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Heavy Metal Resistance in Plasmid-Cured *Bacillus* species

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A B S T R A C T

The study of heavy metal resistant bacteria isolated from polluted site is of interest, as this may provide new isolates and probably new genetic information on heavy metal resistance which could be explored for bioremediation purpose. The aim of this study was to assay for resistance by plasmid-cured *Bacillus* species from polluted soil of a cement factory. Heavy metals used were cadmium, cobalt and zinc. Various biochemical characterizations such as spore staining, gelatin liquefaction, casein liquefaction, starch hydrolysis, hydrogen sulphide production, indole, motility, gas production, voges proskauer, citrate, and various sugar fermentation were carried out on bacterial isolates. Plasmid curing was carried out on bacterial isolates (n=19) using ethidium bromide. Cured bacteria were assayed for resistance to heavy metals such as cadmium (Cd), cobalt (Co) and zinc (Zn) by inoculation on nutrient agar supplemented with salts of various heavy metals at different concentrations (mg/mL). Bacterial isolates were identified as *Bacillus tequilensis*, *Bacillus weidmannii*, *Bacillus subtilis*, *Bacillus toyonensis*, *Bacillus safensis* and *Bacillus cereus*. The UV transilluminator image of the plasmids gel electrophoresis showed that *Bacillus* species were indeed plasmid-cured in that they lost their plasmids. Plasmid curing resulted in loss of heavy metal resistance in some isolates, while some of the plasmid-cured derivatives demonstrated resistance to these heavy metals. The finding of this study showed that heavy metal resistance is both mediated by genetic markers borne on plasmid and chromosomal DNA in these *Bacillus* species.

Introduction

Bacteria developed resistance due to exposure to toxic heavy metals shortly after the start of life in an already heavy metal contaminated environment and also evolve metal resistance in response to recent exposure to heavy metal contamination as stated by Endo *et al.*, (2002). The ever increasing contamination of the environment with anthropogenic sources of heavy metals has necessitated the need for research concerning microbial metal resistant. Bacteria directly or indirectly influence the fate of heavy metals in the environment. Chen *et al.*, (2015) found that, in response to heavy metal pollution in the environment, bacteria have evolved strategies of

metal resistance and detoxification. Some resistance mechanisms are plasmid encoded or borne on the chromosomal DNA and tend to be specific for a particular metal. Others are general, conferring resistance to a variety of metals.

Resistance plasmids in bacteria are known to harbour resistance genes to heavy metals and antibiotics. Curing of plasmids in bacteria is a technique to eliminate the plasmids and determine the heavy metal resistance mediation (Prescott *et al.,* 2008). Plasmid curing occurs naturally in bacteria population during cell division but the occurrence of this curing is extremely low. Also plasmid curing could be induced by treating bacteria cells with chemical or physical mutagens. However, resistance plasmids are extremely stable and require the use of curing agents that might increase the plasmid loss and this forms the basis of artificial plasmid elimination (Elias *et al.,* 2013). There are several methods involving chemical and physical mutagens that have been developed to eliminate plasmids. Chemical mutagens used for plasmid curing in bacteria include acridine orange, ethidium bromide, acriflavine and sodium dodecyl sulphate (Liu *et al.,* 2012).

Also physical mutagens used for curing are ultraviolet light, ionization radiation, thymine starvation, antibiotics and elevated growth temperature (Prescott *et al.,* 2008). The mechanism of plasmid curing starts from the inhibition of plasmid replication resulted from a single nick, outside of the replication origo of the plasmid DNA. The chemical or physical mutagens would then break the superhelical structure of plasmid DNA, subsequently forming an open circular or linear plasmid DNA as reported by Spengler *et al.,* (2006). At the same time, these mutagens allow the replication of bacterial chromosome and the multiplication of bacteria, without the replication of plasmid, which is sensitive to intercalation (Liu *et al.,* 2012). The efficiency of curing generally varies from less than 0.1 % to more than 99 % depending on the bacterial strain and the mode of action of the curing agent.

Plasmid curing in bacteria results in plasmid-cured derivatives. Obtaining plasmid-cured derivatives will make possible a direct comparison between the plasmid-containing and plasmid-cured cells. Loss of heavy metal resistance in plasmid-cured cells, which previously exhibited metal resistance, indicates that resistance determinants are located on plasmids. In addition, exhibition of heavy metal resistance in plasmid-cured cells confirms extra-chromosomal location of resistance determinants (Elias *et al.,* 2013).

It was reported by Sidhu *et al.,* (2015) that heavy metal resistance in bacteria is both plasmid and chromosome mediated. *Bacillus* species in this study, while containing plasmids demonstrated varying degrees of resistance to cadmium (Cd), cobalt (Co) and zinc (Zn) at different concentrations (mg/ml). In order to determine the relevance of chromosome-borne genetic determinants in heavy metal resistance, plasmids of nineteen *Bacillus* species were cured using ethidium bromide and the organisms were subjected to heavy metal resistance assay.

Materials and Methods

The Study Site

The study site was Dangote cement factory (formerly known as Benue cement company Plc), Tse-Kucha, Gboko Local Government Area of Benue State. Dangote cement factory is located at about 5 km along Gboko-Makurdi road.

