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ORIGINAL ARTICLE

Blending *Bacillus* sp., *Lysinibacillus* sp. and *Rhodococcus* sp. for optimal reduction of heavy metals in leachate contaminated soil

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Abstract The present work was designed to evaluate the bioaugmentation potential of directly isolated bacterial species on heavy metals deposited due to leachate pollution of soil. Tailor-made microbial formula used as bioaugmentation agents consisted of nine bacterial species of different classes: γ-proteobacteria (4), α-proteobacteria (1), bacilli (2) and actinobacteria (2). Calculated removal efficiency of the potential bioaugmentation agents was 86, 73 and 71 % for Cu, Zn and Pb, respectively, when only three of the bacterial species (*Bacillus thuringiensis*, *Lysinibacillus sphaericus* and *Rhodococcus wratislaviensis*) were used. The data are suitable for assessing the synergistic effect of microbes on the reduction of extractable heavy metals in contaminated soil, and optimizing their energy flux.

Keywords Leachate pollution · Soil remediation · Bioremediation · Metal reduction

Introduction

Inadequate strategies in waste disposal have left the contemporary researchers with the task of identifying ways to tackle the persistent accumulation of dangerous recalcitrant compounds in the soil. One of such options that are adopted to handle the aforementioned is bioremediation. However, any remediation of soil in a sustainable method involves a technical approach that will not only remove pollutants but have to preserve soil quality and as such its function (Sprocati et al. 2012). Although bioremediation is primarily the use of degrader microbes, but with the degree at which very toxic contaminants accumulate in the environment, it is clear that microorganisms that exist in polluted sites do not basically have the ability to deal with the incessant pollution (Whiteley and Lee 2006) without being engineered or enhanced. Therefore, the nascent study tries to identify the bioremediation potential on polluted soil that will be transformed into effective bioremediation option suitable for tackling some difficulties often encountered in previous bioremediation applications. Among these, the heavy metal contamination of soil which results from different anthropogenic activities that include metal mining, landfilling and other industrial activities has posed a serious environmental concern because the activities of degrader bacteria are hampered by increased concentration of the contaminant.

Leaked leachate is a source of soil, groundwater and occasionally surface water pollution that may persist for many decades (Kennedy and Everett 2001). Raw leachate from landfill is heterogeneous in nature because it does not only contain heavy metals but monocyclic aromatic hydrocarbons, xenobiotic organics and polychlorinated biphenyls, as well (Emenike et al. 2012). Humans and the environment are exposed to significant risk of high metal toxicity, and the potential infiltration of the food chain (Boyd 2010; Ma et al. 2011). Therefore, considering the unavoidable generation of leachate and its potential impacts on the environment, the remedial and recovery options tend to be the significant task of most environmental studies. However, with economic balance and eco-protectionism in mind, every contemporary remedial research is not only weighed on the scale of cost, but also on the after-use impacts on the environment. The condition

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for microbial degradation tends to be jeopardized upon the introduction of high metal concentrations into the environment, and as such, scientists and engineers tend to be saddled with the onerous task of developing removal or immobilization methods for metal contaminated soil (Plociniczak et al. 2013).

Bioaugmentation of contaminated soil with desired microbial strain is one of the methods adopted for tackling heavy metal menace in the environment. Behavioral pattern of microbes in the presence of pollutants tends to vary. This depends on the state of the pollutant; some pollution occurs from single, group or heterogeneous contaminants. These make degrading microbes to respond at varying capabilities. This is the reason why bioaugmentation is influenced by many factors but significantly strains selection, concentration and methods of inoculation and inoculums heterogeneity (Vogel 1996; Sprocati et al. 2012). Survival of inoculants is very important in soil bioaugmentation.

Establishing a suitable ecological background for bioaugmentation is often a barrier to successful performance of bioremediation. Some authors had applied tailor-made consortia which link functionally the in situ microbial community structure with main active pollutants as they meet the ecological criteria (Gentry et al. 2004; Watanabe et al. 2002; Da Silva and Alvarez 2004; Alisi et al. 2009; Sprocati et al. 2012). But this present work deals with inoculation of directly isolated microbes as a way of ensuring concord with ecological background of the microbial community with the pollutants of interest. Therefore, this study was designed to evaluate the bioaugmentation potential of directly isolated bacterial species on heavy metals deposited due to leachate pollution of soil. Essentially, the adopted bioaugmentation technology was based on laboratory trials on the intact soil cores microcosms under ASTM standard guide so as to obtain proximal conditions typical of field situation.

