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Cellulolytic Soil Bacteria Exhibited Tolerance to Heavy Metals

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Abstract: The tolerance ability of cellulolytic soil bacteria to Lead (Pb), Copper (Cu), Mercury (Hg) and Zinc (Zn) was evaluated *in - vitro*. The emphasis was placed on two different dumpsites (Soulos dumpsite and Olusosun landfills) naturally polluted with heavy metals in the city of Lagos, Nigeria. Soil samples were randomly collected from these dumpsites and evaluated for bacteria contents. Out of all the bacteria isolated and identified, *Pseudomonas* spp. *and Bacillus* spp. predominated in comparison to others such as *Klebsiella* spp*., Micrococcus* spp., *Escherichia* spp*., Serratia* spp. and *Enterobacter* spp. Based on evaluation, heavy metal tolerance bacteria with potentials to utilize cellulose were selected. Unfortunately, none of the isolates grow in the presence of Hg even at the lowest concentration level. Despite the challenges of soil physicochemical characteristics, level of metal concentrations and tolerance peculiarities, *Micrococcus roseus* and *Pseudomonas cepacia* were outstanding in their tolerance ability. Specifically, Cu and Zn were the best tolerated metals for *Micrococcus roseus* while *Pseudomonas cepacia* exhibited multi-tolerance ability to Pb, Cu and Zn. Thus, the biodegradation of heavy metal polluted soil could be achieved using indigenous cellulose utilizers.

Key words: Metal Tolerance · Isolates · Heavy Metals · Bacteria · Cellulose · Dumpsites

INTRODUCTION

Heavy metal contamination in the environment especially the soil [1] has become a serious problem due to the increase in the addition of these metals to the environment [2]. They cannot be degraded or destroyed because they are stable and so persistent environmental contaminants are inevitable [3]. The environmental stress caused by heavy metals, generally decreases the diversity and activity of soil microbial populations leading to a reduction of the total microbial biomass, decrease in numbers of specific populations and a shift in microbial community structure [1]. However, traces of these heavy metals are necessary as co-factors of enzymatic reactions, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions [4, 5].

Various microorganisms show a different response to toxic heavy metal ions that confer them with a range of metal tolerance [6]. Soil bacteria which include but not limited to *Paenibacillus* sp. and *Bacillus thuringiensis* have demonstrated tolerance to Cadmium, Copper and Zinc though varied in their pattern of tolerance activity [7]. Similarly, *Pseudomonas stutzeri*, isolated from a foundry soil, was shown to be resistant to the toxic effect of chromium up to 1 mM and anaerobically reduce Cr (VI) up to 100 μM [8].

Though, the heavy metal tolerance ability of many soil bacteria have been established [9-13] but that of cellulolytic soil bacteria [14] have been relegated to the background. Attention on heavy metal tolerance cellulolytic bacteria may offer a beneficial tool for monitoring of pollutants in the environment. Based on this hypothesis, this study, therefore investigated heavy metal tolerance ability of cellulolytic soil bacteria isolated from selected dumpsites in Lagos State, Nigeria in-view to determine their potentials as bioremediation tools for heavy metal contaminated soil.

MATERIALS AND METHODS

Sample Collection, Bacteria Isolation and Identification: The locations namely; Soulos dumpsite (SD), Igando and Olusosun landfill, Ojota (SL) used for this study were

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among the major dumpsites in Lagos State, Nigeria. The dumpsites have been in existence for the past 15 years for dumping any kind of wastes mostly domestic and partly industrial wastes. Soil samples from the dumpsites were randomly collected at different points in three replicates (For each study location) at depths of 8-15cm with sterile hand trowel into well labeled brown paper bags. The soil samples were aseptically transported to the laboratory for physiochemical and bacteriological analysis. A serial-dilution–pour plate technique was used to isolate bacteria [15] on nutrient agar (NA). Inoculated petri plates were incubated at 25± 2?8C for 24 hours. Apart from bacteria load that was determined for each soil sample, isolates differing in morphological appearance on NA were also selected and were streaked onto new plates until pure cultures were obtained. Pure cultures of bacterial isolates were maintained on NA slants and stored at 4°C. Prior to physiochemical analysis of the soil samples and screening of cellulolytic bacteria, identification of the isolates was carried out using colonial (Colonial shape, size, elevation, pigmentation as well as margin) and microscopic morphological characteristics (Based on their cell shape, cell arrangement and retention of the Gram stain). Further identification was carried out using biochemical tests which include tests for catalase, starch hydrolysis, gelatin hydrolysis, indole, oxidase, urease, spore stain, citrate, methyl red, Voges proskauer and sugar fermentation by following standard protocols [16].