Sample Collection

Soil samples were collected by modification of the method of Zaku (2006). Collection was done at 0-30cm depth with the aid of soil auger. Eighty (80) soil samples were randomly collected from industrial, mining and waste disposal sites of Dangote cement factory and control soil site at a different location. Soil samples were collected into sterile polythene bags with proper labeling and immediately conveyed to the Microbiology

Laboratory of University of Mkar, Gboko and analyzed within 24hours of collection.

Isolation of Bacteria from Soil Samples

Bacteria were isolated using streak plate technique. Ten (10) grams of soil samples were aseptically weighed into 90ml of sterile diluents to form an aliquot. Aliquots were properly shaken and a loopful from aliquots of soil samples was streaked on sterile nutrient and blood agar. The plates were incubated at 30^{o}C for 24hours. After incubation, distinct colonies were randomly picked and sub-cultured severally to obtain pure cultures. Pure cultures were stored on agar slants in the refrigerator for further tests.

Cellular and Morphological Characteristics

Gram staining was carried out according to the method described by Adeyinka *et al.,* (2013). To ascertain the shape and Gram reaction of the bacteria, the slides were viewed under microscope using oil immersion objective lens. Malachite green staining procedure was also carried out to determine the presence of spores in bacterial cells (Table 1).

Biochemical Characterization

Bacterial isolates were screened for production of enzymes such as gelatinase using the method described by Madigan *et al.,* (2009), amylase by the method reported by Adeyinka *et al.,* (2013), casein hydrolase and citrase using the method described by Aneja (2003) (Figure 1). The bacterial isolates were also tested for degradative ability on various sugars such as glucose, fructose, maltose, lactose, sucrose, mannitol, xylose, and arabinose using the method described by Aneja (2003) (Table 1). Other biochemical tests carried out were hydrogen sulphide production, indole test, motility test using methods described by Madigan *et al.,* (2009) and Voges-Proskauer test according to the method described by Adeyinka *et al.,* (2013) (Table 1). The biochemical characteristics of the bacterial isolates were benchmarked with Bergey's manual of systematics of archaea and bacteria reported by Contreras-Castro *et al.,* (2018). Bacterial isolates were identified as *Bacillus tequilensis, Bacillus weidmannii, Bacillus subtilis, Bacillus toyonensis, Bacillus safensis* and *Bacillus cereus*.

Extraction of Plasmids DNA from the Uncured *Bacillus* species

Plasmids DNA were extracted from Uncured *Bacillus* species by alkaline lysis method described by Bigot and Charbit (2009). Pure plasmids DNA were electrophoresed for 60 minutes at 100 volts in electrophoresis tank containing Tris-Boric EDTA buffer and the result was read in the UV transilluminator (Figure 2). This was done to detect the presence of plasmids in uncured *Bacillus* species and also served as basis for direct comparison with electrophoresis gel for the plasmid-cured *Bacillus* species.

Plasmid Curing of *Bacillus* species

Plasmid Curing of *Bacillus* species was carried out using ethidium bromide according to Amalesh *et al.,* (2012) at concentration of 0.1mg/ml in Muller Hinton broth. Amended Muller Hinton broth, 1.5ml each was pipette into microcentrifuge tubes and sterilized at 121^{o}C for 15 minutes.

Fresh culture of each *Bacillus* species was inoculated into each sterile amended Muller Hinton broth. Each tube was vortexed for even distribution of cells in the broth. After vortexing, the tubes were incubated at 30^{o}C for 72 hours.

Isolation and Selection of Cured *Bacillus* species after curing treatment

After the period of incubation for curing treatment, a loopful from each *Bacillus* species in a microcentrifuge tube was streaked on each sterile solidified nutrient agar plate and incubated at 30^{o}C for 24 hours. Cured *Bacillus* species were differentiated and selected based on decreased colony size and pale appearance as compared to the

large colony size and bright appearance of the uncured *Bacillus* species.

Plasmids DNA Extraction from Cured *Bacillus* species

Plasmid extraction was carried out according to method described by Bigot and Charbit (2009). Overnight cultures of bacterial isolates in microcentrifuge tubes were centrifuged at 14000 rpm for 5minutes. Each pellet was washed by adding 1.5ml phosphate buffer saline and centrifuged at 14000 rpm. Two hundred (200) microliters (µl) of lysozyme solution was added and incubated at 37^{o}C in echotherm for 30 minutes. *Bacillus* cells were lysed by addition of 400 µl of sodium dodecyl sulphate and lysis halted by adding 300 µl of potassium hydroxide acetate. Cell debris was coagulated by incubation on ice crystals for 5 minutes and centrifuged at 14000 rpm. Clear supernatants were pipetted into clean labeled microcentrifuge tubes and equal volume of phenol-chloroform mixture (1:1) was added to each microcentrifuge tube to extract plasmid DNA and centrifuged at 14000 rpm. Clear supernatants were pipetted into clean labeled microcentrifuge tubes. Equal volume of cold absolute ethanol was added into each microcentrifuge tube to precipitate plasmid DNA and then centrifuged. Each plasmid DNA pellet was washed thrice by adding 1ml of 70 % cold ethanol and centrifuged at14000 rpm for 1minute each time. Plasmid DNA was dried for 15 minutes in a DNA concentrator and eluted by adding 50 µl of Tris-EDTA buffer.

