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Research Article

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Research on the Possibility of Reducing Lead Contamination in Soil Using Microorganisms

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Abstract

In Ulaanbaatar City's soil, the main polluters of the surface soil are Lead, Chromium, and other heavy metals from industrial and mining activities, electric power stations, sewage, and vehicle emissions. Organisms that live in environments polluted by heavy metals do the mechanisms of adaptation to survive. Some microorganisms are resistant to metal and can accumulate it in their cells or detoxify it by reacting with its metabolic products. The purpose of the work was to identify bacteria that can resist the action of lead salts and to determine some activity. In 18 city soil samples, Lead was found at 5.9-5132 mg/kg, and 3 points exceeded the maximum permissible level. The agar dilution method determined that resistant bacteria isolate to varying concentrations of heavy metals such as Lead, Zinc, Ferric, Copper, Chromium, and Cadmium. Lead-resistant bacteria were isolated on a medium with PbCl2. Four strains could resist nine mM of Lead, two mM of Zinc, two mM of Chromium, and one mM of Iron. In liquid media, our strain B.thuringiensis MN01 can reduce the lead concentration by up to 46 % within 1 month. Consortium bacteria have been determined in active laboratory conditions to reduce the concentration of 1 mM of Lead to 0 mM after 1 month.

Keywords: Ulaanbaatar City, Heavy Metals, Bacteria, Resistance.

Introduction

Heavy metals are important both industrially and biologically due to their non-biodegradable nature. They are steady in the environment and pose a serious threat to it, and their excessive deposition in the soil can cause severe damage to the soil ecosystem. Lead is one of the major pollutants and needs an immediate concern [1]. Familiar anthropogenic sources of lead contamination in the environment include smelting of ores, burning of coal, mining, effluents from battery industries, automobile exhausts, metal plating, leather tanning, finishing operations, fertilizers, and pesticides [2-4]. Of all the heavy metals found in the environment, dangerously increased amounts of Cd and Pb are the biggest concern, i.e., ballast elements that are entirely unnecessary for living organisms [5-8].

Studies have shown that these elements cause changes in the cell cycle, carcinogenesis or apoptosis [9].

Urbanization and contamination of agricultural soil by Pb (II) have severely affected the health of higher animals as lead-containing compounds enter the food chain. Its increasing level has severely affected the soil microbial diversity and the growth and metabolism of plants. Besides that, it can accumulate in different parts of plants and enter the food chain. Lead can affect any organ in the body, but the most sensitive parts are the developing nervous system, hematological and cardiovascular system, reproductive system and kidney [10, 11].

Although many regulatory steps are being taken to limit the discharge of lead compounds into the ecosystem, Pb (II) contamination is still increasing [12]. Various physicochemical methods have been employed to remediate Pb (II) contaminated soil. These include excavation, landfill, thermal treatment, and electro-reclamation. However, these methods are not suitable for the remediation of Pb (II) contaminated sites at large scale due to their high cost and low efficacy [13, 14]. In addition, these methods destroy soil fertility, structure, and other properties. In 2015, the United Nations (UN) set a key sustainable development objective to reduce diseases and deaths associated with soil contamination by 2030 [15]. *B. thuringiensis* CASKS3 strain isolated from the Vellar River on the southeastern coast of India could reduce mercury (HgCl2) by 43-95%. A maximum mercury concentration of 200 mg-l was tolerated [16].

Bioremediation is a practical and eco-friendly approach that can reduce environmental heavy metal contamination. Bioremediation agents include bacteria of the genus *Bacillus among* others. The best-described species in terms of the bioremediation potential of *Bacillus spp*. Are *B. subtilis, B. cereus, or B. thuringiensis.* This bacterial genus has several bioremediation strategies, including biosorption, extracellular polymeric substance (EPS)-mediated biosorption, bioaccumulation, or bioprecipitation. Due to the above-mentioned strategies, *Bacillus spp.* Strains can reduce the amounts of metals such as lead, cadmium, mercury, chromium, arsenic or nickel in the environment. Moreover, strains of the genus Bacillus can also assist phytoremediation by stimulating plant growth and bioaccumulation of heavy metals in the soil. Therefore, *Bacillus spp.* is one of the best sustainable solutions for reducing heavy metals from various environments, especially soil [17].