Physicochemical Analysis of Soil Samples: Based on each location, collected soil samples were manually pooled together to form a composite sample. The samples were sieved through 0.5 - 2 mm wire mesh so as to obtain fine sand grains specifically to determine the moisture content, pH, total hydrocarbon content (THC), total organic carbon (TOC), heavy metals, potassium, phosphorus and nitrogen contents. The moisture content was determined using the constant dry weight method as described by Fawole and Oso [17]. For pH, air-dried soil sample of about 20g was weighed into a beaker and 20ml of distilled water was added into the same beaker and allowed to stand for 30minutes with stirring using a glass rod. The electrode of the pH meter was then inserted into the partly settled suspension and the pH was measured [17]. The THC, TOC and available phosphorus (P) were determined as described by APHA [18]. Heavy metals and potassium (K) [19-21] were determined by digesting 5g of dried soil sample with 10ml of nitric acid in a conical flask and heated until the brown flames disappear. The sample was allowed to cool after which distilled water was added to make up to 50ml in the flask. The solution obtained was filtered and the filtrate was analyzed using the atomic absorption spectrometer. Total nitrogen (N) was determined by the micro-kjeldahl digestion method [22].

Screening for Cellulolytic Soil Bacteria: Pure culture of each bacterium isolate was inoculated onto cellulose Congo red agar medium. The plates were incubated at 25±2°C for 48 hours. Cultured plates showing discoloration of Congo red were taken as positive cellulose-degrading bacteria isolates [23]. Cellulose-degrading potential of the positive isolates was quantitatively estimated by calculating its hydrolytic capacity (HC), that is, the ratio of diameter of clearance zone and colony [24].

Evaluation of the Potentials of Isolated Bacteria for Heavy Metal Tolerance: Isolates found to be cellulolytic were further evaluated for their ability to utilize heavy metals such as Pb, Cu, Hg and Zn. Their salts PbSO4, $CuSO_4.5H_2O$, $HgCl_2$ and $ZnSO_4.7H_2O$ were used in preparing stock solution. A minimal medium supplemented with increasing concentration of the metal salts was prepared [25, 26]. Briefly, prior to heavy metal tolerance test, McFarland standard corresponding to 0.5 was prepared [27, 28]. Turbidity was confirmed to have optical density (OD) of 0.08 - 0.10 at 625nm using photo-electric colorimeter. A stock solution of each metal salt was prepared in their increasing concentrations (0.25g, 0.5g, 1g and 2g) and was separately added to 100ml of the minimal medium. To complement this, 1ml of the standardized bacteria suspensions was added to 9ml of the prepared stock solution with respect to their increasing concentrations in test tubes and then incubated at 25±2°C for 48 hours. After incubation, the bacterial culture was serially diluted to 10^{-5} in distilled water before 0.1ml of the culture was spread on NA plates. The plates were incubated at 25±2°C for 48 hours and observed for growth.

RESULTS

Bacterial Load, Identification and Occurrence of Isolates: The bacterial loads from the two study locations were numerically different from each other. Soil samples collected from SD had a higher bacterial load in comparison to that of SL. The total bacterial load from the SD soil sample ranged from 164×10^5 to 185×10^5 CFU/ml while that of the SL soil sample ranged from 114×10^5 to 149×10^5 CFU/ml (Fig. 1). The cultural, morphological and

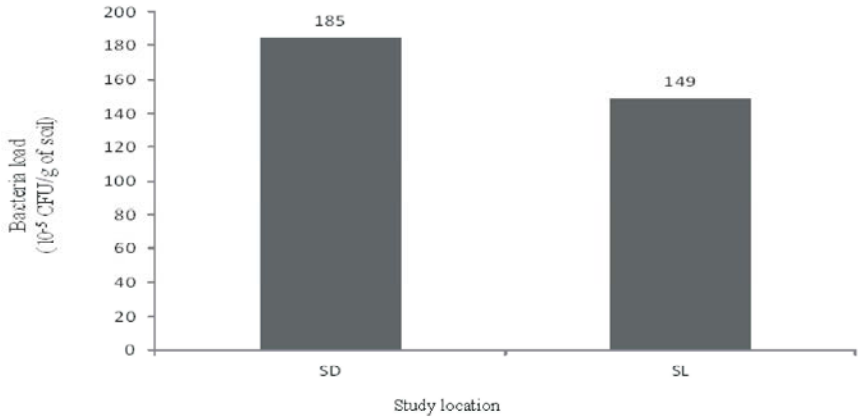

Fig. 1: Bacteria load from study locations at 10^{-5} CFU/g of soil.
SD = Soulos dumpsite, SL = Olusosun landfill

