ORIGINAL RESEARCH ARTICLE

Bio stimulation of indigenous microorganisms with Gomeya: a bioremediation technique

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ABSTRACT

The burden of heavy metals in the environment can be reduced using organic amendment stimulated bacterial remediation. This study employed cattle manure slurry stimulated bacterial inoculum to treat heavy metals-contaminated soil. Samples of contaminated soil and cattle manure were collected from the area surrounding a steel rolling mill and a commercial animal pen respectively. Bacteria were isolated using pour plate technique; identified using various biochemical tests and screened for resistance to heavy metal salts by incorporating heavy metal salts into agar plates. The contaminated soil and manure slurry were analysed for heavy metals and then sterilised separately. Five kilogram of the sterilised contaminated soil was weighed and mixed with 100g of sterilized cow dung slurry and aseptically packed into plastic nursery bags. Bacterial samples showing high tolerance to heavy metal salts were introduced into the bags singly and as a consortium for bioremediation exercise. Thirty-six bacterial isolates were obtained from the contaminated soil. Chemical analysis revealed that the soil was heavily contaminated especially with lead and chromium with concentrations of 1505.1-2333.6 and 1526.0-1678.7 mg/kg, respectively. *Alcaligenes faecalis, Pseudomonas azotoformans* and *Bacillus mycoides* exhibited high tolerance to heavy metals salt and were selected for bioremediation. Post bioremediation analysis of the soil samples revealed a reduction in the concentration of heavy metals concentration with major reduction in the concentration of chromium in groups treated with *P. azotoformans*. Biostimulation of microorganisms with organic amendment effectively remediated heavy metals contaminated soil and can be employed in the treatment of such contaminated environments.

Biological: Microbiology. Keywords: *Cattle manure slurry, biomagnification, stimulated bacterial inoculum*

INTRODUCTION

Some important heavy metals such as cadmium, copper, lead, chromium and mercury are also important environmental contaminants and they are found in high concentration especially in areas with high anthropogenic activities (Suruchi and Khanna, 2011). Though some of these heavy metals are also soil micronutrients; the extent of soil pollution by these heavy metals and base

metal ions is alarming. As a result of pollution, it has been observed that the larger the urban area, the lower the quality of the environment (Eddy, 2004a). The various sources through which heavy metals are released into the soil environment include but are not limited to natural means such as emissions from volcanoes, transport of continental dust and the weathering of metal-enriched rocks (Ernst, 1998) or as a result of various anthropogenic activities such as exploration of mines and smelters, the application of manures, fertilizers, metal based pesticides and metal-enriched sewage sludge in agriculture, combustion of fossil fuels, metallurgical industries, military training, manufacturing, usage and disposal of electronics.

Heavy metal uptake by plants grown on polluted soils has been studied to a considerable extent (Suruchi and Khanna, 2011; Navarro et al., 2008; Dixit et al., 2015; Sukreeyapongse et al., 2002, Yusuf et al., 2003). Heavy metal uptake via roots from contaminated soils and surface water, and direct deposition of heavy metal contaminants from the atmosphere unto plant surfaces can lead to contamination of plant by heavy metals. When these metals exceed the physiological demand of plant, they may not only be toxic to the plants, but oftentimes enter into the food chain, become biomagnified and pose serious health concern to humans (Sugiyama, 1994; Odoh and Kolawole, 2011). The biotoxic effects of heavy metals on plants depend upon the concentrations and oxidation states of heavy metals, its source and mode of deposition (Duruibe et al., 2007).

Though various conventional technologies such as chemical oxidation, precipitation, ion exchange, soil washing, incineration, solidification and stabilization are usually employed in the remediation of heavy metals contaminated soil (FRTR, 2000; Gomes et al., 2012) biological treatment of heavy metal-contaminated soil is often more attractive than direct chemical or physical treatment. One of the most promising technological approaches to the problem of heavy metal contamination in the environment is bioremediation. This can be attributed to the ability of the microorganisms to directly sequester contaminants rather than merely transferring them from one medium to another (USEPA, 1995). Some organisms which have been involved in bioremediation processes include *Pseudomonas aeruginosa, P. ambigua, P. fluorescens,* other species such as *Bacillus cereus, B. subtilis, E. coli* (ATCC 33456), *Achromobacter Eurydice, Micrococcus roseus, Enterobacter cloacae, Desulfovibriode sulfuricans* and *D. vulgaris. Shewanella algae* BrY-MT have been reported to be effective in bioremediation of various contaminants (Guha et al., 2012; Camargo et al., 2003). Comparing the effectiveness of conventional methods of metal removal with biological methods of metal removal, it has been observed that the use of biomass of microorganisms has distinct advantages over conventional methods due to the fact that they are highly selective and cost effective, have diversity of active binding site (Ahluwalia and Goyal, 2007; Green-Ruiz et al., 2008). For instance, microorganisms can assimilate heavy metals actively (bioaccumulation) and/or passively (adsorption) (Hussein et al., 2001). The bacterial cell walls, which consist mainly of polysaccharides, lipids and proteins, offer many functional groups that can bind heavy metal ions, and these include carboxylate, hydroxyl, amino and phosphate groups (Randhawa and Kullar, 2011). Algae, fungi, yeast, protozoa and bacteria have been employed in the removal of heavy metals from industrial waste waters by using the microorganisms in whole and/or using products of their metabolism such as enzymes and biosurfactant (Congeevaram et al., 2007; Özdemir and Kılınc, 2012; Özdemir et al., 2012).

Cattle manure slurry/gomeya usually referred to as a waste product can enhance the degradation of contaminants in the environment. Cattle dung slurry is a cheap and easily available rich source of organic amendment. It is a mixture of cattle dung and urine in a ratio of around 3:1 respectively (Randhawa and Kullar, 2011). According to Adedokun and Ataga (2007), soil amendments or

additives are needed to increase the activities of microbes and for effective bioremediation of polluted soil.

Corchorus olitorius commonly called jute or Jew mallow belongs to the Tiliaceae family. The choice of *C. olitorius* for this study is based on the fact that it is one of the most popular vegetables in every home; hence it is grown in nearly all home gardens, market gardens near the city and truck gardens around the world (Aluko et al., 2014).

The aim of this study was to bioremediate heavy metals contaminated soil using organic amendment stimulated bacterial remediation. This study was therefore designed to study the ability of bacteria isolated from heavy metal-contaminated soil to effectively remove heavy metals in order that the bioremediated soil can support plant growth.