Microbial remediation of Pb (II) polluted sites has been considered a cost-effective, promising, and environment-friendly approach as these microbes such as Acinetobacter junii Pb1, Pseudomonas stutzeri M-9, Vibrio harveyi M-11, and Ralstonia metallidurans CH34 are known to be resistant to heavy metals to a very high concentration. Various mechanisms are known in microbes, which help them to oppose the toxic effect of Pb (II). These mechanisms include sequestration by exopolymers, bioaccumulation, efflux, immobilization, and precipitation [18-22]. However, more work needs to be done to understand the lead-microbe interactions and test the practicality of the microbial strategy.

The present study focusses on the ability of A. junii Pb1 to tolerate high concentration of Pb (II) without growth inhibition up to 500 mg l−1. The resistance pattern of A. junii Pb1 towards Pb (II) was studied by analyzing cell viability by FM, FCM, and Spectrofluorimetry using fluorescence dye PI and LG AM dye, which confirm its intracellular accumulation by the bacterium. FCM is a rapid, excellent means to detect the physiological changes in bacteria cells under stress. Dead (PI stained) cell population was insignificant at 100, 250, and 500 mg l−1 Pb (II) concentration, thereafter (1000 mg l−1) it significantly increased, as observed by FCM studies. Physiological changes in the bacterium following Pb (II) exposure included increased cell size and granularity. Because of its capacity to survive and grow at highly toxic Pb (II) concentrations, the strain A. junii Pb1 can be used for the bioremediation of Pb (II) contaminated sites [23].

Thanks to modern research, the now-improved bioremediation methods using suitable microbial species (that can act alone or support the action of hyperaccumulators) are becoming more common in environmental protection [24].

One way of bioremediation may be the use of bacteria of the genus Bacillus [25-29]. They are Gram-positive, spore-forming, rod-shaped, and aerobic or facultative anaerobes. Overall, the genus Bacillus is most commonly found in soil but can also be isolated from other sources, e.g., water, air, water, vegetables, and food, as well as human and animal intestines [30-33].

The unique trait of *Bacillus spp*. is the ability of spore-forming under extreme conditions. Due to their specific structure, the spores can resist significant environmental stresses, including high temperatures, drought, humidity, and radiation. This characteristic gives them an advantage over other bacteria and makes them eagerly used commercially in various fields of industry and agriculture [34]. Moreover, bacteria capable of precipitating lead into lead phosphate ($Pb_3(PO_4)_2$) also include *B. thuringiensis* 016 [35].

Furthermore, Molokwane et al. observed Cr (VI) reduction by precipitation after the application was enriched by a mixed culture of bacteria consisting of bacteria of the genus *Bacillus,* including *B. cereus, B. thuringiensis,* and related genera, such as Paenibacillus and Oceanobacillus. The highest reduction of Cr (VI) in aerobic cultures was obtained at a high concentration of 200 mg L−1, after incubation for 65 h. To our knowledge, there have been no studies to date describing the possibility of precipitation of other heavy metals (important from the point of view of pollution) by bacteria of the genus Bacillus [36]. Organisms that live in environments polluted by heavy metals do the mechanisms of adaptation to survive [37].

Methods

Methods for Collecting Soil Samples

Eighteen soil samples were collected from different parts of Ulaanbaatar city, and we performed microbiological analysis. Soil samples were determined according to MNS3298-90 and heavy metal content was determined according to the MNS5850:2008 standard. When selecting a sample, first select soil with a high lead content. In order not to be exposed to external contamination, take 300-500 g of the sample from a depth of 3-20 cm of the soil using sterile tools [38, 39].

Method for Determining Lead in Soil Samples

Lead analysis of soil and water was determined by iCAP7400 ICP-OES instrument according to the MNS (ISO) 11885:2011 standard method at “SCV-LAB in Mongolia”. The soil to be an-