Agarose Gel Electrophoresis of Cured Plasmid DNA

Electrophoresis was carried out according to the method described by Bigot and Charbit (2009). First, 0.8g Agarose powder was dissolved in 100ml of Tris-Boric EDTA (TBE) buffer and boiled to obtain a clear solution. Then cooled, 4 µl of ethidium bromide was added. The agarose solution was poured to make a gel containing twenty wells. The gel was placed in the electrophoresis tank containing TBE buffer. The DNA ladder was loaded into the first well of the gel and plasmid DNA samples mixed with loading dye were loaded into the remaining nineteen wells. Electrophoresis was run for 60 minutes at 100 volts and the result was read in the UV transilluminator (Figure 3).

Heavy Metal Resistance Assay for Cured *Bacillus* species

Heavy metal resistance assay was carried out for cured *Bacillus* species by modification of the method reported by Amalesh *et al.*, (2012). Three heavy metals were used namely cadmium (Cd), cobalt (Co) and Zinc (Zn) at concentrations of 0.00, 0.75, and 1.00 mg/mL respectively. Sterile nutrient agar supplemented with different salts of cadmium chloride hemidihydrate ($CdCl_2.2½H_2O$), cobalt chloride hexahydrate ($CoCl_2.6H_2O$) and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) was aseptically poured into sterile Petri-dishes and allowed to solidify. Pure isolates were spot inoculated on the heavy metal supplemented nutrient agar. The Petri- dishes were incubated at 30^{o}C for 48 hours. After the period of incubation, the plates were examined for growth and results were recorded (Table 2-4).

Results and Discussion

Morphological and biochemical characteristics of *Bacillus* species are presented in Table 1. Various biochemical characteristics such as presence of spore, hydrogen sulphide production, motility, indole, gas production, voges-proskauer, citrate, starch hydrolysis, gelatin liquefaction, casein hydrolysis and various sugar fermentation tests were compared with standards to identify *Bacillus tequilensis*, *Bacillus weidmannii*, *Bacillus subtilis*, *Bacillus toyonensis*, *Bacillus safensis* and *Bacillus cereus.*

Bacillus species were screened for enzyme activity as presented in Figure 1. Eighteen *Bacillus* produced both gelatinase and citrase while fifteen *Bacillus* produced casein hydrolase and seven *Bacillus*

produced amylase. Analysis of plasmid profile was first carried out for the uncured *Bacillus* species to verify if they contain plasmids DNA before plasmid curing treatment was carried out on *Bacillus* species. Plasmid profile of uncured *Bacillus* species is shown in Figure 2. Agarose gel's well labeled L contained molecular markers in the regions of 23130 kilo base pair (kbp), 9416 kbp, 6557 kbp, 4361 kbp, 2322 kbp, and 2027 kbp.

Other wells labeled 1 to 19 were loaded with plasmid DNA of uncured *Bacillus* species. The bands representing plasmids of uncured *Bacillus* species mostly appeared in the region of 23130kbp of molecular marker. This is a clear indication that uncured *Bacillus* species harbour high molecular markers of resistance determinants. This gives us baseline information and basis for comparison after curing treatment.

Figure 3 presents plasmid profile of cured *Bacillus* species. Agarose gel's well with label L contained molecular markers in the regions of 23130 kilo base pair (kbp), 9416 kbp, 6557 kbp, 4361 kbp, 2322 kbp, and 2027 kbp. Other wells labeled 1 to 19 were loaded with plasmid DNA of cured *Bacillus* species. Unlike Figure 2, there were no bands seen and this is a proof that the plasmids were indeed cured by the treatment. Then, the cured *Bacillus* species were subjected to heavy metal resistance assay.

Cadmium resistance assay on cured *Bacillus* species at 0.00, 0.75 and 1.00 mg/mL concentrations is presented in Table 2. Absence of growth is represented by (-) indicating total inhibition of the growth of the organisms by cadmium metal, while scanty growth is depicted by (+). Moderate growth is denoted by (++) and exuberant growth is indicated by (+++) showing no inhibition on the growth of the organisms by cadmium metal.

Cobalt resistance assay by cured *Bacillus* species at 0.00, 0.75 and 1.00mg/mL concentrations is showed in Table 3. Complete inhibition of growth of the organisms is represented by (-) indicating total cobalt resistance on the growth of the organisms, while scanty growth of the organisms in cobalt supplemented agar is depicted by (+). Moderate growth is denoted by (++) and exuberant growth is indicated by (+++) representing no inhibition of the growth of the organisms by cobalt.

Zinc resistance assay on cured *Bacillus* species at 0.00, 0.75 and 1.00 mg/mL concentrations is presented in Table 4. Complete inhibition of growth of the organisms is represented by (-) indicating total zinc inhibition of the growth of the organisms, while scanty growth of the organisms incubated in zinc supplemented agar is depicted by (+). Moderate growth is denoted by (++) and exuberant growth is indicated by (+++) indicating no inhibition of the growth of the organism by zinc metal.