Materials and methods

Soil samples collection and leachate characterization

The soil samples used in this study were collected in July and August 2012, from closed non-sanitary landfill (contaminated; 3°13.78′N, 101°39.72″E) and University of Malaya (non-contaminated; 3°7′24.15′N, 101°39′16.79″E), respectively, in Kuala Lumpur, Malaysia. Soil from the landfill was already soaked with leachate due to leakage from the waste cells, and was taken to laboratory in order to isolate the resident microbes. Intact soil cores from both sources were scooped (0–20 cm from surface) into sterile soil bags and immediately transferred to the laboratory in accordance with ASTM guidelines E-1197 (2004), "standard guide for conducting a terrestrial soil-core microcosm test." Portion of soil from the non-contaminated soil was air-dried and used for heavy metal analyses. Raw leachate samples were also collected and characterized according to APHA (1998) and USEPA (2000) standards.

Microbial isolation and identification

One gram of the landfill soil was mixed with saline water (0.9 % NaCl) and the suspension vortexed for 2 h at 150 rpm using Lab-Line 3521 orbit shaker (LabLine Instruments, Inc, Maharashtra, India). Serial dilutions were plated (Kauppi et al. 2011) on nutrient agar (NA) and subsequently incubated for 48 h at 33 °C. Single colonies were re-streaked separately on freshly prepared NA to obtain individual pure culture suitable for identification.

Isolated bacteria were identified using the Biolog GEN III MicroPlate protocol. The GEN III MicroPlate™ test panel provided a standardized micromethod using 94 biochemical tests (Bochner 1989a, b). The cells were freshly regrown (16–24 h) in order to avoid the loss of viability and metabolic vigor which is typical of most organisms at stationary phase. Using inoculation fluid (IF), inoculums of each target cell were prepared with Protocols A (IF-A catalog no. 72401) and B (IF-B catalog no. 72403) at turbidity range of 95–98 % T.

An 8-channel automated pipettor was used to dispense 100 µL of the suspension into each of the wells in the MicroPlate (Catalog no. 1030). The wells (Table 1) contain 71 carbon source utilization assays (columns 1–9) and 23 chemical sensitivity assays (columns 10–12); hence, they can be identified at the species levels based on the "Phenotypic Fingerprint" of the microorganisms provided by the test panel. These MicroPlates were placed in Omnilog reader, where they were read using Biolog's Microbial Identification Systems software. Identified microbes were recorded.

Microbial formulation

Microbial formula used in the bioaugmentation experiment comprised of nine strains isolated from the landfill soil. Each strain was grown as a pure culture in NA plates at 33 °C for 2 days before being inoculated in nutrient broth E and grown to stationary phase in a rotating shaker at 29 °C and 150 rpm. Individual suspensions at the same physiological phase (1.3 ABS at 600 nm) were then pooled in equal proportions to set up inoculums for bioaugmentation.

Table 1 Layout of assays for MicroPlate (GEN III)

A1 Negative control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Celloblose	A6 Gentiblose	A7 Sucrose	A8 D-Turanose	A9 Staychose	A10 Positive control	A11 pH 6	A12 pH 5
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
D-Rafflinose	α-D-Lactose	D-Melibiose	β-Methyl-D-Glucoside	D-Salicin	*N*-Acetyl-D-Glucosamine	*N*-Acetyl-β-Mannosamine	*N*-Acetyl-D-Galactosamine	*N*-Acetyl Neuraminic acid	1 % NaCl	4 % NaCl	6 % NaCl
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1 % Sodium lactate	Fusidic acid	D-Serine
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
D-Sorbitol	D-Mannitol	D-Arabitol	Myo-inositol	Glycerol	D-Glucose-6-PO_4	D-Fructose-6-PO_4	D-Aspartic acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Gelatin	Glycl-L-Proline	L-Alanine	L-Arginine	L-Aspartic acid	L-Glutamic acid	L-Histidine	L-Pyroglutamic acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Pectin	D-Galacturonic acid	L-Galactonic acid lactone	D-Gluconic acid	D-Glucuronic acid	Glucuronamide	Mucic acid	Quinic acid	D-Saccharic acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
p-Hydroxy-Phenlyacetic Acid	Methyl Pyruvate	D-Lactic acid methyl ester	L-Lactic acid	Citric acid	α-Keto-Glutaric acid	D-Malic acid	L-Malic acid	Bromo-Succinic acid	Nalidixic acid	Lithium Chloride	Potassium tellurite
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Tween 40	γ-Amino-Butyric acid	α-Hydroxy-Butyric acid	β-Hydroxy-D,L-Butyric acid	α-Keto-Butyric acid	Acetoacetic acid	Propionic acid	Acetic acid	Formic acid	Aztreonam	Sodium butyrate	Sodium bromate