alyzed was dried at 40ºC for 24 hours and prepared for processing. Approximately 0.1 g of each soil sample was weighed on an analytical balance (Adventurer OHAUS, USA) and placed in a sample processing vessel (Vessel), and 2 ml of 65% concentrated nitric acid (HNO3); 37% concentrated hydrochloric acid (HCl) was added. The samples were decomposed by heating in a microwave oven (BioBase, China) at 130ºC, 150ºC, and 180ºC in ascending order of temperature. The standard solution containing 1000mg/L ± 4mg/L of cadmium (Fluka, Japan) used to construct the calibration curve was diluted with 2% nitric acid to 0.2 mg/L; 0.4 mg/L; 0.6 mg/l; 0.8 mg/l; 1.0 mg/l were prepared and used in this study. In this study, using the flame method, we calculated the results using the MSoolar program on an atomic absorption spectrometer (Thermo Scientific™ iCE™ 3500, USA). A hollow cathode lamp Pb (Thermoscientific, USA) with a wavelength of 217.0 nm was used for lead detection, a D2 (deuterium) lamp was used for background correction, an air-acetylene flow rate of 0.9 l/min, and a 50 mm long titanium burner. We calculated the results as the average of three measurements. Quality control was performed using Certified Reference Material BAM U-112a, and the confirmed lead value was 198 mg/kg and the analyzed value was 190 mg/kg.

Method for Isolating Heavy Metal-Resistant Bacteria

In order to isolate heavy metal-resistant bacteria, the concentration of heavy metal salts is adjusted to 1-9 mM in a Nutrient agar medium [40]. Inoculate by surface method and incubate at 37^{0}C for 24-48 hours. Record the morphology of the colonies growing on the surface of the nutrient medium. Increase the concentration of heavy metals in the nutrient medium little by little to determine the ability of the culture gas bacteria to withstand the highest concentration of that heavy metal [41]. To determine the growth phase of the cells, a Nutrient broth medium was used to measure the spectrophotometer at a wavelength of λ= 600nm. By gradually increasing the concentration of heavy metals in the culture medium, bacteria that tolerate the highest concentrations were determined [44]. When determining the tolerance of the concentration of lead salt, 0.1M, 0.2M, 0.4M, 0.6M, and 0.8M were inoculated into Nutrient Agar medium with 1M NaCl and incubated at 37°C for 24-48 hours. To determine the temperature dependence, after inoculation in Nutrient Agar medium, 4°C, 25°C, 30°C, 37°C, and 42°C were incubated for 24-48 hours [42].

Genomic DNA Extraction and PCR Preparation

The extraction of bacterial DNA was performed using a commercial genomic DNA extraction kit (Bio Basic Inc.; Ontario, Canada). Proteinase K and RNase A were added to remove protein and RNA contaminations and obtain good-quality DNA according to the manufacturer's instructions. The extracted DNA was quantified by gel electrophoresis using lambda (λ) DNA as the marker; it was then stored at −20 °C for further use. The PCR master mixture was prepared as a 50 µL solution containing 25 µL of 2 × concentrated solution (Thermo Scientific, United States), 3 µL each of forward primer (27F, 5′- AGAGTTTGATCCTGGCTCAG -3′) and reverse primer (1492R, 5′- TACGGYTACCTTGTTACGACTT -3′) for amplification of 16s rDNA universal sequence, 4 µL of template, and 15 µL of nuclease-free water. DNA sequences obtained were compared to sequences available online in the GenBank database (http://www.ncbi.nlm.nih.gov). Homology search was performed using bioinformatics tools available online, BLASTn [43].