Table 1: Identity of bacteria isolated from the selected dumpsite locations

SD		SL	
Genus	Species	Genus	Species
Pseudomonas	*cepacia* (SD3, SD8, SD12), *caryophylli* (SD4, SD6, SD7 and SD17).	*Pseudomonas*	*cepacia* (SL1, SL2, SL9, SL10, SL12, SL13, SL14 and SL15).
Bacillus	*cereus* (SD2 and SD11), *polymyxa* (SD10 and SD18), *licheniformis* (SD13), *megaterium* (SD16).	*Bacillus*	*cereus* (SL4, SL5 and SL11), *megaterium* (SL3 and SL7).
Micrococcus	*roseus* (SD5 and SD15).	*Micrococcus*	*roseus* (SL6 and SL16).
Serratia	*marcescens* (SD9)	*Serratia*	-
Klebsiella	*ozaenae* (SD1)	*Klebsiella*	-
Escherichia	*coli* (SD14)	*Escherichia*	-
		Enterobacter	*aerogenes* (SL8)

SD = Soulos dumpsite, SL = Olusosun landfill

Table 2: Occurrence of bacterial isolates based on locations

Isolate	SD	SL	Total number	% of occurrence
Pseudomonas	7	6	13	38
Bacillus	6	7	13	38
Micrococcus	2	2	4	12
Enterobacter	-	1	1	3
Serratia	1	-	1	3
Klebsiella	1	-	1	3
Escherichia	1	-	1	3

SD = Soulos dumpsite, SL = Olusosun landfill

biochemical characteristics revealed isolates identity as *Klebsiella ozaenae*, *Micrococcus roseus*, *Pseudomonas caryophylli*, *Pseudomonas cepacia*, *Bacillus polymyxa*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus cereus*, *Escherichia coli*, *Serratia marcescens* and *Enterobacter aerogenes* (Table 1). Based on their frequency of occurrence, *Pseudomonas* occurred more than any other bacteria in the SD soil sample while *Bacillus* occurred more in the SL soil sample. Generally, *Bacillus* and *Pseudomonas* were the most frequently occurring isolates, followed by *Micrococcus* and the least were *Enterobacter*, *Serratia*, *Klebsiella* and *Escherichia* (Table 2).

Soil Physicochemical Analysis: The SD soil sample was more alkaline (pH of 8.51) in comparison to SL that was slightly alkaline (pH 7.72). The moisture content had a wide variation with respect to the two soil samples. The SD soil sample had 17.7% moisture content in comparison to SL that was relatively low (4.0%). The TOC, N and Zn

Table 3: Physicochemical analysis of collected soil samples based on location

	Soil sample location	
Physiochemical Parameters	SD	SL
Moisture %	17.70	4.0
pH	8.51	7.72
TOC %	1.73	1.40
THC (mg/kg)	210.53	655.17
P mg/kg	214.50	47.03
N %	0.31	0.25
K (mg/kg)	3.70	4.20
Pb (mg/kg)	0.02	0.04
Zn (mg/kg)	4.09	2.17
Cu (mg/kg)	1.66	1.92

P = phosphorus, TOC = Total organic carbon, THC = Total hydrocarbon content, N = Nitrogen, K = Potassium, Pb = Lead, Zn = Zinc, Cu = Copper. SD = Soulos dumpsite, SL = Olusosun landfill.

Table 4: Cellulolytic activity of bacterial isolates

SD isolate	Hydrolytic capacity	SL isolate	Hydrolytic capacity
SD1	2.8	SL1	1.86
SD2	2.0	SL2	1.57
SD3	1.6	SL3	2.0
SD4	1.89	SL4	2.67
SD5	2.33	SL5	2.17
SD6	3.0*	SL6	1.64
SD7	2.14	SL7	2.43
SD8	2.5	SL8	1.71
SD9	1.67	SL9	2.0
SD10	2.25	SL10	3.25*
SD11	2.50	SL11	1.8
SD12	2.40	SL12	7.75*
SD13	2.0	SL13	4.6*
SD14	1.73	SL14	2.6
SD15	1.67	SL15	2.6
SD16	2.33	SL16	3.0*
SD17	2.0		
SD18	1.86		

* Indicate hydrolytic capacity = 3. SD = Soulos dumpsite, SL = Olusosun landfill

content of the SD soil were higher than those of the SL soil. However, THC, K, Pb and Cu content of the SL soil were higher when compared to that of SD soil (Table 3). Interestingly, the P content was relatively high in the collected SD soil, even to the extent of 4 fold higher than the SL soil (Table 3).