Materials and Methods

Sample collection

The study location was densely contaminated with heavy metals contained in effluent released as a result of the activities of a steel rolling company in South-western Nigeria. Consequently, plants grown in the vicinity of the company could not thrive which had an impact on the livelihood of people living in that area as majority of them were peasant farmers. The study location was visited during the dry and rainy season to carry out *in situ* analysis in order to obtain background knowledge of the seasonal variations in the physical and chemical properties of the location. Physical observation was done to observe changes in physical characteristics such as colour, texture, odour and deposition of effluents from the rolled steel industry which were considered as indicators for pollution. Soil sample was collected from different points of the location using a soil auger. The soil was transported to the Department of Microbiology, University of Ibadan where microbiological and chemical analysis were conducted within 24 h of collection.

Analysis of heavy metal-contaminated soil

The soil samples collected were thoroughly mixed using a hand trowel to obtain a composite sample before subjecting it to various analyses. Soil hydrogen ion concentration (pH 1:1 H_2O) was determined using a glass electrode pH meter (Hanna instruments HI2210) following the methods described by Bates (1954). The exchangeable acidity was determined using the KCl extraction method following the method of Mclean (1965), the organic matter in the soil sample in the form of carbon was determined using the Walkey-Black wet oxidation method as described by Page (1982), the total nitrogen in the composite soil sample was determined using the macro-Kjeldahl method as described by Page (1982), the phosphorus in the composite soil sample was analysed using the vanado-molybdate method (AOAC, 2012). The calcium and magnesium content of the soil was determined using methods described by Mehlich (1953) and Watanabe and Olsen (1965). The concentration of heavy metals such as cadmium, iron, copper, lead, chromium, zinc, nickel, manganese and cobalt present in the soil sample was determined using the wet digestion procedure (SSSA, 1971). This was carried out by weighing 0.5 g of the 0.5 mm sieved soil into a 100 mL Berzellius beaker, 5 mL HNO_3 and 2 mL $HClO_4$ was added and covered with a watch glass. This was digested in a fume cupboard by heating it to a final volume of 3 to 5 mL. Ten to fifteen millilitres of water was added and the digest solution was filtered through an acid washed filter paper into a 50 mL volumetric flask. It was diluted to volume with deionized water and the filter paper was washed with water. The filtrate was used to determine the concentration of heavy metals present in the sample using Buck Scientific 210/211 VGP Atomic Absorption Spectrophotometer (AAS).

Isolation of microorganisms

The determination of the total viable bacteria count (TVBC) was carried out in triplicates. The agar medium and the diluents used were sterilized at 121^{o}C for 15 minutes. One gram of the thoroughly mixed composite soil samples was suspended in 9 mL of sterile distilled water and serially diluted (Olutiola et al., 2000). One millilitre of appropriate dilutions was inoculated into sterile Petri dishes and already prepared and cooled nutrient agar (Lab M, United Kingdom) was added to it using the pour plate technique as described by Olutiola et al. (2000). Inoculated plates were incubated at 37^{o}C for 24 h after which distinct bacteria colonies were counted. Morphologically distinct bacteria colonies were subcultured by streaking on fresh nutrient agar plates until pure bacteria colonies were obtained. Pure cultures of each bacteria strain were stored on nutrient agar slants at 4 ^{o}C for further studies. Pure bacterial isolates were subjected to various biochemical tests to aid their identification.

Molecular Characterisation of Bacterial Isolates

16S rRNA based identification

Isolation of 16S rRNA gene of the bacterial isolates were carried out using QIAamp DNA Mini Kit (250) cat no 51306 after which the sequences were amplified using Applied Biosystems Thermocycler, model 9800. Sequencing of the 16S rRNA was carried out using a 16-well Applied Biosystems sequencing plate following the manufacturer's instructions. The obtained sequences of bacterial 16S rRNA were analysed using Sequence Scanner (Applied Biosystems) software and the 16S rRNA sequence contigs were generated using Chromas Pro. The online program BLASTn was used to find out the related sequences with known taxonomic information in the databank at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to accurately identify the bacterial strain. The data obtained from the molecular characterization was used in constructing a phylogenic tree for the bacterial isolates and also submitted to DNA Data Bank of Japan (DDBJ) for accession numbers.

Phylogenetic Analyses of bacterial strains

The 16S rRNA gene sequences obtained from the GenBank database of the National Centre for Biotechnology Information (NCBI) were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6 following the method described by Hall (2013) and Tamura et al. (2013). The evolutionary history of the bacterial isolates was inferred using the Neighbor-Joining method as described by Saitou and Nei (1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and in the units of the number of base substitutions per site.

Determination of the susceptibility of bacterial isolates to heavy metal salts

The susceptibility of the bacterial isolates to increasing concentration of chromium, cadmium, lead, copper, cobalt, nickel and zinc was determined quantitatively using the agar diffusion method following the method described by Narasimhulu *et al.* (2010). Concentration of heavy metals in nutrient agar medium was gradually increased from 100-500 µg/mL. The screening was done by streaking a 24 h old culture of the test organism on nutrient agar plate supplemented with 100 µg/mL of the salt of the heavy metals of interest and was incubated for five days. Isolates that grew at this concentration were subcultured to nutrient agar plates supplemented with higher concentration of the heavy metal salts until 500 µg/mL concentration of heavy metal at increasing level of 50µg/mL was attained. Isolates which were observed to have high tolerance to heavy metal

salts were maintained on agar slants and stored at 4 ^{o}C to be used for bioremediation of the contaminated soil samples.

Sterilization of soil sample

Composite soil samples collected from the heavy metal-contaminated steel rolling site were air dried in the sunlight for a day and then sieved using a 0.5 mm nylon mesh sieve. The soil sample was then sequentially sterilized using hot air oven at 105 ^{o}C for one hour, after which it was aseptically packaged by weighing 5 kg into sterile polythene bags for the planting exercise as described by Saeed and Rafique (1980) and Iqbal *et al.* (2011). To check for sterility of the soil sample, the soil sample was subjected to the method used for isolation of microorganisms as described above.

Collection of Corchorus olitorius seeds

For the purpose of this study, seeds of *Corchorus olitorius* already treated with scarification method (in order to break the dormancy) were obtained from Agronomy Department, Faculty of Agriculture, University of Ibadan, Nigeria.