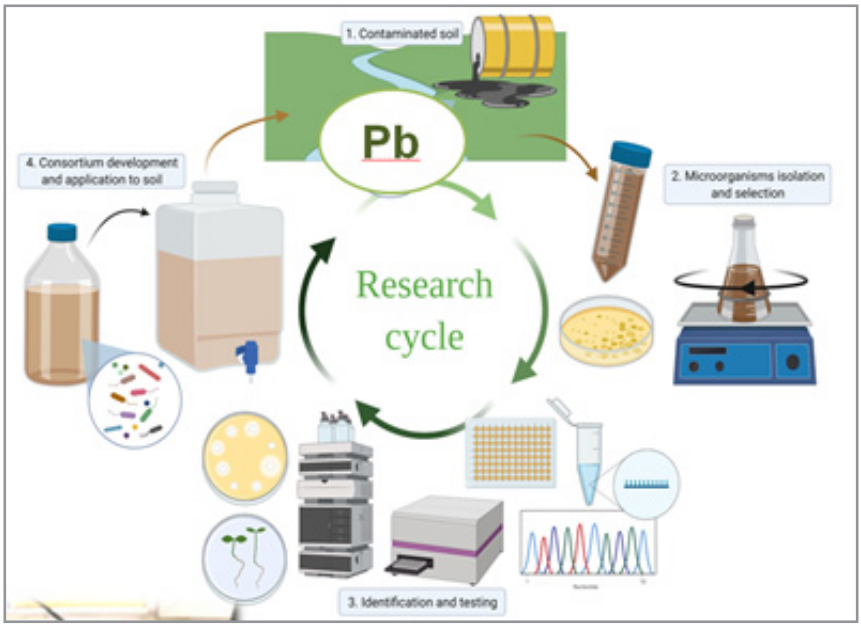

Fig. 1: Schematic showing the sequence of work for the isolation of lead-resistant bacteria [44].

Results

Soil Heavy Metal Result

The first sixteen samples appear less polluted than Mongolia's standard of soil pollution (the maximum allowable amount is 100 mg/kg), but there is a certain amount of pollution. Six points (11th district of ChD, Denjiin 1000, Khuchit shonkhor market, Nalaikh district (inside and outside the lead factory yard), Darkhan's metallurgical dump point) have an above-average lead content in the soil (Figure 2).

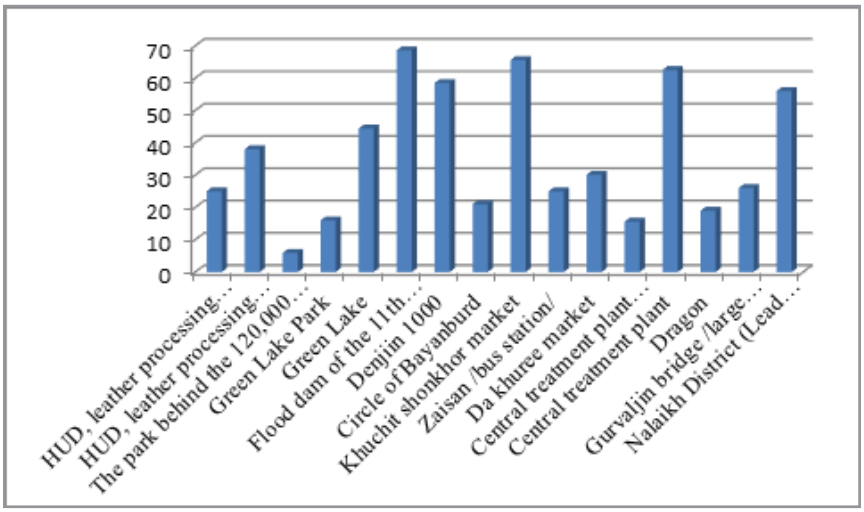

Fig. 2: Amount of Lead Detected in Soil (mg/kg)

Two soil samples showed more than the maximum permissible level. 16.5 times the toxic lead concentration and 1.4 times the dangerous concentration of lead were detected in the soil of the raw material collection yard of the Darkhan Metallurgical located in District of Songinokhairkhan, while the soil inside the yard of the Lead factory located in District of Nalaikh had 51.3 times the harmful lead concentration, 10.3 times more lead than dangerous concentration was detected (Figure 3).

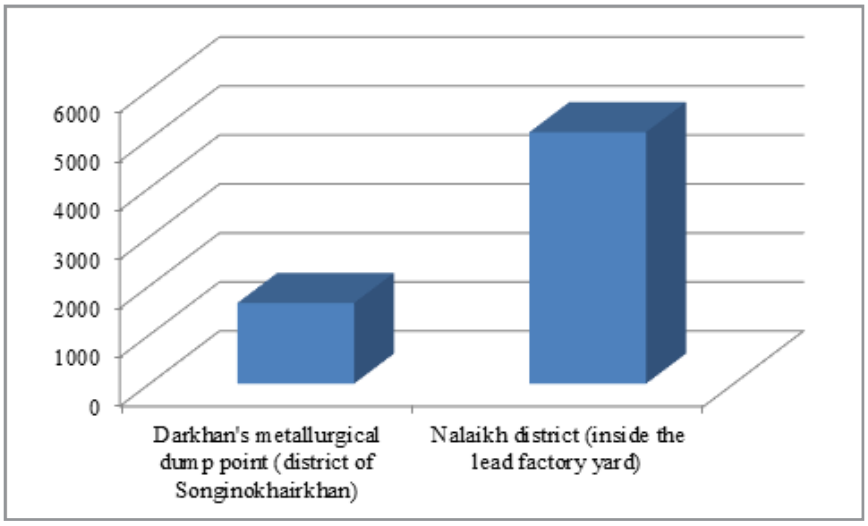

Fig. 3: The highest level of lead detected in soil (mg/kg)

