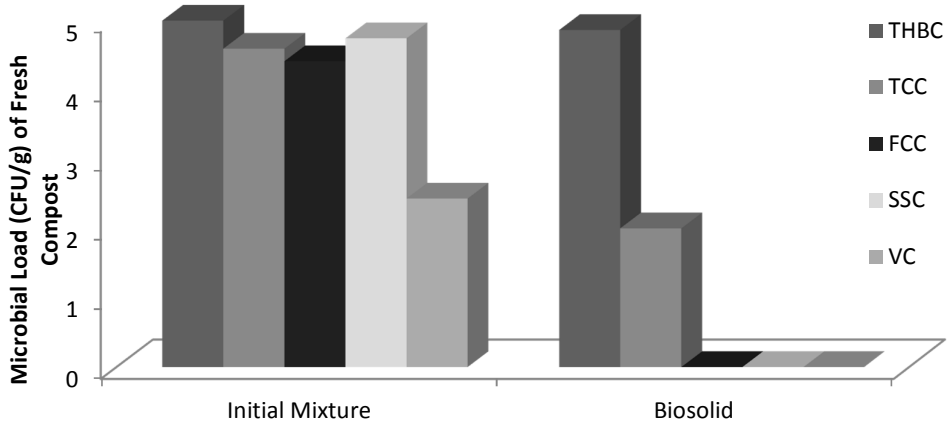
The virulence factor assay was carried out as described by Pasquale *et al*. (2012). Factors assayed included; Urease activity, gelatin liquefaction test, hemolysin production, extracellular protease, DNase production, lecithinase production and lipase activity. The isolates were screened for enzymatic activity by culturing them on media supplemented with the substrate specific for the exo-enzyme of interest as described by James and Natalie (2008) and Pasquale *et al*. (2012).Haemolysin production was determined on blood agar medium as described by Cheesbrough (2006). Antibiotic susceptibility profile of potential pathogenic isolates was carried out using Modified Kirby-Bauer disc diffusion technique as described by Cheesbrough (2006). Commercially available discs specific for both Gram positive and Gram negative bacteria were used for the assay.

RESULTS AND DISCUSSION

The initial mixture (sewage sludge/ sawdust) haboured 5.0 $\log_{10}$CFU/g of heterotrophic bacteria, 4.59 $\log_{10}$CFU/g of coliform bacteria and 4.74 $\log_{10}$CFU/g of *Salmonella* and *Shigella*

species in fresh compost. The microbial density was significantly (p=0.05) reduced after 40 days of composting to 4.86 and 2.0 log_{10}CFU/g of heterotrophic bacteria and coliforms respectively while no viable cell of faecal coliforms, Salmonella/Shigella sp and *Vibrio* sp were detected in the final compost after 40 days (Figure 1). However,*Vibrio* species survived the in-vessel composting process for 30 days. The comparative status of bacterial loads in the fresh and treated (biosolid) sludge samples is presented in Figure 1. The above results have shown that composting had a positive influence on the bacterial quality of sludge. Its effect however varied with the diverse bacterial groups and their activities which are often affected by exhaustion of nutrients, pH and temperature changes (Fatunla *et al.,*2017). In this study, temperature was at its peak (65^{o}C, thermogenic phase) at day 10 of composting (Fig.2). Maintaining the temperature at 60^{o}C for the first 15days of composting caused a significant elimination of total aerobic heterotrophs, yeasts and moulds as well as the pathogenic group of *Salmonella* and *Shigella* and *Vibrio* sp. At this temperature, only few days are required to eliminate almost all pathogens (Dumontet *et al.,* 1999). Although aerobic bacteria (bacilli) are very often active between 60^{o}C and 65^{o}C, composting temperatures should not exceed 75^{o}C, which would irreversibly denature the bacterial enzymes (Tuomela *et al.,* 2000). The intense microbial activity induced very significant transformations of the mixture of sewage sludge with sawdust used in composting.

The biochemical attributes of the bacterial isolates have shown that a total of 12 species were encountered (Table 1). This comprises 6 Gram positive and 6 Gram negative bacterial species. Prominent amongst the isolates were; *Bacillus cereus, Bacillus megaterium, Bacillus polymyxa, Bacilus subtilis, Corynebacterium kutsceri, S. aureus, Pseudomonas aeruginosa P. flourescens and Citrobacter diversus, Vibrio sp, Aeromonas hydrophila* and *Klebsiella pneumoniae* (Table 1). Of the twelve (12) bacterial isolates, only eleven (11) exhibited virulence factors. *Bacillus cereus* had the highest 6 (100%) number of virulence factors while *B. subtilis, P. flourescens and Citrobacter diversus* had 1 (16.7%) each (Figure 3). The antibiogram of virulence elaborating isolates revealed that *B. cereus* was susceptible to 4 (40%) of the antibiotics used, while S. *aureus* was susceptible to 6 (60%).*Vibrio sp* was susceptible to 4 (40%) of the antibiotics used, while *P. aeruginosa* was also susceptible to 4 (40%) (Table 2 and 3).