Bioremediation of contaminated soil sample

Five kilogram of already sterilized soil sample were aseptically weighed and mixed with one hundred gram of sterilized cow manure slurry. This was then packed into perforated polythene bags (nursery bags) to be used for the bioremediation and planting exercise. The bioremediation and planting exercises were conducted in a screen house. Working solution for the bioremediation exercise was prepared using a modified method of Ayotamuno *et al.* (2009) by inoculating each of the bacterial isolates into peptone water broth and incubating until a cell density of 7.6×10^{11}cfu/mL was obtained, however, for bioremediation exercise using mixed culture, the working solution was allowed to reach a cell density of 1.5 x 10^{12}cfu/ml as described by Okparanma *et al*. (2009). Twenty millilitres of the working solution of the bacterial isolate was pipetted into each experimental setup. The experiment was laid out in a Completely Randomized Design (CRD) having eight treatments with five replicates making a total of forty pots.

Treatments:

A Sterilized soil treated with *Alcaligenes faecalis* (LC349889.1) and manure

B Sterilized soil treated with *Pseudomonas azotoformans* (LC349894.1) and manure

C Sterilized soil treated with *Bacillus mycoides* (LC349697.1) and manure

AB Sterilized soil treated with consortia of *Alcaligenes faecalis* (LC349889.1) and *Pseudomonas azotoformans* (LC349894.1) and manure

AC Sterilized soil treated with consortia of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1) and manure

BC Sterilized soil treated with consortia of *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) and manure

ABC Sterilized soil treated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) and manure control Sterilized soil alone (without bacterial inoculums and manure).

Planting of *Corchorus olitorius*

Planting of *C. Olitorius* was done immediately after introducing the bacterial isolates. The pots were watered twice daily (100 mL/time) for the first two weeks of planting and later reduced to once daily so as to prevent the leaching of nutrient from the treatments. This was done till the experiment was terminated seven weeks after planting (WAP). Plant height was measured from the rhizoplane to the apical tip of the plant in centimetres starting from 2WAP till the seventh week when the experiment was terminated, stem diameter measured using a vernier caliper below the first nodes of the plant and number of leaves obtained by counting the number of leaves per plant manually Elings (2000).

Analysis of harvested C. olitorius

At the end of the experiment, the plants were harvested by uprooting the plantings and pooled together per treatment group. The roots were washed to remove attached soil debris and then subjected to various analyses. The wet and dry weight, moisture content, dry matter, % crude protein, % ash, % ether extract (fat), % crude fibre were determined using standard analytical methods as described by AOAC (2012). The heavy metal content of the plant samples was determined following the method described by SSSA (1971) using Buck Scientific 210/211 VGP Atomic Absorption Spectrophotometer (AAS).

Analysis of the bioremediated soil samples

Upon termination of the experiment and harvest of the plants, the five soil replicates in each treatment group were pooled together and mixed well in order to obtain a composite sample which was analysed for pH, nitrogen, organic carbon, exchangeable acidity, available phosphorus, calcium, potassium, sodium, magnesium, manganese, iron, copper, zinc, lead, cadmium, chromium, cobalt and nickel content following the methods described earlier.

Data Analysis

Data obtained were analysed and reported as mean ± standard deviation of five measurements and analysed using univariate analysis of variance and Duncan Post Hoc test to determine significant differences ($p \geq 0.05$) between treatments using Statistical Package for Social Science Research version 17 (SPSS).

Results

The collected soil samples were dark brown in colour with a characteristic choking odour which is peculiar to heavy metal contaminated sites. Table 1 shows the result of the initial *in situ* analysis carried out to determine the physical, chemical and heavy metals properties of the composite soil samples collected during the rainy and dry season. It was observed that the soil from the study site had higher physical, chemical and heavy metal content during the dry season than the rainy season. For instance, heavy metals such as cadmium, lead, cobalt, nickel and chromium had concentrations of 3.0, 2333.6, 13.7, 40.6 and 1678.7 mg/kg, respectively during the dry season and concentrations of 0.5, 1505.5, 10.5, 31.5 and 1526.0 mg/kg, respectively during the rainy season.

Table 1: Physical and chemical properties of the heavy metals contaminated soil sample during dry and rainy seasons

Parameters	Dry season	Rainy season
Ph	6.5	7.0
Exchangeable Acidity(meq/100g)	0.4	0.4
Mineral content		
Total organic carbon (TOC) (g/kg)	42.4	40.8
Total Nitrogen (g/kg)	4.0	3.9
Available Phosphorus(mg/kg)	14.0	13.3
Ca (cmol/kg)	2.6	2.7
K (cmol/kg)	0.3	0.4
Na (cmol/kg)	0.5	0.3
Mg (cmol/kg)	0.5	0.4
Heavy metals (mg/kg)		
Mn	99.4	98.5
Fe	24.8	23.8
Cu	2.8	1.0
Zn	2.6	1.4
Cd	3.0	0.5
Pb	2333.6	1505.5
Co	13.7	10.5
Ni	40.6	31.5
Cr	1678.7	1526.0

Thirty-six bacterial isolates were obtained from the contaminated soil sample and they were distributed as follows: *Pseudomonas* sp (52.77%), Proteus *mirabilis* (13.89%), *Alcaligenes faecalis* (13.89%), *Enterobacter* sp (8.33%), *Providencia* sp (5.56%) and *Bacillus* sp (5.56%).

However only three of these isolatesviz: *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) exhibited high tolerance to heavy metal salts, therefore the three bacterial isolates were selected for bioremediation exercise. Figures 1-3 shows the phylogenetic tree constructed for each of the isolates based on the molecular data obtained when they were identified using molecular characterization (isolates appear in bold format).