Bacterial Heavy Metal Resistance Result

The effects of heavy metals were determined by culturing in a medium with six heavy metals. *Bacillus thuringiensis* was characterized by its habitat, and it was found dominantly in soil contaminated with car maintenance oil, soil contaminated with iron waste for many years, and soil highly contaminated with lead. *Bacillus thuringiensis* was exposed to 9 mM (2503 mg/L) of Lead (PbCl2), two mM (273 mg/L) of Zinc (ZnCl2), and two mM (304 mg/L) of Chromium (Cr2O3), resistant to 1mM (159 mg/l) concentration of iron (Fe2O3) (Table 1).

Table 1: Result of Heavy Metal Resistance Activity

Heavy metal	Pb			Zn		Fe		Cu		Cr		Cd	
Conc	7mM	8mM	9mM	1mM	2mM	1mM	2mM	1mM	2mM	1mM	2mM	1mM	2mM
TS-4	++	+	+-	+	+-	+	-	-	-	+	+-	-	-

Note: Resistance to heavy metals: ++ very good; + good; +- bad; - did not endure.

Result of Bacterial Biochemical Activity

The pure culture of the Ts-4 bacteria that we isolated is a Gram-positive bacillus, and according to the results of the VITEK®2 instrument analysis, it was determined to be a local strain of *Bacillus thuringiensis* with a probability of 93%. The culture is active in breaking down D-mannose, D-glucose, and D-dribose carbohydrates as well as amino acids such as Leucine, Phenylalanine, and Glycine, breaks down Tyrosine, Arylamidase, Esculin, resistant to Renicillin and Polyb-R antibiotics, 6.5% Able to grow in NaCl environment.

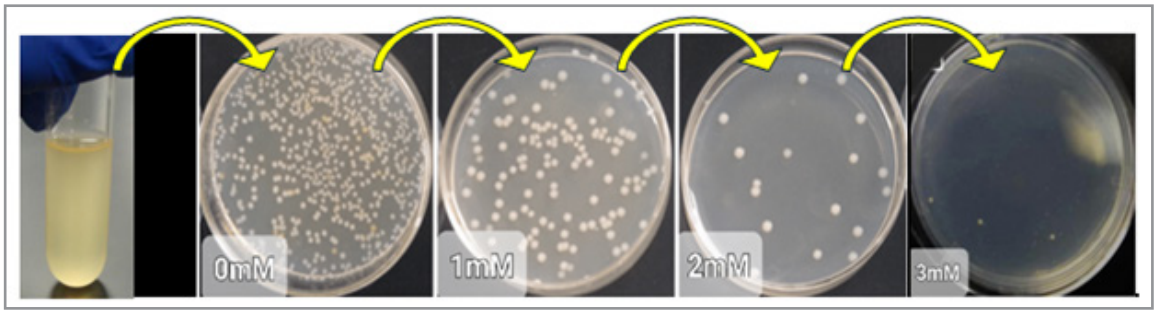

Fig 4: Bacterial Heavy Metal Resistance Activity on Petri Dish (Maximium Resist)

Note: The bacteria did not tolerate a lead concentration of 4 mM, and it tolerated the maximum concentration of 3 mM, so the maximum tolerance for this bacterium is 3 mM. This method is a tolerance method by transferring the bacteria directly from the medium with the concentration they are accustomed to tolerating to the next concentration or increasing the concentration little by little.

Graphs 1 and 2 show the relationship between the culture medium's pH and temperature and the growth dynamics measured by a spectrophotometer at a wavelength of ƛ= 600 nm.