Experimental design

The contamination of soil (from University Malaya) was done according to the ASTM guidelines: characterized leachate was evenly dispersed into each microcosm to attain 10 % v w^{-1} concentrations. Three microcosms in triplicates were set up under different conditions: LSA, contaminated soil amended with all the nine isolated bacteria strains; LSB, soil contaminated and amended with three strains; and LSC, contaminated soil without any amendment. The microbial formula was introduced into the designated microcosm's soils on the third day after contamination of soil with leachate (Sprocati et al. 2012) to mark the start of the bioremediation experiment.

Microcosms LSA and LSB were watered with 100 mL of the inoculums (obtained from equal volumes of pooled discrete species) each containing about 3×10^9 CFU g^{-1}. Soil moisture content was maintained by regular watering with distilled water. Caution was applied to prevent excess watering to avoid leaching that will cause loss of metals contents. Portions of the soil microcosms were used every 20 days (until 100 days) for onward metal analysis and assessment of total count of microbial population. Duration (100 days) reported in the study was to capture the most active period of the microbes before addition of inocula if continued remediation is required.

Chemical analyses

To 0.5 g of experimental soil samples, HNO_3 and H_2O_2 were added (Hseu et al. 2002) before using Multiwave 3000 microwave digester (Perkin-Elmer/Anton Paar) for sample digestion. The elemental concentrations were measured using Optima 5300 DV (Perkin-Elmer, Massachusetts, USA). Evaluation of a procedure blank was always carried out. Every labware utilized in the experiment was soaked with diluted nitric acid overnight before being rinsed in double deionised water and experiments were duly replicated.

Determining reduction of extractable heavy metal

Concentrations of the heavy metals in the discrete microcosms were recorded after analyses at 20 days interval for 100 days. Results were evaluated for significance using ANOVA at $P < 0.05$. Therefore, data were processed to calculate the percentage of heavy metal removal from each treatment as stated;

$$\% \text{ of heavy metal removal } = \left(\frac{C_{0(x)} - C_{F(x)}}{C_{0(x)}}\right) \times 100\ \%$$

where $C_{0(x)}$ = initial concentration of metal × in the soil at the start of experiment; $C_{F(x)}$ = final concentration of metal × at the end of experiment.

The data were further processed to determine the rate constant of heavy metals removal via the use of first-order kinetic model as stated;

$$K = -\frac{1}{t}\left(\ln\frac{C}{C_0}\right)$$

where K = first-order rate constant for metal uptake per day; t = time in days; C = concentration of residual metal in the soil (mg kg^{-1}); and C_0 = initial concentration of metal in the soil (mg kg^{-1}).

Result and discussion

Leachate and soil characterization

Characterized leachate refers to raw leachate sample, collected from municipal solid waste (MSW) landfill for the experiment. The physicochemical properties of the leachate included BOD (27,000 mg L^{-1}), COD (51,200 mg L^{-1}), pH 7.35, TDS (1,730 mg L^{-1}), monocyclic aromatic hydrocarbons and heavy metals (Table 2). It was evident that the raw leachate samples were highly heterogeneous in nature. This was typical of raw leachate from MSW landfills in Malaysia (Emenike et al. 2012, 2013; Fauziah et al. 2013). Such can cause difficulties in handling pollution associated with leachate since discrete pollutants react differently especially during biological treatments. The microbial load of the heterotrophic population was 3×10^6 CFU g^{-1} of soil. The nine species isolated from the cultivable fraction of the heterotrophic population are shown in