Key: THBC =Total Heterotrophic Bacterial Count; TCC =Total Coliform Count; FCC = Faecal Coliform Count; SSC =Salmonella Shigella Count; VC = Vibrio count.

Figure 1: Microbial load (CFU/g) of the initial fresh compost mixture and the final biosolid

Salmonella and *Shigella* and *Vibrio* were not detected in the final compost (Figure 1). The absence of these pathogens in the final compost was mostly as a result of temperature rise above 60^{o}C between the 5th and 10th day of composting and it's usually an indication of the

efficacy of the process. Composting of sewage caused a significant (p=0.05) reduction in the number of enteric bacteria in the biosolid. Although coliforms were not completely eliminated, their levels fell within acceptable limits of <100CFU/25g of coliforms in the final compost meant for land application (USEPA, 1999). The ability of pathogenic bacteria to cause disease in a susceptible host was determined by multiple virulence factors acting individually or together at different stages of infection (Wu *et al.,* 2008).

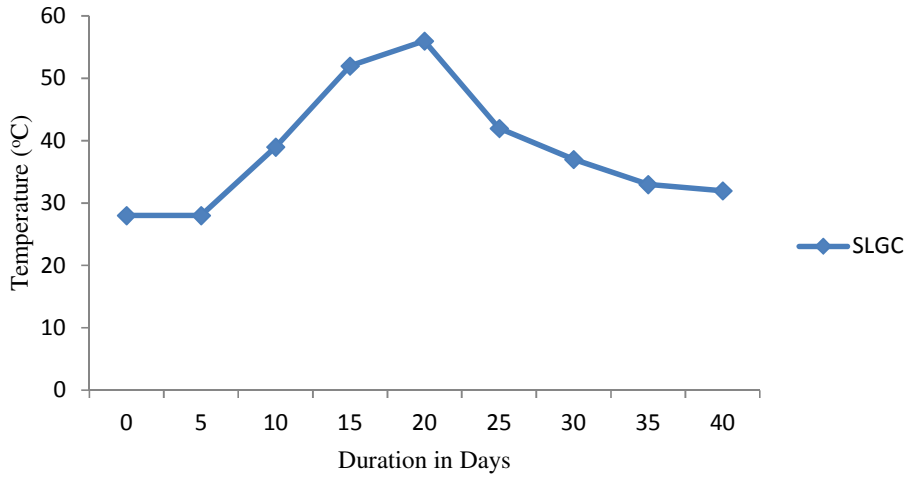


Figure 2: Temperature changes in sewage sludge feedstock during composting

Isolates	G.R	Shape	Motility	Catalase	Coagulase	Starch hydrolysis	Urease	Oxidase	Indole	MR	VP	Citrate	H_2S	Glucose	Lactose	Dextrose	Maltose	Mannitol	Probable Organism
1	+	R	+	-	-	+	+	-	-	-	+	+	-	A	-	A	A	-	*Bacillus cereus*
2	+	S	-	+	+	+	+	-	-	+	-	+	-	A	-	A	A	A	*Staphylococcus aureus*
3	+	R	+	+	-	+	-	-	-	+	-	+	-	A	A	A	A	AG	*Bacillus megaterium*
4	-	R	+	-	-	+	+	-	-	+	-	+	-	A	A	A	A	A	*Aeromonas* hydrophila
5	-	R	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	*Vibrio* sp
6	+	R	+	+	-	+	-	-	-	+	-	+	-	A	A	A	A	AG	*Bacillus subtilis*
7	-	R	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	A	*Pseudomonas aeruginosa*
8	-	R	+	+	-	-	-	+	-	-	+	+	-	-	-	-	A	A	*Pseudomonas fluorescens*
9	+	R	+	+	-	+	-	-	-	+	-	+	-	A	A	A	A	AG	*Bacillus polymyxa*
10	-	R	-	+	-	-	+	-	-	+	-	+	+	A	A	A	A	A	*Klebsiella pneumoniae*
11	+	R	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Corynebacterium kutsceri*
12	-	R	+	+	-	+	-	-	-	+	-	+	-	A	A	A	A	A	*Citrobacter diversus*

Table 1: Morphological and Biochemical Characteristics Bacterial of Isolates

Key: G.R = Gram Staining; MR. = Methyl Red; VP = Voges Proskauer; + = Positive; - = Negative; R = Rod; S = Spherical; A = Acid only; AG = Acid and Gas produced.