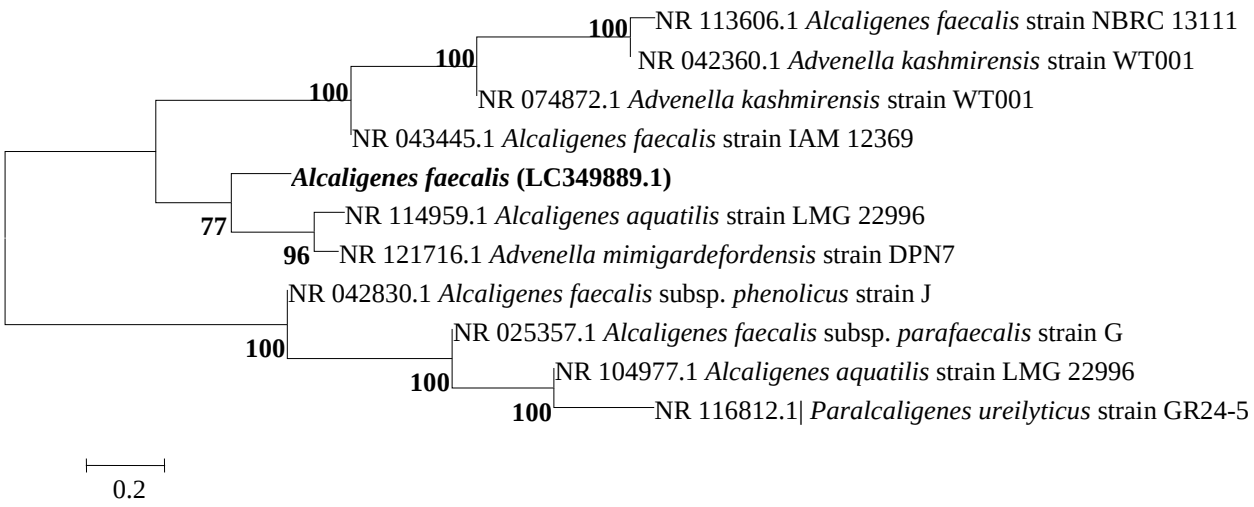

Figure 1: Evolutionary relationship of *Alcaligenes faecalis* (LC349889.1)

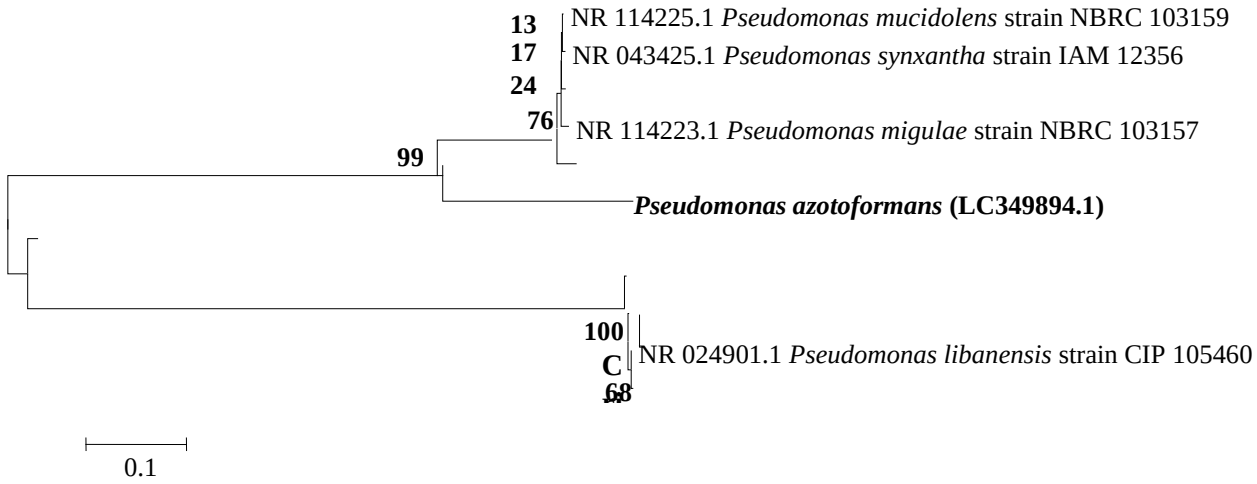

Figure 2: Evolutionary relationship of *Pseudomonas azotoformans*(LC349894.1)

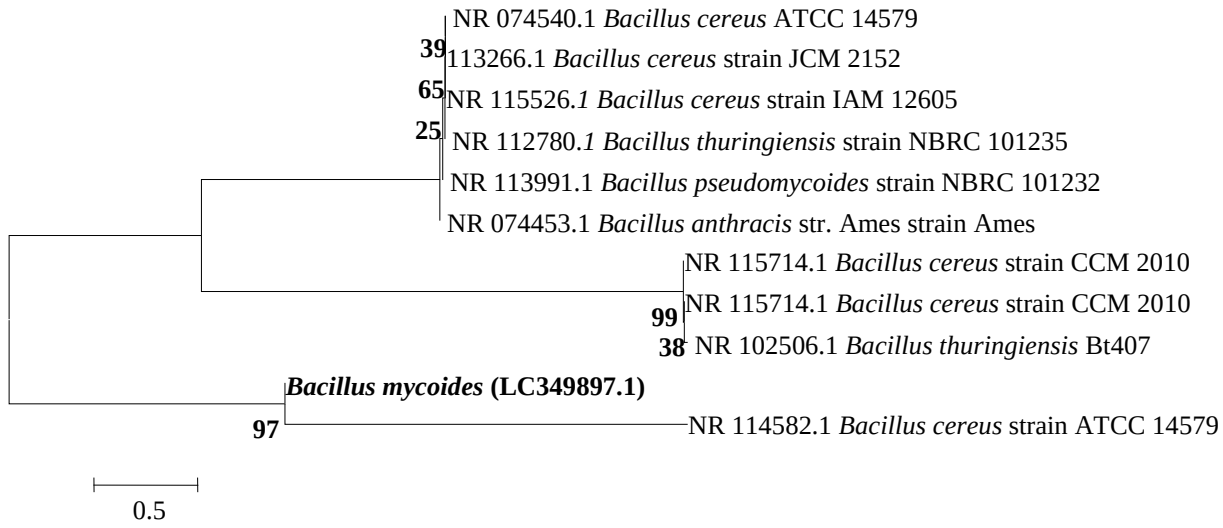

Figure 3: Evolutionary relationship of *Bacillus mycoides* (LC349897.1)

The result obtained for the monitored agronomic parameters is as shown in Figures 4-6. There was significant difference (at p≥0.05) in the plant height of the treatments starting from 2WAP to 7WAP when the experiment was terminated as shown by Figure 4. It was observed that the plant response in the various treatment groups varied in their performance. It was observed that *C. Olitorius* harvested from soil bioremediated with a mixed culture of *Pseudomonas azotoformans* (LC349894.1)and *Bacillus mycoides* (LC349897.1) had the least height of 2.76±0.15 cm at 2 WAP, however at 6 WAP, the highest height was observed in *C. Olitorius* harvested from soil bioremediated with a consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) with a height of 7.84±0.69 cm, while the least height was observed in *C. olitorius* harvested from soil bioremediated with *Pseudomonas azotoformans* (LC349894.1) having a height of 4.68±0.41 cm, and this trend was maintained till the experiment was terminated. The control had the highest height of 4.10±0.55 cm as at 2WAP and at 7WAP it had a height of 5.86±0.57 cm.