Table 2 Leachate analysis

Parameters	Values
pH	7.35 ± 0.2
BOD	27,460 ± 460
COD	51,200 ± 1320
Chloride	4500 ± 118
Ammoniacal nitrogen	880 ± 74
TDS	1730 ± 48
Copper	13.59 ± 1.9
Mercury	2.1 ± 0.08
Chromium	25.27 ± 0.03
Zinc	827.7 ± 0.02
Manganese	540.6 ± 34
Benzene	0.00022 ± 0.00014
Toluene	0.0012 ± 0.00006
Ethyl benzene	0.00086 ± 0.00001
o-Cresol	0.00009 ± 0.00002
p-Cresol	0.00006 ± 0.00001

All units in mg L^{-1} except for pH

Table 3. Isolated species belong to four genera of class γ-proteobacteria (Pseudomona, Stenotrophomonas, Flavimonas and Acinetobacter), two genera class of Bacilli (Bacillus, Lysinibacillus), two genera of the class Actinobacteria (Microbacterium, Rhodococcus) and a genera of the class α-proteobacteria (Brevundimonas). It showed that isolated microbes were reflection of native community of bacteria often represented in phylogenetic tree (Sprocati et al. 2012), and as such, their use in the microbial formula was expected to show increased metabolic potential without any significant alteration in structure.

Reduction of extractable heavy metals

The heavy metal composition of the soil microcosms were analyzed via ICP-MS. From the inception of the experiment, the LSA, LSB and LSC microcosms were spiked with leachate that contained heavy metals. After 100 days, some fractions of the heavy metals, namely Pb, Cu and Zn, were still detectable (Fig. 1). At the end of the experiment, the reduction results (expressed as percentage of heavy metals removal efficiency, Fig. 2) depicted efficiency of the bioaugmentation.

Table 3 Isolated bacterial species and distribution in microcosms for bioaugmentation

Treatment LSA	Treatment LSB	Treatment LSC (Control experiment)
Bacillus thuringiensis	*Bacillus thuringiensis*	NU
Pseudomonas putida biotype B	NU	NU
Stenotrophomonas maltophilia	NU	NU
Flavimonas oryzihabitans	NU	NU
Lysinibacillus sphaericus	*Lysinibacillus sphaericus*	NU
Acinetobacter schindleri	NU	NU
Brevundimonas vesicularis	NU	NU
Microbacterium maritypicum	NU	NU
Rhodococcus wratislaviensis	*Rhodococcus wratislaviensis*	NU

"NU" means not used (meaning that such bacterial in the treatment LSA was not used)