Al-Khashman and Shawabkeh (2006) reported that cement production is an important emission source of heavy metals such as cadmium (Cd), chromium (Cr), copper (Cu), cobalt(Co), lead (Pb) and zinc (Zn) into the environment, air, water and soil. Cement is manufactured through a closely controlled chemical combination of metallic compounds. Cement factory releases cement dust into the surrounding soil through the industrial processes. Also mining activity that involve excavation of earth crust using gigantic metallic trucks and the use of explosives to get the major raw materials such as limestone and shale results in geological redistribution of heavy metals and subsequent pollution of the soil environment. Wastes like unused furnace slag, clinker and accumulated cement dust containing heavy metals are dumped on soil at waste disposal site, thus resulting in heavy metal pollution of soil.

Eighteen bacterial isolates from the mining, industrial, waste site soil of Dangote cement factory and one bacterial isolates from control sites were identified as *Bacillus* species (Table 1) by comparing their biochemical characteristics with standards in Bergey's manual of systematics of archaea and bacteria according to Contreras-Castro *et al.*, (2018). Similar studies have reported isolation of *Bacillus* species in heavy metal contaminated

sites. Kamala-Kannan *et al.,* (2006; 2007) reported the presence of *Bacillus cereus* in heavy metal contaminated sediment from the pulicat lake and ennore creek both in the South East India. Also, Kamala-Kannan and Lee (2008) found that *Bacillus* species isolated from Sunchon Bay Sediments in South Korea, demonstrated resistance to heavy metals: zinc, chromium, manganese, magnesium, mercury and cobalt. Furthermore, Singh *et al.,* (2013) posited that *Bacillus* species isolated from industrial effluent demonstrated resistance to multiple heavy metals such as chromium, cadmium, cobalt, lead, zinc and mercury. *Bacillus* species were screened for production of various enzymes such as gelatinase, amylase casein hydrolase and citrase. These organisms produced these enzymes at various levels in that 95% *Bacillus* species produced both gelatinase and citrase. Also 79% *Bacillus* species produced casein hydrolase and 37% *Bacillus* species produced amylase. This corroborates the findings of Hussein and Joo (2013). These authors reported that heavy metal resistance strains of *Bacillus subtilis* kh and *Pseudomonas putida* KNU5 produced antioxidant enzymes such as catalase, peroxidase and ascorbate peroxidase. In addition Manzar *et al.,* (2022) reported production of extracellular enzymes such as amylase, beta-lactamase, protease, lipase, gelatinase, and urease in heavy metal and antibiotic resistant *Proteus vulgaris*.

The plasmid profile analysis was carried for the nineteen uncured *Bacillus* species. This was necessary to find out the presence of plasmids in the uncured *Bacillus* species and to provide baseline information to serve as reference for plasmid curing result for *Bacillus* species. Plasmid profiling revealed that most of the *Bacillus* species harbour high molecular weight plasmid DNA (23130kbp). Several authors have reported presence of plasmids in *Bacillus* species isolated from contaminated environment. Kamala-Kannan and Lee (2008) reported the presence of mega plasmid of molecular weight of 23310 kpb in heavy metal resistant *Bacillus* species isolated from Sunchon Bay Sediments, South Korea. Amalesh *et al.,* (2012) investigated heavy metal resistance of *Bacillus* species isolated from municipal waste in India and reported presence of plasmid DNA in the organism. Another authors found that heavy resistant *Bacillus* strains isolated from soil in Rize, Turkey contained plasmid DNA by Sevim and Sevim (2015). Sumathy and Lekha (2017) opined that heavy metal resistant *Bacillus* species isolated from coal mining site of Neyveli were found to contain plasmid DNA.

In order to determine if heavy metal resistance is mediated by resistance determinants borne on plasmid or chromosome, plasmid curing was carried out for all the *Bacillus* species. Many authors have also reported plasmid curing for *Bacillus* species (Kamala-Kannan and Lee, (2008); Amalesh *et al.,* (2012); Sumathy and Lekha (2017)). In this present study, the curing of plasmids in *Bacillus* species was successful as shown by Figure 3. The mega plasmids (23130 kbp) previously haboured by these organisms before curing as shown in Figure 2 were completely absent in Figure 3 after curing. The loss of plasmids by *Bacillus* species in the present study underscores the fact that ethidium bromide inhibited the replication of plasmids DNA during cell division which also corroboratates similar research by Amalesh *et al.,* (2012). The process of inhibition of plasmids DNA replication was that ethidium bromide intercalated between the purine-pyrimidine pairs in plasmid DNA of uncured *Bacillus* species during incubation in broth containing ethidium bromide. The intercalation broke the superhelical form of plasmid DNA, subsequently forming an open circular or linear plasmid DNA according to Spengler *et al.,* (2006). This is in accordance with the findings of Amalesh *et al.,* (2012) that ethidium bromide cured plasmids in heavy metal resistant *Bacillus* species isolated from municipal waste.