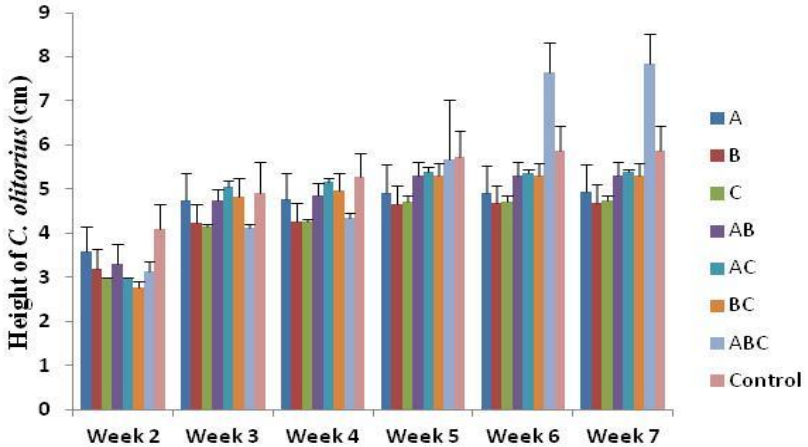

Fig 4: Height of *C. olitorius*

Keys: A- *C. Olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B- *C. Olitorius* harvested from sterilized soil bioremediated with *Pseudomonas*

azotoformans(LC349894.1), C- *C. Olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. Olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. Azotoformans* (LC349894.1), AC- *C. Olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. Olitorius* harvested from sterilized soil bioremediated with mixed culture of *P. Azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. Olitorius* harvested from sterilized soil bioremediated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. Olitorius* harvested from sterilized soil alone.

The results obtained for number of leaves/plant revealed that there was significant difference (at $p \leq 0.05$) in the number of leaves per plant among the treatments starting from 2WAP as shown by Figure 5. *C. Olitorius* harvested from soil bioremediated with *Alcaligenes faecalis* (LC349889.1) had the highest number of leaves/plant from 2WAP till 4WAP having an average of 4.00±0.00 and 5.20±0.45, respectively but by 5WAP till the 7WAP when the experiment was terminated, *C. Olitorius* harvested from soil bioremediated with a consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) had the highest number of leaves/plant with an average of 5.80±0.45.

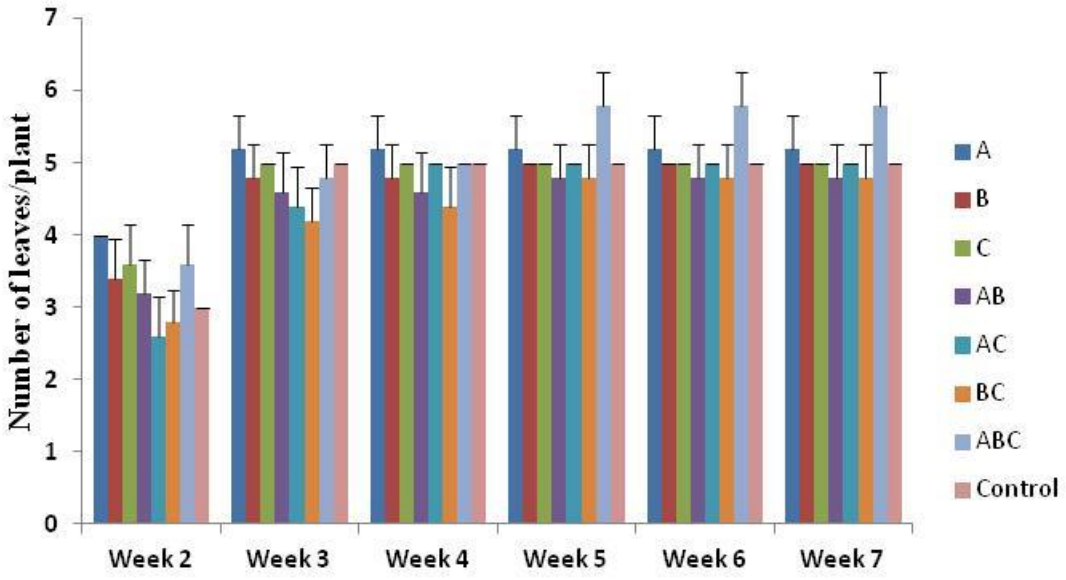

Fig 5: Number of leaves/*C. olitorius*

Keys: A- *C. olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B- *C. olitorius* harvested from sterilized soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), C- *C. olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. azotoformans* (LC349894.1), AC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *P. azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. olitorius* harvested from sterilized soil bioremediated with

consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. olitorius* harvested from sterilized soil alone.
There was significant difference at $p \leq 0.05$ in the plant stem diameter of harvested *C. Olitorius* starting from 2WAP till 7WAP except at 3WAP as shown in Figure 6. By 7WAP, the highest stem diameter was observed in *C. Olitorius* harvested from soil bioremediated with a consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) which had an average of 1.35±0.00 mm while the smallest stem diameter was observed in *C. Olitorius* harvested from soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Pseudomonas azotoformans* (LC349894.1), mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1) and mixed culture of *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) with each having an average of 1.20±0.00 mm each.