Cellulolytic Activity of Bacterial Isolates: The hydrolytic capacity of SD and SL isolates ranged from 1.6 - 3.0 and 1.57 - 7.75 respectively. SD6 and SL12 had the highest hydrolytic capacity in comparison to SD3 and SL2 that were the lowest in their respective soil samples (Table 4). Isolates SD6, SL12, SD3 and SL2 were all identified as *Pseudomonas* (Table 1, 4). Generally, it was observed that 25% of the isolates from SL soil sample exhibited hydrolytic capacity ≥ 3 which is extremely good for cellulose utilizing bacteria. On the contrary, isolates from SD soil samples did not exhibit good cellulose utilization ability since only 0.56% of the isolates had hydrolytic capacity ≥ 3.

Heavy Metal Tolerance of Bacterial Isolates: Isolates that showed hydrolytic capacity ≥ 3 were selected (Table 4) and were further evaluated towards their heavy metal tolerance ability. The isolates were *Pseudomonas caryophylli* (SD6), *Pseudomonas cepacia* (SL10, SL12 and SL13) and *Micrococcus roseus* (SL16). Specifically, with the exception of isolate SL12 that tolerated 0.76mM of Pb, other isolates were unable to tolerate Pb at higher concentration levels. Interestingly, both isolates SL12 and SL16 sequentially tolerated Cu at a concentration of 0.63mM and 1.25mM. Further observation showed that

Table 5: Tolerance activity of SD and SL isolates to Pb, Cu, Hg and Zn

	Pb			
Isolate	0.76 mM/ml	1.52 mM/ml	3.03 mM/ml	6.06 mM/ml
SD6	-	-	-	-
SL10	-	-	-	-
SL12	1.8 x 10^5 CFU/ml	-	-	-
SL13	-	-	-	-
SL16	-	-	-	-
	Cu			
	0.63 mM/ml	1.25 mM/ml	2.50 mM/ml	5 mM/ml
SD6	-	-	-	-
SL10	-	-	-	-
SL12	9.6 x 10^5 CFU/ml	8.0 x 10^5 CFU/ml	5.8 x 10^5 CFU/ml	-
SL13	-	-	-	-
SL16	5.8 x 10^5 CFU/ml	3.6 x 10^5 CFU/ml	-	-
	Hg			
	0.68 mM/ml	1.36 mM/ml	2.72 mM/ml	5.43 mM/ml
SD6	-	-	-	-
SL10	-	-	-	-
SL12	-	-	-	-
SL13	-	-	-	-
SL16	-	-	-	-
	Zn			
	0.72 mM/ml	1.44 mM/ml	2.87 mM/ml	5.75 mM/ml
SD6	-	-	-	-
SL10	-	-	-	-
SL12	3.3 x 10^5 CFU/ml	2.0 x 10^5 CFU/ml	-	-
SL13	-	-	-	-
SL16	6.4 x 10^5 CFU/ml	4.7 x 10^5 CFU/ml	-	-

SD = Soulos dumpsite, SL = Olusosun landfill

SL12 isolate still utilized Cu at 2.5mM concentration level. Apart from the fact that SL12 and SL16 isolates could not tolerate Cu at higher concentration (5mM), other isolates (SD6, SL10 and SL13) were unable to utilize Cu even at the lowest concentration levels. None of the isolates could tolerate Hg at both lower (0.68mM and 1.36mM) and higher (2.72mM and 5.43mM) concentrations. Zn out rightly discouraged the growth of SD6, SL10 and SL13 isolates. However, Zn concentration at 0.72mM and 1.44mM encouraged the growth of SL12 and SL16 isolates. Generally, it was observed that as the metal concentrations increases, the bacteria biomass decreases (Table 5). Also, isolates from SL soil sample possesses the ability not only to utilize cellulose, but they can as well tolerate heavy metals (Table 4, 5) than isolates from SD soil.

DISCUSSION

The heavy metal - bacteria interaction is a complex global issue. This global phenomenon was re-confirmed in our study with respect to the soil samples collected from the selected dumpsites in Lagos State, Nigeria. Heavy metals can affect microorganisms in soil multisidedly: they can shift the structure of microbial populations, impoverish their diversity and affect species composition, reproduction and activity of indigenous microorganisms [29-31].