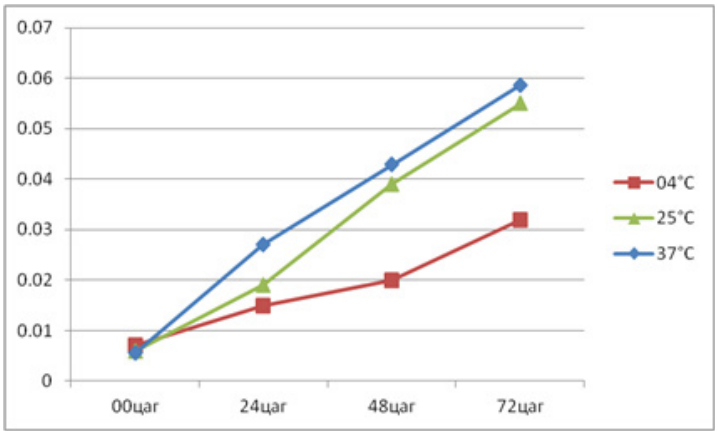

Fig. 5: Temperature Dependence (OD 600nm)

Under facultative aerobic conditions, it can grow up to 24.8*106 cells/ml at +40C, 44*106 cells/ml at +250C, and 47.7*106 cells/ml at +370C after 72 hours, and the ideal temperature range is 25–370C.

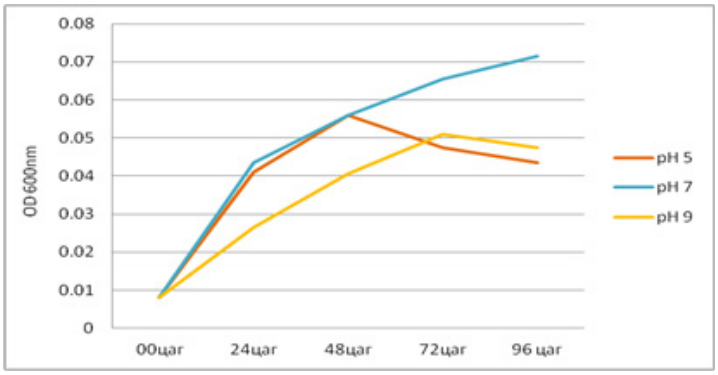

Fig. 6: Dependence of Environmental pH (OD 600 nm, +370C)

The Ts-4 local strain grew stably at pH 5 for 48 h and at pH 9 for 72 h before decreasing in growth, while at pH seven it was still actively growing after 96 h (Figure 6).

PCR and Sequence Analysis

The results of determining the species relationship of *Bacillus thuringiensis* Ts-4 local strain by molecular biology method: The DNA product was amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) of the pure bacterial culture Gel confirmed by electrophoresis (Figure 7).

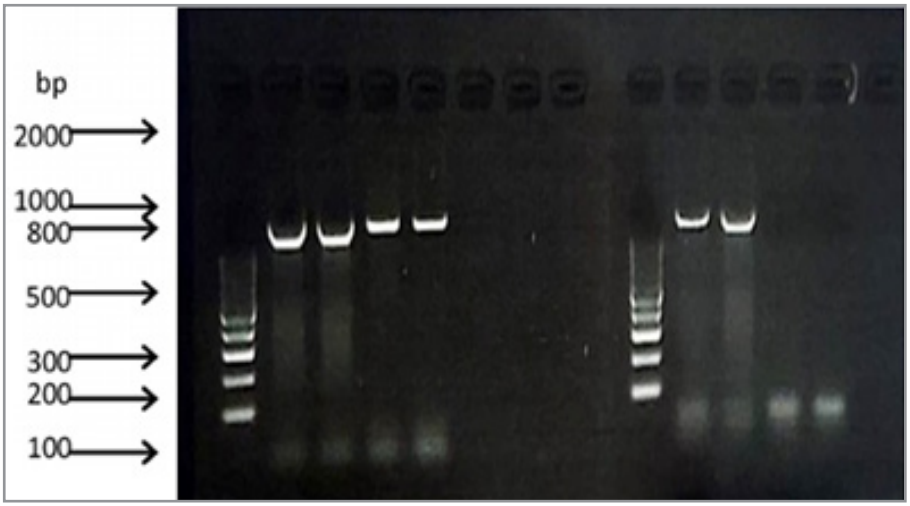

Fig. 7: PCR Results Verified by Electrophoresis

A phylogenetic tree was constructed based on the 16S rRNA gene sequences to determine the evolutionary relationship between strain Ts-4 and closely related Bacillus species. As shown in Figure 8, strain Ts-4 formed a distinct clade separate from other Bacillus species, including *Bacillus cereus, Bacillus anthracis, Bacillus wiedmannii, Bacillus toyonensis, and Bacillus thuringiensis*. The branch supporting Ts-4 exhibited a bootstrap value of 22%, indicating a moderate level of phylogenetic distinctiveness.