Curing of plasmids in bacteria aims at eliminating plasmids and to determine the heavy metal resistance mediation (Prescott *et al.,* 2008). Now that the plasmids DNA and its genetic determinants were absent in plasmid-cured *Bacillus* species, this provides an opportunity to test chromosomal genetic determinants in these plasmid-cured *Bacillus* species for heavy metal resistance mediation.

Table.1 Biochemical Characterization of *Bacillus* species

Isolate	Gram reaction	Shape	Spore staining	H_2s production	Indole	Motility	Gas production	Voges proskauer	Citrate	Gelatin liquifaction	Casein hydrolysis	Starch hydrolysis	Xylose	Arabinose	Glucose	Sucrose	Lactose	Mannitol	Organism
IMSS1	+	rods	C	-	-	+	+	-	+	-	-	-	AG	AG	AG	AG	AG	AG	*Bacillus subtilis*
IMSS3	+	rods	C	-	-	-	-	+	+	+	+	+	AG	A	AG	AG	A	AG	*Bacillus subtilis*
IMSS4	+	rods	C	-	-	-	-	+	+	+	+	+	-	A	AG	AG	A	AG	*Bacillus subtilis*
IISS7	+	rods	C	-	-	-	-	-	+	+	-	-	-	-	AG	A	-	-	*Bacillus cereus*
IISS11	+	rods	C	-	-	+	+	+	+	+	+	-	AG	AG	AG	AG	A	AG	*Bacillus subtilis*
IISS13	+	rods	C	+	-	-	-	+	+	-	-	-	AG	A	AG	AG	-	A	*Bacillus subtilis*
IISS18	+	rods	C	-	-	+	-	-	+	+	+	-	-	-	A	A	-	-	*Bacillus cereus*
IISS19	+	rods	C	-	-	-	-	+	-	+	+	-	-	-	AG	A	-	-	*Bacillus cereus*
IWSS1	+	rods	C	+	-	-	-	-	+	+	+	+	AG	A	AG	AG	-	AG	*Bacillus subtilis*
IWSS2	+	rods	C	-	-	+	-	-	+	+	+	-	AG	A	A	-	A	AG	*Bacillus cereus*
IWSS3	+	rods	C	-	-	+	-	-	-	+	+	+	AG	-	AG	A	A	AG	*Bacillus toyonensis*
IWSS4	+	rods	C	-	-	-	-	-	+	+	+	-	-	-	AG	-	A	AG	*Bacillus safensis*
IWSS5	+	rods	C	-	-	+	-	-	+	+	+	-	-	-	A	A	-	-	*Bacillus safensis*
IWSS6	+	rods	C	-	-	+	-	-	+	+	-	-	-	-	A	A	-	-	*Bacillus safensis*
IWSS18	+	rods	C	+	-	-	-	+	+	+	+	+	AG	AG	AG	A	-	-	*Bacillus safensis*
IWSS13	+	rods	C	-	-	+	+	+	+	+	+	+	AG	A	AG	AG	A	AG	*Bacillus subtilis*
IWSS17	+	rods	C	-	-	+	-	-	+	+	+	-	-	-	AG	AG	A	AG	*Bacillus wiedmannii*
IWSS18	+	rods	C	-	-	-	-	+	+	+	+	+	-	-	AG	AG	-	AG	*Bacillus tequilensis*
ICSS8	+	rods	C	-	-	+	-	-	+	+	-	-	-	-	A	A	-	AG	*Bacillus tequilensis*

Key: IMSS = Isolate from Mining Site Soil; IISS = Isolate from Industrial Site Soil; IWSS = Isolate from Waste Site Soil; ICSS = Isolate from Control Site Soil; AG = Acid and Gas Production

Table.2 Cadmium Resistance Assay for Cured *Bacillus* species

	Concentration(mg/mL)		
Isolate	0.00	0.75	1.00
IMSS1	+++	+++	-
IMSS3	+++	-	-
IMSS4	+++	-	-
IISS7	+++	++	-
IISS11	+++	++	++
IISS13	+++	++	+
IISS18	+++	++	-
IISS19	+++	++	++
IWSS1	+++	++	++
IWSS2	+++	-	-
IWSS3	+++	-	-
IWSS4	+++	-	-
IWSS5	+++	++	-
IWSS6	+++	-	-
IWSS11	+++	++	++
IWSS13	+++	++	++
IWSS17	+++	++	-
IWSS18	+++	+++	++
ICSS8	+++	-	-

Key: IMSS =Isolate from Mining Site Soil, IISS= Isolate from Industrial Site Soil, IWSS=Isolate from Waste Site Soil, ICSS = Isolate from Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth, - = No Growth

Table.3 Cobalt Resistance Assay for Cured *Bacillus* species

	Concentration (mg/mL)		
Isolate	0.00	0.75	1.00
IMSS1	+++	+++	++
IMSS3	+++	-	-
IMSS4	+++	-	-
IISS7	+++	++	+
IISS11	+++	-	-
IISS13	+++	-	-
IISS18	+++	+++	++
IISS19	+++	+++	-
IWSS1	+++	+++	++
IWSS2	+++	-	-
IWSS3	+++	-	-
IWSS4	+++	-	-
IWSS5	+++	-	-
IWSS6	+++	++	+
IWSS11	+++	+++	++
IWSS13	+++	++	-
IWSS17	+++	+++	-
IWSS18	+++	+++	-
ICSS8	+++	-	-