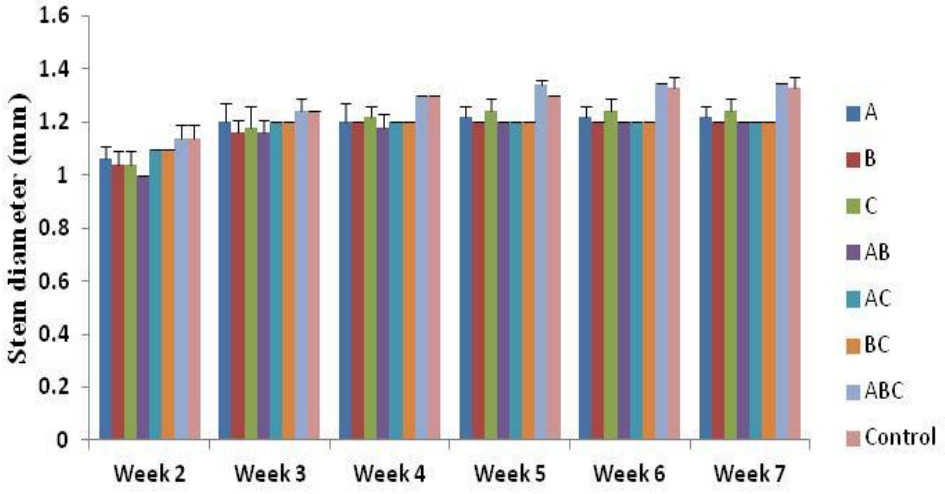

Fig 6: Stem diameter/ *C. olitorius*

Keys: A- *C. olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B- *C. olitorius* harvested from sterilized soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), C- *C. olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. azotoformans* (LC349894.1), AC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *P.azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. olitorius* harvested from sterilized soil bioremediated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. olitorius* harvested from sterilized soil alone.

Tables 2 and 3 shows the result obtained for the post-bioremediation and harvest analysis harvested of proximate matter and heavy metals content of *C. olitorius* respectively. It was observed that there were significant differences in the responses of the various treatment groups.

Table 2: Proximate matter of harvested *C. olitorius*

Treatment	Proximate matter							
	Fresh weight (g)	Dry weight (g)	% Moisture	Dry matter	% Crude protein	% Ash	% Ether extract (fat)	% Crude fibre
A	0.22±0.05^{c}	0.10±0.01^{f}	55.00±7.07^{g}	45.00±7.07^{h}	12.24±1.90^{i,j}	19.06±1.47^{o}	1.40±0.15^{s}	22.62±0.55^{t}
B	0.26±0.03^{c}	0.10±0.03^{f}	61.90±6.79^{g}	38.10±6.79^{h}	7.92±1.17^{jkl}	20.08±0.11^{no}	1.66±00.22^{rs}	19.78±0.53^{vw}
C	0.36±0.09^{c}	0.14±0.04^{f}	62.00±0.14^{g}	38.00±0.14^{h}	15.82±1.90^{i}	14.97±0.69^{p}	1.93±0.25^{qr}	18.72±0.25^{w}
AB	0.33±0.13^{c}	0.15±0.08^{f}	56.25±8.84^{g}	43.75±8.84^{h}	10.39±3.18^{j,k}	17.88±1.62^{o}	1.91±0.15^{qr}	19.62±0.54^{vw}
AC	0.38±0.05^{c}	0.14±0.01^{f}	61.10±3.25^{g}	38.90±3.25^{h}	11.03±2.08^{j}	18.16±1.48^{o}	1.35±0.22^{s}	18.95±0.96^{vw}
BC	0.29±0.07^{c}	0.13±0.04^{f}	51.95±26.36^{g}	48.05±26.38^{h}	6.58±0.03^{k,l}	21.65±0.78^{n}	2.00±0.05^{qr}	19.88±0.47^{vw}
ABC	2.54±0.57^{a}	0.99±0.17^{d}	60.75±2.05^{g}	39.25±2.05^{h}	4.44±1.33^{l}	24.57±0.62^{m}	2.00±0.20^{q,r}	21.72±0.99^{tu}
Control	1.10±0.13^{b}	0.63±0.24^{e}	43.65±15.34^{g}	56.35±15.34^{h}	5.57±0.80^{l}	22.56±0.63^{m} ^{n}	2.11±0.06^{q}	20.61±0.78^{uv}

******values with the same letters on each column are not significantly different from each other at p≤0.05***

Keys: A- *C. olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B-*C. olitorius* harvested from sterilized soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), C- *C. olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. azotoformans* (LC349894.1), AC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *P.azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. olitorius* harvested from sterilized soil bioremediated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. olitorius* harvested from sterilized soil alone.

Table 3: Heavy metal content of harvested *C. Olitorius*

Treatment	Heavy metals							
	Iron	Copper	Zinc	Cadmium	Lead	Cobalt	Chromium	Nickel
A	25.35±0.49 ^{c}	36.05±2.05^{d}	17.45±1.34^{g}	35.55±8.56^{i}	37.70±0.85^{l}	30.50±1.56^{n}	27.00±0.71^{p}	29.00±13.29^{s}
B	26.95±0.64^{bc}	31.70±2.26^{de}	16.50±0.85^{g}	36.45±4.60^{i}	37.65±5.02^{l}	27.70±1.41^{n}	20.75±1.48^{q}	29.00±14.57^{s}
C	28.40±1.70^{b}	29.15±1.34^{de}	17.00±0.28^{g}	32.95±6.43^{i}	34.55±2.47^{l}	28..35±6.86^{n}	24.80±3.82^{pq}	26.70±8.63^{s,t}
AB	24.95±1.77^{c}	30.05±3.60^{de}	16.10±0.85^{g}	34.85±8.13^{i}	36.30±4.81^{l}	29.35±1.06^{n}	24.40±2.97^{pq}	29.35±11.52^{s}
AC	28.35±1.77^{b}	30.55±6.01^{de}	15.90±0.71^{g}	27.85±11.67^{i,j}	32.50±2.55^{l}	28.55±1.48^{n}	26.40±2.40^{p}	27.70±9.76^{s,t}
BC	28.45±049^{b}	31.00±3.68^{de}	16.10±0.85^{g}	12.00±12.02^{jk}	31.85±1.77^{l}	26.80±0.99^{n}	26.50±2.55^{p}	31.25±11.10^{s}
ABC	37.95±1.20 ^{a}	20.20±4.95^{f}	4.25±0.21^{h}	4.40±0.28^{k}	10.90±0.85^{m}	6.30±0.71^{o}	4.15±0.64^{r}	3.20±0.85^{t}
Control	38.10±0.85^{a}	25.80±0.85^{ef}	5.95±0.78^{h}	4.80±0.71^{k}	9.15±0.64^{m}	5.90±0.71^{o}	4.00±0.14^{r}	3.25±0.63^{t}

******values with the same letters on each column are not significantly different from each other at p≤0.05***

Keys: A- *C. olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B- *C. olitorius* harvested from sterilized soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), C- *C. olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. azotoformans* (LC349894.1), AC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *P.azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. olitorius* harvested from sterilized soil bioremediated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. olitorius* harvested from sterilized soil alone.