Higher bacterial load was observed in these study locations. The bacterial load might have been initiated by different wastes dumped at the sites, thus enhanced conducive environment for microbial proliferation and biological activity [12, 32]. When waste is dumped on

land, soil microorganisms (Bacteria and fungi) readily colonizes the waste carrying out degradation and transformation of cellulose and other organic materials in the waste [33]. The identified isolates were members of *Pseudomonas* spp., *Escherichia coli*, *Bacillus* spp., *Enterobacter* spp., *Klebsiella* spp. and *Micrococcus* spp. Similar bacteria were isolated and identified by Oviasogie *et al.* [34] and Anyanwu *et al*. [35] from different dump sites. Out of all the isolates, *Pseudomonas a*nd *Bacillus* species were the most prevalent in the two dump sites. The frequency of occurrence of *Pseudomonas* could be attributed to its define relationship with dumpsites as reported by Sandhu *et al*. [36] while that of *Bacillus* could be related to its association with soil and can thrive under harsh environmental conditions [37]. *Klebsiella* spp., *Escherichia coli*, *Serratia* spp. and *Enterobacter* spp. had the least frequency of occurrence. Probably these isolates could not utilize the carbon sources in the environment as building energy in their metabolic pathways [38]. In addition, anthropogenic impacts such as changes in nutrient composition have the potential to directly or indirectly affect the microbial composition of the soil [39]. The isolated bacteria showed an appreciable degree of cellulose utilization *in-vitro*. This had been established by previous investigators [40, 41] especially on *Pseudomonas* spp*., Bacillus* spp*., Micrococcus* spp., *Klebsiella* spp., *Escherichia coli*, *Serratia* spp. and *Enterobacter* spp.

Based on our benchmark of hydrolytic capacity (= 3), many of the selected isolates were unable to utilize Pb, Cu, Hg and Zn. This observation agreed with the work of Lugauskas *et al.* [10] who stated that high concentrations of metals can exert a harmful effect on microorganisms. Reduction in the number of cellulose utilizing isolates is an indication that essential functions have been affected, resulting in the influence on the whole metabolism [42, 43]. Among all the heavy metals tested, Hg was the most toxic. It inhibited all the isolates. In this context, the toxic effect of metals may include blocking of functional groups of biologically important molecules, the displacement and substitution of essential metal ions from biomolecules, conformation, modification, denaturation and inactivation of enzymes and disruption of cellular and organelle membrane integrity [31, 44].

Out of all the isolates, only two cellulose-degrading bacteria withstood the impact of Pb, Cu and Zn very well, though based on hydrolytic capacity of ≥ 3. Specifically, only SL12 (*Pseudomonas Cepacia*) and SL16 (*Micrococcus roseus*) isolates shows potentials to utilize heavy metals. The heavy metal tolerance ability of SL16 was highly remarkable for Zn in comparison to Pb, Cu and Hg. Apart from the fact that the selective response to a specific heavy metal can influence shift to a certain group of microorganisms [10] certain cellulose utilizing bacteria, such as *Micrococcus roseus* can exhibit potentials to tolerate different types of heavy metals. In addition, this may be as a result of the fact that adapting peculiarities (physiological or genetic) are very important in microbial survival strategy under stress conditions [10]. Furthermore, our findings show that *Pseudomonas* spp. tolerated more of Cu and Zn in comparison to the work of Shakibaie *et al.* [45]. Also, the tolerance ability of SL12 isolate was even far better than what was obtained in the work of Hussein *et al.* [46] that Cu (II) inhibited the growth of Pseudomonas putida strain at concentration of >4 mmol/L. More so, the SL12 tolerance ability to Pb (3mM) was quite outstanding and incomparable to what was obtainable by Atuanya *et al*. [47]. This is a welcome development for Nigeria, especially in industrial locations where soil is contaminated with Pb substances. Similarly, the multiple tolerance ability of SL12 to Cu, Zn and Pb is unique. Our observation on multi-tolerance ability of SL12 corroborated with the work demonstrated by Sharma *et al.* [48] on exposure of *Pseudomonas fluorescence* ATCC 948 to three different heavy metals. On the other hand, the physico-chemical characteristics of SL and SD soil samples may as well influence the variation observed among the heavy metal tolerance bacteria because the physico-chemical properties of a particular environment determine metal speciation and consequently their biological availability and toxicity [49-52].

CONCLUSIONS

Generally, the present study revealed that *Pseudomonas cepacia* (SL12) and *Micrococcus roseus* (SL16) can tolerate Pb, Cu and Zn to a certain level. Thus, biodegradation of heavy metal polluted soils could be achieved in the city of Lagos, Nigeria using indigenous cellulose utilizing bacteria.

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