The relatively long branch length associated with Ts-4 suggests considerable genetic divergence from its closest relatives. In contrast, other species such as *B. cereus and B. anthracis* clustered closely together with high sequence similarity (99.79–99.93%) and higher bootstrap support. Notably, Ts-4 did not cluster tightly with any previously identified Bacillus strains, implying that it may represent a novel species or subspecies within the Bacillus genus.

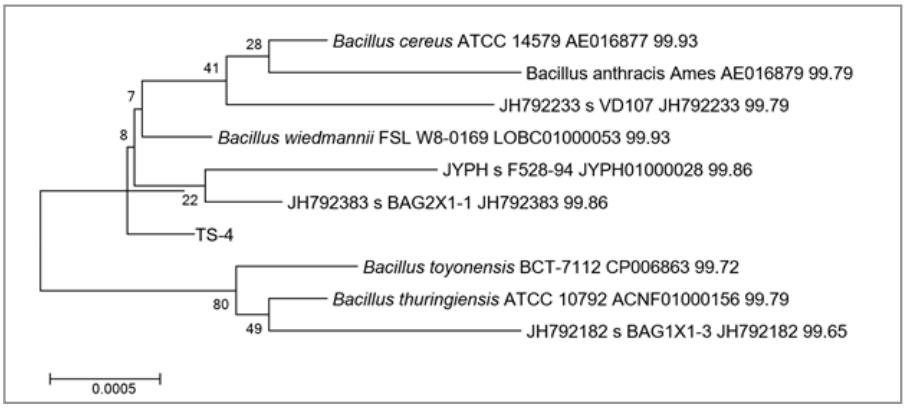

Fig. 8: Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain TS-4 and closely related Bacillus species.

Note: The scale bar represents 0.0005 nucleotide substitutions per site, reflecting the relatively low genetic distances among the examined strains.

Initial phylogenetic analysis based on partial 16S rRNA gene sequences indicated that strain Ts-4 was genetically distinct from closely related Bacillus species. Subsequent full-length sequencing and database comparison identified Ts-4 as *Bacillus thuring-*

iensis SUB12233956 MN01, registered under the accession number OP744527 in the National Library of Medicine (NIH)

Bacterial Cell Growth and Biological Activity

The strain forms 3-7 mm white flat colonies with uneven wavy cloudy margins on the surface of solid media (Figure 9). The cells are 0.5-1.0×2-5 μm in size, form motile bacilli and spores, are Gram-positive, facultative aerobic catalase positive, and oxidase negative, can grow and multiply between pH 5-9, and are active in the temperature range of +20 to +40oC (Figure 5).

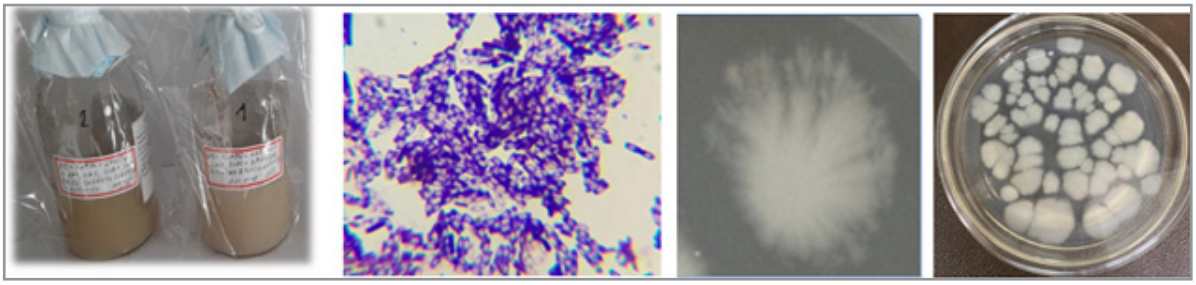

Fig. 9: *B.Thuringiensis* Cell and Spores. Light Microscope BEL Photonics (2000X)

The results of bacterial dry biomass extraction: *Bacillus thuringiensis* SUB12233956 MN01 local strain culture was dried by lyophilization method to obtain dry biomass (Figure 10). Eight types of supplements were used as protective medium, and the protective medium was made in a 1:1 ratio of biomass. After deep freezing the biomass at -200C and -400C for 2 days and drying at -600C at one Pascal pressure for 98 hours, the dry biomass was obtained.