Key: IMSS =Isolate from Mining Site Soil, IISS= Isolate from Industrial Site Soil, IWSS=Isolate from Waste Site Soil, ICSS = Isolate from Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth, - = No Growth

Table.4 Zinc Resistance Assay for Cured *Bacillus* species

Isolate	Concentration (mg/mL) 0.00	0.75	1.00
IMSS1	+++	-	-
IMSS3	+++	+++	-
IMSS4	+++	-	-
IISS7	+++	+++	+++
IISS11	+++	++	+
IISS13	+++	++	++
IISS18	+++	++	++
IISS19	+++	++	-
IWSS1	+++	++	++
IWSS2	+++	-	-
IWSS3	+++	-	-
IWSS4	+++	++	-
IWSS5	+++	++	-
IWSS6	+++	+++	++
IWSS11	+++	-	-
IWSS13	+++	+++	+++
IWSS17	+++	++	-
IWSS18	+++	++	++
ICSS8	+++	-	-

Key: IMSS =Isolate from Mining Site Soil, IISS= Isolate from Industrial Site Soil, IWSS=Isolate from Waste Site Soil, ICSS = Isolate from Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth, - = No Growth

Fig.1 Screening for Enzymatic Activity in *Bacillus* species

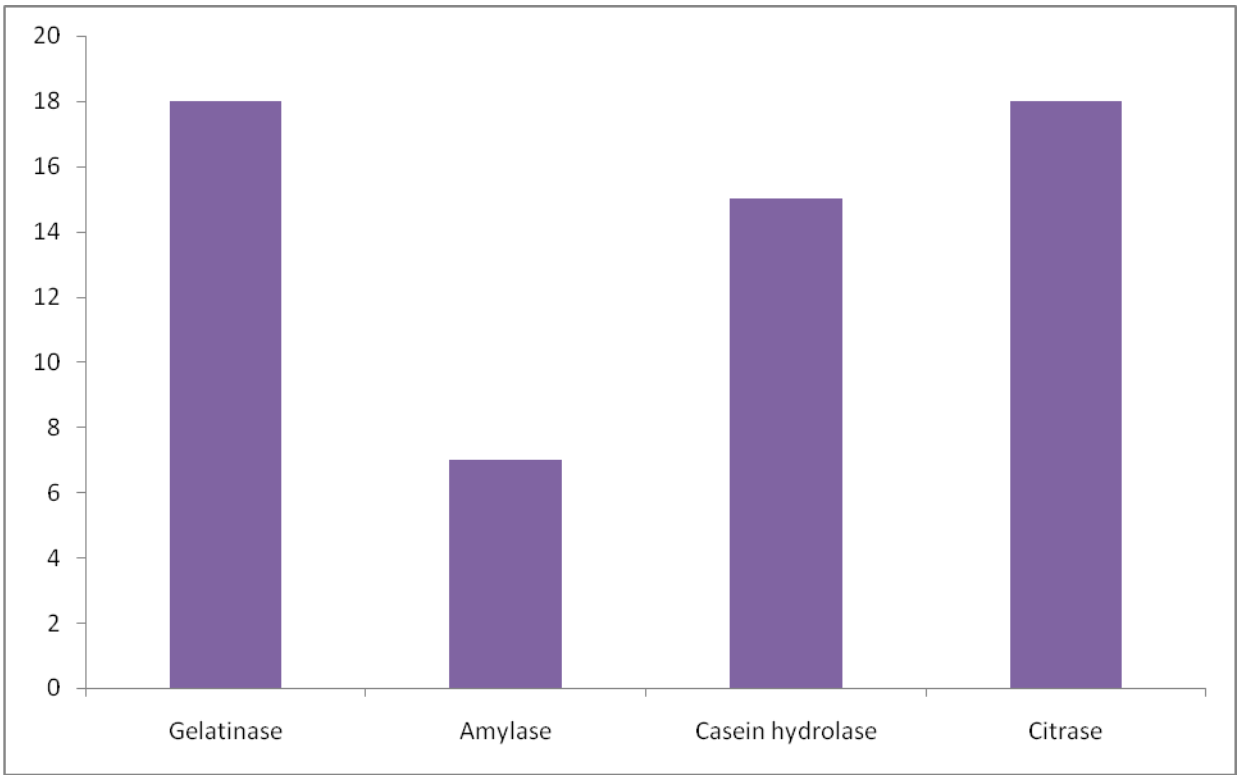
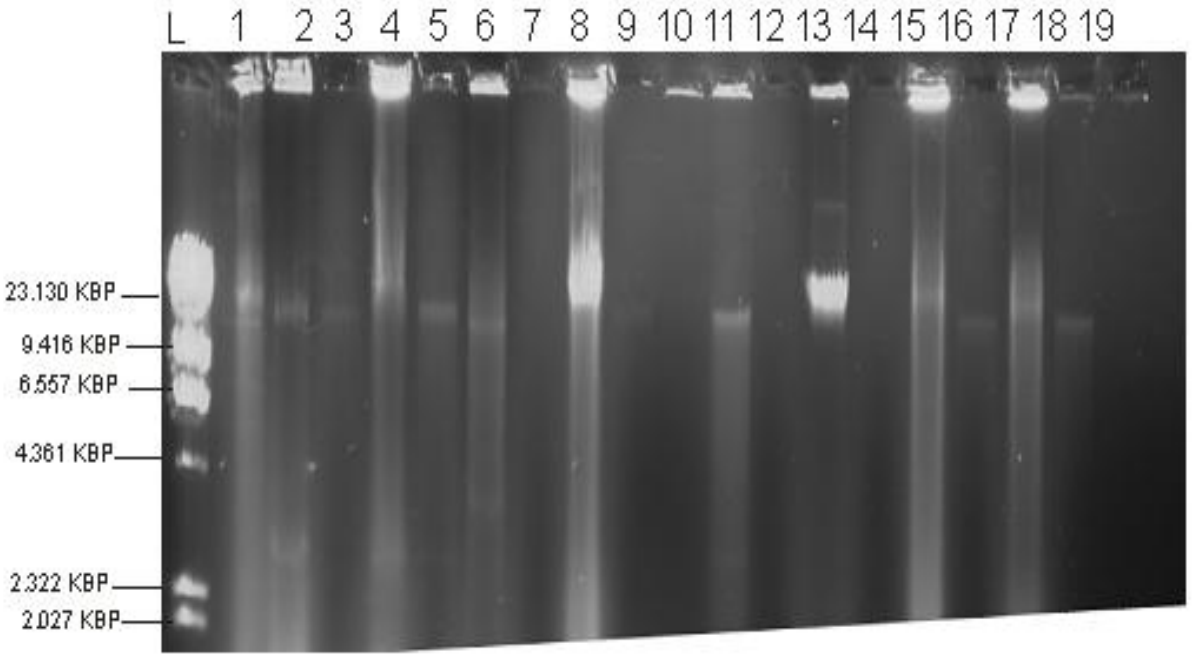