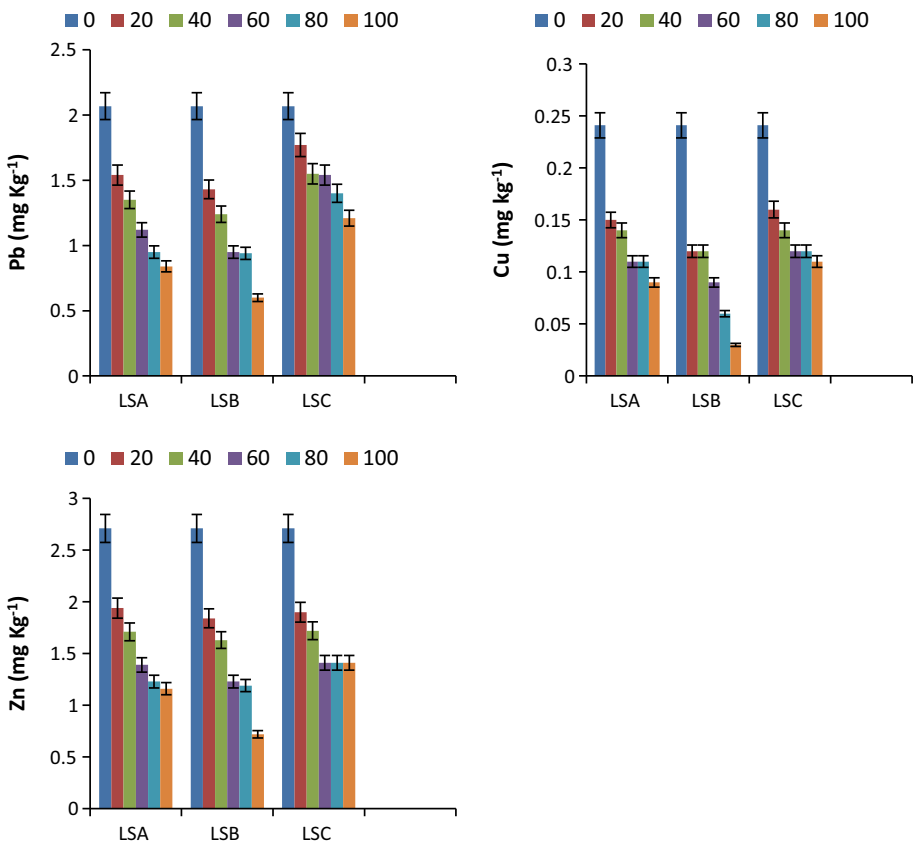

Fig. 1 Heavy metals (Pb, Cu and Zn) concentrations in the microcosms at the beginning of the experiment (0 day) and across the 100 days of treatments: LSA, with the addition of inocula composed of nine bacterial species; LSB, with the addition of three bacterial species; and LSC, without any bacterial formula (control experiment)

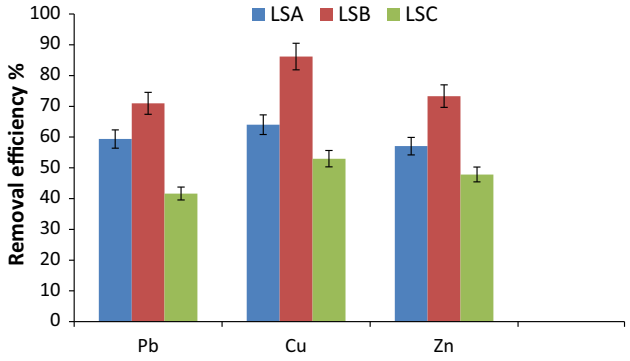

Fig. 2 Removal efficiency (%) of heavy metals in the contaminated soil after 100 days in different conditions: LSA, with the addition of inocula composed of nine bacterial species; LSB, with the addition of three bacterial species; and LSC, without any bacterial formula (control experiment)

In the microcosm without the microbial formula (LSC), the reduced percentage of heavy metals are attributable to the "bioattenuation" phenomenon which was natural and ranged from about 42 to 53 % depending on the heavy metal. Only Cu was abated to above 50 % of the initial concentration. On the other hand, an effective bio-removal occurred in the LSA and LSB microcosms: in particular, 64–86 % of Cu was removed and showed a significant difference between the microcosms ($P = 0.013$) at 95 % confidence using one-way ANOVA. The presence of the microbial formula improved the reduction of Pb and Zn (59–73 % in LSA and LSB vs. 42–48 % in LSC). Though Pb and Zn removals from the LSA and LSB microcosms were above 50 %, there was negligible difference ($P = 0.175$) between the microcosms over Pb removed, whereas a significant difference was observed at Zn removal ($P = 0.011$). Therefore, it is worthy to note that the heterogeneous nature of the raw leachate did not inhibit the heavy metal bioremoval potential of the microbial community.

However, the highest removal efficiency was found in the LSB microcosm despite being formulated with only three bacterial species (*Bacillus thuringiensis*, *Lysinibacillus sphaericus* and *Rhodococcus wratislaviensis*) from the isolated lot, as it is evident from the removal rate constants calculated (Table 4) (expressed as K = constant day^{-1}). Removal rate constant was highest during Cu removal (0.0212 day^{-1} in the LSB microcosm). This might suggest that Cu removal was the prioritized potential of the microbial community used in this experiment for heavy metals reduction.

Previous studies (Babu et al. 2013; Rani et al. 2009) had presented that selecting microbes for bioaugmentation avail high potential for heavy metals removal (most cases above 65 % removal efficiency) from contaminated soil. But the additional findings in this study considered the heterogeneous nature of MSW leachate, as against situations where heavy metal is the sole contaminant in the test soil. The experimental system represents an effort to attain conditions as close as possible to "field situations" so as to directly transfer results to the field (Pritchard and Bourquin 1984; Sprocati et al. 2012). Literature has shown that previous work describing the same approach, and it is worth noting that the removal efficiency obtained in this work was higher than those described in experimental systems that are even larger than the batch scale (Oves et al. 2013; Aniszewski et al. 2010) which used similar microbes.