Table 4 shows the result obtained for heavy metal content of the soil after bioremediation and harvesting of *C. Olitorius* have been conducted. It was observed that the concentration of heavy metals present in the soil was significantly reduced compared to the concentrations observed in the contaminated soil during rainy and dry seasons.

Table 4: Physical and chemical properties of bioremediated soil after harvesting *C. olitorius*

Parameters	A	B	C	AB	AC	BC	ABC	CONTROL
Ph	8.50	8.41	8.52	8.49	8.50	8.41	8.51	8.44
Exchangeable Acidity (meq/100g)	0.40	0.30	0.50	0.40	0.50	0.30	0.40	0.40
Mineral content								
T.O.C (g/kg)	63.52	59.98	65.11	60.74	61.93	67.89	67.09	63.92
T/N (g/kg)	6.57	6.20	6.74	6.28	6.41	7.02	6.94	6.61
Available Phosphorus (mg/kg)	42.87	33.20	66.48	39.30	39.78	31.46	25.70	49.67
Ca (Cmol/kg)	86.20	105.66	128.11	107.41	99.80	125.62	94.84	101.42
K (Cmol/kg)	2.75	2.78	14.32	2.06	2.42	1.99	0.97	6.04
Na (Cmol/kg)	8.04	8.70	12.39	10.22	9.13	8.91	9.35	8.91
Mg (Cmol/kg)	0.53	1.17	1.08	1.17	0.86	1.23	0.90	1.19
Heavy metals (mg/kg)								
Mn (mg/kg)	450.0	515.0	738.0	867.0	581.0	756.0	558.0	910.0
Fe (mg/kg)	17.4	13.5	17.2	15.2	10.2	12.1	14.1	23.5
Cu (mg/kg)	4.12	1.61	2.04	0.97	1.07	2.01	1.51	5.84
Zn (mg/kg)	3.84	1.04	1.68	1.12	0.93	1.68	0.99	4.92
Cd (mg/kg)	2.2	2.6	2.6	2.2	1.8	2.1	2.0	4.8
Pb (mg/kg)	21.3	20.8	40.5	24.3	21.1	20.8	22.6	1360.56
Co (mg/kg)	1.04	1.51	1.68	1.86	1.04	1.33	1.75	8.72
Ni (mg/kg)	2.23	1.06	0.93	1.22	0.86	1.04	1.43	1.21
Cr (mg/kg)	1.96	1.81	3.36	3.04	2.17	2.23	3.06	1240.87

Keys: A- *C. olitorius* harvested from sterilized soil bioremediated with *Alcaligenes faecalis* (LC349889.1), B- *C. olitorius* harvested from sterilized soil bioremediated with *Pseudomonas azotoformans* (LC349894.1), C- *C. olitorius* harvested from sterilized soil bioremediated with *Bacillus mycoides* (LC349897.1), AB- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *P. azotoformans* (LC349894.1), AC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), BC- *C. olitorius* harvested from sterilized soil bioremediated with mixed culture of *P.azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), ABC- *C. olitorius* harvested from sterilized soil bioremediated with consortia of *Alcaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), Control- *C. olitorius* harvested from sterilized soil alone.

Discussion

The area surrounding the study site was heavily contaminated with heavy metals as a result of the release of effluent by a steel rolling industry in the area into the surrounding soil. It was observed that the soil could not support plant growth and some plants which could adapt to the heavy metal in the environment were either drying up or had a yellow colour; all these deviations from normal

physical characteristics of a healthy soil and plant indicated a high degree of pollution of the study area by heavy metals. This is in agreement with the report of Revathi *et al.* (2011), who reported that plant growth is not sustained in soils heavily polluted with heavy metals. The observed deviations in the physical characteristics of the soil samples were in agreement with changes observed in a soil contaminated by acid mine drainage water as reported by Bitala *et al.* (2009).

Analysis of the contaminated soil sample done before treatment revealed that the concentration of the heavy metals were higher during the dry season than during the wet or rainy season. This can be attributed to the effect of leaching which is more evident during the wet or rainy season compared to the dry season and also the rates of deposition of suspended particles are generally higher during the dry season compared to the wet or rainy season. This is in agreement with the findings of Kilicel (1999).

Concentrations of some heavy metals in the contaminated soil were found to exceed the concentrations recommended by WHO and USEPA. For instance, heavy metals such as cadmium, lead, chromium and cobalt had concentrations of 0.50-2.98 mg/kg, 1505.50-2333.55 mg/kg, 1526.00-1678.67 mg/kg and 10.50-13.65 mg/kg, respectively which exceeded the recommended concentrations of 0.003, 10, 2, and 8 mg/kg by WHO and USEPA for each of the respective heavy metals (Parizanganeh *et al*., 2012; Ezejiofor *et al*., 2013); whereas minerals such as calcium, potassium, magnesium and sodium had a concentration of 2.56-2.68, 0.34-0.36, 0.41-0.50 and 0.30-0.50 cmol/kg respectively which were below the recommended values of 10-20, 0.6-1.2, 3-8 and 0.7-1.2 cmol/kg (Parizanganeh *et al*., 2012; Ezejiofor *et al*., 2013). These minerals are essential to plant growth and development, a reduction in their bioavailability often leads to reduced plant growth. A major reason for the reduction in bioavailability of these important minerals could be attributed to the high contamination of the soil with heavy metals. According to Chibuike and Obiora (2014), the presence of heavy metals in a soil may affect the availability of other element especially the minerals in the soil.

The result of the molecular characterization of the isolates used in this study revealed high similarity between the isolated organisms and other related organisms in their genus as revealed by the evolutionary tree. The pH of the treated soil obtained in this study ranged between 8.41 and 8.52, this was suitable for the growth and yield of *C. olitorius*. According to Facciola (1990), *C. Olitorius* is able to grow well in acid, neutral and basic (alkaline) soils; it tolerates soil pH of 4.5 to 8.0. However, extreme pH conditions have the tendency to reduce the availability of iron in the soil which can cause yellowing between leaf veins (Palada and Chang, 2003).

A mean plant height of 7.84 cm was observed in the group treated with the consortia of was *Alkaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1), at 7 WAP, this height low compared to that obtained by Ogunrinde and Fasimirin (2011) in which they obtained a mean height of 105.03 cm at 7WAP for *C. Olitorius* planted in an uncontaminated soil. The stunted growth of plants observed in this study may be as a result of the effect of heavy metals contamination on the soil as indicated by Kabir *et al.* (2009). Adenipekun *et al.* (2013) reported similar observation in a study in which they observed a height range of 4.083-11.183 cm in *C. Olitorius* grown on a soil contaminated with oil and remediated using *Pleurotus pulmonarius* at 5WAP.