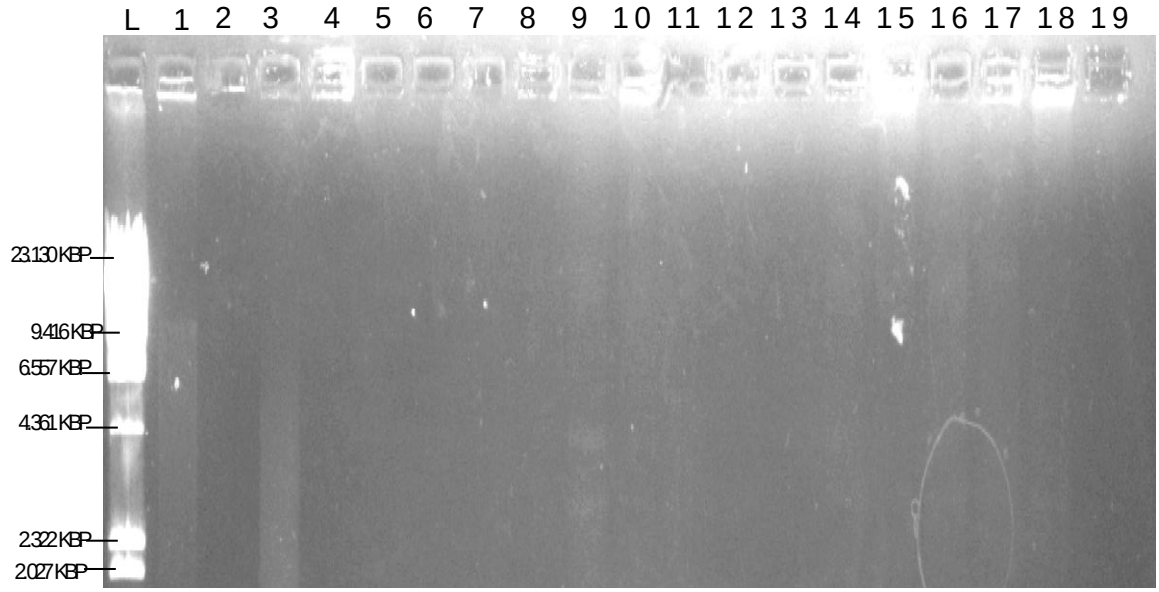
Fig.2 Plasmids Electrophoresis Profile of *Bacillus* species



Key: L = Ladder, 1 = IMSS1, 2 = IMSS3, 3 = IMSS4, 4 = IISS7, 5 = IISS11, 6 = IISS13, 7 = IISS18, 8 = IISS19, 9 = IWSS1, 10 = IWSS2, 11 = IWSS3, 12 = IWSS4, 13 = IWSS5, 14 = IWSS6, 15 = IWSS11, 16 = IWSS13, 17 = IWSS17, 18 = IWSS18, 19 = ICSS8