Table 4 Removal rate of metals across the microcosms due to bioaugmentation

Heavy metal	Removal rate day^{-1}		
	LSA	LSB	LSB
Pb	0.0089	0.0124	0.0053
Cu	0.0099	0.0212	0.0078
Zn	0.0084	0.013	0.0065

Function and efficiency of the microbial formula inoculated as bioaugmentation agents

When defining the bioaugmentation approach, a very critical factor to consider is how to interpret the biodiversity to be introduced via bioaugmentation. In the present work, the microbial formula used as bioaugmentation agent was established according to a few ecological considerations. First, the isolation of microbes from the polluted environment suggested a prevalence of microbial metabolic activity. Also, based on the principle that only a small portion of the organisms consume a major part of energy flux (Pareto's law), bacteria playing this role in a community are preferred candidates as inoculants for bioaugmentation considering the fact that they play quantitative key roles in the system, are stable and as such have become adapted to actively survive in similar conditions (Dejonghe et al. 2001; Sprocati et al. 2012). Therefore, bioaugmentation should aim toward the rearrangement of the groups of microbes dominantly involved in the overall energy flux so that specific catabolic traits pivotal for the cleanup of pollutants are part of those active groups (Dejonghe et al. 2001). Hence, increasing the right metabolic competences for the given experimental conditions was the core essence of the bioaugmentation, as against just microbial species diversity.

Therefore, it might seem to be a surprise that the highest percentage and rate of Pb, Cu and Zn removals were most pronounced in soil amended with only three bacteria species (LSB microcosms), whereas it is easy to hypothesize that the LSA microcosm which contained all the nine

isolated bacteria species will show higher bioremediation potential (considering that increased microbial diversity will enhance more rapid metabolic activities). However, mechanism behind the result is found in interactions that exist among microbes when concentrations and diversity are manipulated. *Bacillus thuringiensis* which was found in the two microcosms (LSA and LSB) is known to have heavy metal removal capacity and had removed 77 % Pb, 64 % Zn and 8 % Cu, from a mine extract[21]. Hence, this ability is enough to have enhanced the removal of the heavy metals in the LSA and LSB microcosms, but seemed that the best interaction that yielded the optimal removal for the studied heavy metals existed among *Bacillus* sp., *Lysinibacillus* sp. and *Rhodococcus* sp., only. This argument can be buttressed further by the fact that it is expected that the LSA would have shown the optimal Cu removal considering the implication of *Pseudomonas putida biotype B*, *Brevundimonas vesicularis* and *Stenotrophomonas maltiphilia* in previous studies of Cu removing potential (Choudhary and Sar 2009; Plociniczak et al. 2013). But, the selective use of the three bacteria species in the LSB microcosm had shown special form of interaction especially as they were all gram-positive bacteria. Also considering that *Lysinibacillus sphaericus* possessed a hexhistidine tag (His_6-tag) at the C-terminus of its S-layer protein SbpA, it is possible that the metal binding property of the His_6-tag was better expressed when in association with *Bacillus* sp. and *Rhodococcus* sp., hence providing the bioremediation edge for treatment B. The study can infer that bacterial species that belong to the same form (gram positive or negative) appear to blend easily and ensure optimized removal of heavy metals when used as bioaugmentation agent than when used as combination of both species forms (gram positive and gram negative).

Conclusion

This study shows that the microbial consortia formulated and introduced as bioaugmentation agents were able to enhance heavy metals removal in soil (86 %), under the pressure of MSW leachate. The tailor-made microbial formula composed of species of bacteria previously isolated from soil persistentely polluted with leachate, and selected to be functionally associated with the existing pollutants in the test soil, which was able to initiate positive metabolic competences without disrupting the indigenous microbial community. The use of only selected microbes from the isolated lot optimized the energy flux which enhanced its full potential for bioreduction. Hence, the study concludes that utilizing directly isolated bacterial species isolated from leachate polluted soil as bioaugmentation agent is capable of allaying the fear of option required for heavy metal remediation in the presence of other pollutants. However, optimal efficiency is enhanced from selection based on ecological consideration.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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