A mean of 5.80 was obtained for the for number of leaves at 7 WAP in the group treated with the consortia of *Alkaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and

Bacillus mycoides (LC349897.1), this is similar to that obtained by Adenipekun *et al.* (2013), in which they observed a range of 4.500-10.666 number of leaves in *C. Olitorius* grown on a *Pleurotus pulmonarius* remediated soil contaminated with oil at 5WAP.

The biggest stem diameter observed among the different treatment groups in this study was found in the group treated with the consortia of *Alkaligenes faecalis* (LC349889.1), *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) having a mean diameter of 1.35 mm. This is comparable to that obtained by Adenipekun *et al.* (2013), in which they observed a range of 0.128-1.05 mm for stem diameter of *C. Olitorius* grown on a *Pleurotus pulmonarius* remediated soil contaminated with oil at 5WAP.

Analysis of the harvested *C. olitorius* plants revealed that for proximate matter such has % crude fibre, ether extract (fat) and fresh weight most of the treatment groups performed well having a range of 18.72-22.62; 1.35-2.00 and 0.22-2.54 respectively compared to values of 20.30; 0.12 and 1.11 respectively obtained in some previous studies (Adenipekun *et al.*, 2013, Ndlovu and Afolayan, 2008; Yekeen *et al.,* 2013). Other proximate matters such as % ash, % crude protein and % moisture had a range of 14.97-24.57; 4.44-15.82 and 43.65-62.00 respectively and these were low compared to values of 21-21.40; 21.12 and 84.28 respectively obtained by Onwordi *et al.* (2009) and Acho *et al.* (2014). High ash content in food is a measure of high deposit of mineral contents (Akpabio *et al*., 2012). The value obtained in this study suggests that the *C. Olitorius* harvested was moderately rich in mineral elements. One major reason why the harvested *C. Olitorius* is not high in fibre content is because the harvested plants were observed to absorb heavy metals and this could have affected its mineral content. Dietary proteins are important for natural synthesis and maintenance of body tissues, enzymes and hormones as well as other substances required for healthy functioning of the body system (Hayat *et al*., 2014). The protein value obtained from this study 4.44±1.33 to 15.82±1.90 suggests that *C. olitorius* can effectively contribute to the daily protein needed. Even though the value is low compared to protein values from other reports, Gqaza *et al.* (2013) however said that any plant food that provides more than 12 % of its caloric value from protein is considered a good source of protein. The moisture content obtained for *C. Olitorius* in this study though low when compared to that obtained by some previous authors, shows that the harvested *C. olitorius* can be easily susceptible to spoilage by micro-organisms during storage (George, 2003).

The analysis of the harvested *C. Olitorius* plant revealed the presence of heavy metals in the leaves; this is an indication that there was an uptake of these heavy metals by the roots of *C. olitorius* and this was followed by its translocation which is in agreement with Peralta-Videa *et al.* (2002). According to earlier studies by Kashem and Singh (2004) and Rieuwerts *et al.* (2006), it was reported that at pH ranges between 4.0-8.5, metal cations are mobile while anions tend to transform to oxide minerals, thus increasing their concentration in the environment, the pH of the treated soil samples were found to be between 8.41 and 8.52, this could be one of the reasons the heavy metals were easily absorbed and translocated in the plant. According to Muhammad *et al.* (2008), leafy vegetables grown in heavy metal contaminated soils, accumulate higher amounts of metals than those grown in uncontaminated soils. This is because they are capable of absorbing these metals through their roots. As earlier reported by Akan *et al.* (2009), vegetables accumulate heavy metals in their edible and non-edible parts as can be seen in the accumulation of heavy metals in the leaves and stems of *C. Olitorius* in this study. The analysis of the harvested *C. Olitorius* plant revealed that the content of iron (Fe) and zinc (Zn) in the harvested plants was low having a range of 24.95-

38.10 and 4.25-17.45 mg/kg respectively compared to the recommended standard of 60 mg/kg (Sanyaolu *et al*., 2011; Ayejuyo *et al*., 2014).

The post bioremediation and post-harvest analysis of the treated soils revealed an increase in the pH, total organic carbon, total nitrogen and available phosphorus of the treated soils. The pH and available phosphorus observed in the treated soil in this study were higher than the pH of 5.1-6.5 and available phosphorus of 20 mg/kg observed in studies by Brady and Weil (2008) and Holland *et al.* (1981) respectively. An increase was observed in the concentration of minerals such as calcium, potassium and sodium of the treated soils. The isolates used in this study were able to achieve high reduction in heavy metal concentration in the soil. For instance, there was 57.99% reduction in the concentration of iron in the group treated with a mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* (LC349897.1), groups treated with *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) and a mixed culture of *Pseudomonas azotoformans* (LC349894.1) and *Bacillus mycoides* (LC349897.1) had a 98.92% reduction on the concentration of lead, groups treated with *Alcaligenes faecalis* (LC349889.1) and a mixed culture of *Alcaligenes faecalis* (LC349889.1) and *Bacillus mycoides* had a 91.39% reduction on the concentration of cobalt, group treated with *Bacillus mycoides* (LC349897.1) had a 97.42% reduction on the concentration of nickel while the group treated with *Pseudomonas azotoformans* (LC349894.1) had a reduction of 99.89% on the concentration of chromium. This is comparable to results from earlier studies. For instance, Chang *et al.* (1997) reported a *Pseudomonas aeruginosa* PU21 (Rip64) strain with a metal uptake efficiency of 80% within 2 days while Roane *et al.* (2001) reported a *Bacillus* strain H9 with a metal uptake efficiency of 36% within 48 hours. Magyarosy *et al.* (2002) also reported a *Pseudomonas* spp with a metal uptake efficiency of 98% within 4 days. Though this isolates did not work as rapid as those earlier cited, this can be attributed to the fact that soils contaminated with heavy metals are poor in nutrients and bacterial diversity which results in impeded rates of remediation (White *et al.*, 2006).

Conclusion

This study has shown that stimulation of bacterial isolates with organic amendments such as cow manure slurry/gomeya has great potentials in bioremediation. It has also been observed that the use of bacterial consortia rather than individual microorganism works more effectively in bioremediation of heavy metals.

Conflict of Interest

The authors declare that there is no conflict of interest.

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