IMSS = Isolates from Waste Site Soil; IISS = Isolates from Industrial Site Soil; IWSS = Isolates from Control Site Soil

Fig.3 Plasmid Electrophoresis Profile of Cured *Bacillus* species



Key: L = Ladder, 1 = IMSS1, 2 = IMSS3, 3 = IMSS4, 4 = IISS7, 5 = IISS11, 6 = IISS13, 7 = IISS18, 8 = IISS19, 9 = IWSS1, 10 = IWSS2, 11 = IWSS3, 12 = IWSS4, 13 = IWSS5, 14 = IWSS6, 15 = IWSS11, 16 = IWSS13, 17= IWSS17, 18 = IWSS18, 19 = ICSS8

IMSS = Isolates from Waste Site Soil; IISS = Isolates from Industrial Site Soil; IWSS = Isolates from Control Site Soil

The results of heavy metal resistance assay (Table 2 to 4) revealed that plasmid curing led to loss of heavy metal resistance in some isolates. For cadmium resistance assay, the percentage loss of resistance recorded was 32% in that bacterial isolates: IMSS3, IMSS4, IWSS2, IWSS3, IWSS4, IWSS6 and ICSS8 completely lost heavy metal resistance at concentrations 0.75 and 1.00 mg/mL. Also in cobalt resistance assay, 41% metal resistance loss was recorded. The following bacterial isolates IMSS3, IMSS4, IISS11, IISS13, IWSS2, IWSS3, IWSS4, IWSS5 and ICSS8 completely lost heavy metal resistance at concentrations 0.75 and 1.00 mg/mL. Furthermore, bacterial isolates : IMSS1, IMSS4 IWSS2 IWSS3, IWSS11 and ICSS8 lost metal resistance (27 %) at concentrations 0.75 and 1.00mg/mL in zinc resistance assay, The least percentage loss of resistance was recorded in zinc (27%) followed by cadmium (32%) and then cobalt (41%). This is an indication that the genetic markers to heavy metals in these *Bacillus* species are exclusively borne on plasmids and not found on chromosomes. This finding agrees with the report of Amalesh *et al.,* (2012) that after plasmid curing of heavy metal resistant *Bacillus* species isolated from municipal waste, they completely lost resistance to heavy metals.

Moreover, there were some plasmid cured bacterial isolates that retained metal resistance at 0.75mg/mL but lost at 1.00mg/mL concentration. For cadmium assay, the isolates were IMSS1, IISS7, IISS18, IWSS5 and IWSS17. Also, for cobalt metal, the isolates were IWSS13, IWSS17 and IWSS18. Lastly for zinc, the isolates were IMSS3, IISS19, IWSS4, IWSS5 and IWSS17. All these isolates would not have retained metal resistance even at 0.75mg/mL, if the resistance determinants for respective heavy metal were exclusively borne on plasmids DNA. This suggests that the resistance determinants of these bacterial isolates are borne on chromosomal DNA. The inability of these isolates to survive 1.00mg/mL of various heavy metals could be that this concentration was the minimum inhibitory concentration for their chromosome-borne resistance determinants. The decreased microbial growth as metal concentration increases could be due to increased toxicity of heavy metals on metabolic activities of microorganisms including growth as opined by Amalesh *et al.,* (2012) and Eghomwanre *et al.,* (2016)

Alternatively, many of the plasmid-cured *Bacillus* species exhibited resistance to various heavy metals. In cadmium assay, the following isolates IISS11, IISS13, IISS19, IWSS1, IWSS11, IWSS13 and IWSS18 demonstrated to cadmium metal at 0.75 and 1.00mg/mL concentrations. This represents 33% resistance. For cobalt, 29% of bacterial isolates showed resistance at 0.75 and 1.00mg/mL concentrations. The isolates were IMSS1, IISS7, IISS18, IWSS1, IWSS6 and IWSS11. Lastly, in zinc assay, IISS7, IISS11, IISS13, IISS18, IWSS1, IWSS6, IWSS13 and IWSS18 were cured isolates accounting for 38% that exhibited resistance at both concentrations. This is a clear indication that resistance markers to these heavy metals are borne on chromosomal DNA in these isolates. The highest resistance was recorded in zinc (38%) followed by cadmium (33%) and cobalt (29%). This finding also agrees with Kamala-Kannan and Lee (2008) in a similar research with a report that after plasmid curing all *Bacillus* species exhibited resistance to heavy metals.

The present study shows clearly that the genetic resistance determinants responsible for heavy metal resistance mediation are borne on both plasmid and chromosomal DNA in bacteria. Similar studies have also reported this finding (Sidhu *et al.,* (2015); Kamala-Kannan and Lee (2008)).

Microorganisms that are able to survive well in high concentration of heavy metals are of great importance as bioremediation agents. Cured *Bacillus* species in the present study survived at high concentrations (0.75 and 1.00mg/mL) of these heavy metals. These bacteria could be used to develop remediation processes to these heavy metals both in the cement factory and those of natural origin. The production of antioxidant enzymes such as gelatinase, amylase, citrase and casein hydrolase is

another biotechnological benefit from these heavy metal resistant cured *Bacillus* species.

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