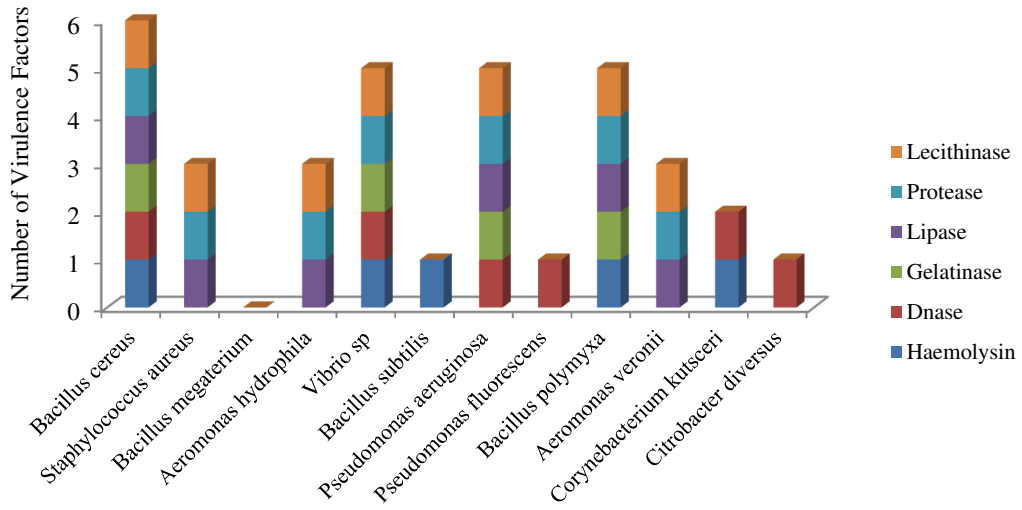


Figure 3: Distribution of Virulence factors among bacterial isolates

Table 2 Antibiotic susceptibility profile of Gram positive pathogenic isolates

	CPX	CH	NB	S	AMX	RD	APX	CN	LEV	E
C. kutsceri	30(S)	- (R)	- (R)	- (R)	17 (I)	- (R)	- (R)	20 (I)	21 (S)	16 (I)
B. subtilis	14(R)	- (R)	20 (I)	19 (I)	-(R)	16 (I)	- (R)	16 (I)	17 (I)	18 (I)
B. polymyxa	18 (I)	28 (S)	- (R)	16 (I)	- (R)	15 (R)	16 (I)	- (R)	28 (S)	17 (I)
B. cereus	14(R)	- (R)	- (R)	30 (S)	- (R)	18 (I)	- (R)	21 (S)	18 (I)	- (R)
S. aureus	25(S)	35 (S)	- (R)	22 (S)	16 (I)	12 (R)	- (R)	20 (I)	25 (S)	- (R)

S = Sensitive/Susceptible (≥ 21 mm); R = Resistant (≤15 mm); I = Intermediate (16 to 20mm

Table 3: Antibiotic Susceptibility Profile of Gram negative Pathogenic Isolates

	OFX	PEF	CPX	AUG	CN	S	CEP	NA	SXT	PN
A. hydrophila	22(S)	18 (I)	17 (I)	- (R)	24 (S)	- (R)	- (R)	17 (I)	- (R)	- (R)
Vibrio sp	- (R)	- (R)	13 (R)	16 (I)	20 (I)	18 (I)	23 (S)	- (R)	- (R)	- (R)
A. veronii	29(S)	28 (S)	29 (S)	18 (I)	15 (R)	13 (R)	23 (S)	13 (R)	15 (R)	15 (R)
P. aeruginosa	28(S)	- (R)	25 (S)	- (R)	28 (S)	21 (S)	- (R)	- (R)	- (R)	- (R)
C. diversus	21(S)	20 (I)	19 (I)	18 (I)	17 (I)	21 (S)	25 (S)	20 (I)	- (R)	- (R)

S = Sensitive/Susceptible (≥ 21 mm); R = Resistant (≤15 mm); I = Intermediate (16 to 20mm)

Virulence factors are often involved in direct interactions with the host tissues or in concealing the bacterial surface from the host's defense mechanisms (Wu *et al*., 2008). Some of the isolated microorganisms exhibited one or more virulence attributes (Figure 2) as exemplified by *Bacillus cereus* (6), *Vibrio* sp (5), *Pseudomonas aeruginosa* (5), *Bacillus polymyxa* (5) and *S. aureus* (3) all of which have been implicated in infectious disease initiation in humans. Although *S. aureus* is not included as a pathogen of concern in sewage sludge, this study has revealed its capability of initiating a disease process in the case of direct or indirect human exposure to untreated or improperly treated sludge meant for land application. Similar finding has earlier been reported by Ong *et al,* (2011).Although biosolid is not expected to harbour pathogens, heterotrophic organisms that survives the composting process (*Bacillus* species) could pick up these virulence attributes through transformation and other gene transfer techniques. The antibiotic susceptibility assay results (Table 2 and 3) have shown that most of the pathogenic isolates were susceptible to more than 50% of antibiotics tested. Direct or

indirect exposure to these emerging pathogens in biosolid could therefore be managed or prevented with the right choice of antibiotics.

CONCLUSION AND RECOMMENDATION

This study has shown that although the treated sludge (biosolids) met the permissible limits of microbiological standards recommended by United States Environmental Protection Agency (USEPA) for land application of compost, efforts should be made towards monitoring of the stored biosolid to prevent disease outbreak associated with contamination with putative pathogens.

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