Fig. 10: Dry *Biomass of Bacillus Thuringiensis* Strain Capable of Lead Reduction

In the dry biomass, there are 2.4*10+8 cell/g bacteria. Bacterial viability is 96%.

In the National Center for Biotechnology Information, "Heavy Metal Resistant and Active to Reduce Organic Pollutants:

- *Bacillus thuringiensis* SUB12233956 MN01 OP744527;
- *Bacillus cereus* SUB13292374 MN OQ938267;
- *Bacillus cereus* SUB13292374 MN2 OQ938268;
- *Sphingomonas paucimobilisstrain* SUB13302645 MN01 OQ938543 BCT 13-MAY-2023, National Library of Medicine. Registered with NCBI, NIH.

Experiments in Liquid Medium in LAB

$PbCl_2$ was prepared at 4mM in NB medium, and the experiment was conducted in 2 ways. Ten cultivars tolerant to high lead concentrations were selected. Pure bacterial cultures were isolated and pre-incubated in broth for 48 hours, then 1 ml of the supernatant of each culture was centrifuged and inoculated into 200 ml of broth prepared in 500 ml flasks prepared with four mM lead concentration. They were incubated at 37°C in an incubator with a 160 rpm shaker, and the total number of microorganisms and lead content were determined at 00 hours, 10 days, 20 days, and 30 days. As a control, 4mM (800mg/l) lead was added to 200ml broth in the same 500ml flask. This experimental research work is not finished and will be confirmed after several repetitions of the experiment to determine the growth dynamics of each culture and to reduce the concentration of lead in the liquid medium (Table 2):

1. 200ml NB + 10ml culture (ublf1, ublf3, ubmf2, ubts4, sub5, subz4, sagpo1, wutso1)
2. 200ml NB + 10ml culture (ublf1, ublf3, ubmf2, ubts4, sub5, subz4, sagpo1, wutso1) + 0.1g vitamin complex to support bacterial growth
3. Control (200 ml NB)

Table 2: Amount of Bacterial Lead Neutralization

	Culture biomass (cell/ml)				Total of Pb (mg/L)		
Time	00 hour	10 day	20 day	30 day	10 day	20 day	30 day
Test -1	1*105	6.75*108	8*108	10*1012	722	485.2	20
Test -2	1*105	9*108	11*108	12*1012	713.1	379	0
Control	0	0	0	0	787.2	765.8	736

Note: MNS (ISO) 11885:2011 standart, iCAP7400 ICP-OES analyzator. The used to create the Consortium bacteria included two different subspecies of *Bacillus cereus*, three different species of *Bacillus spp*., and additional strains of *Sphingomonas paucimobilis* and *Pseudomonas fluorescens* (ubts4 is Ts-4).

Discussion

In 2020, the *B. thuringiensis* HM-311 strain was detected in the environment in China, and it was found that the bacterial cells have genes resistant to heavy metals such as zinc, copper, nickel, cadmium, and manganese International studies after 1975 did not find research papers investigating the activity of *B* [45]. *thuringiensis* to tolerate lead [46]. However, the strain we isolated was resistant to lead, cadmium, chromium, and iron. In 2016, the strain of *B. thuringiensis* BT-MN01 was isolated from infected and dead Siberian moths in order to kill pests with biological fertilizers in Mongolia. The researchers determined that the product decomposes completely and dies in 15-30 days after being sprayed against harmful insects in nature, does not produce toxic substances in soil and water, and does not affect soil microorganisms. No adverse effects on the environment or warm-blooded animals were observed. However, it is prohibited to transport or store it with food. In an American study, bacteria of the type *Bacillus sp*. reduced lead up to 66% [47]. *Bacillus subtilis* X3 bacterium can withstand the maximum concentration of lead salt of 2000 mg/l [48]. Our isolated *Bacillus sp*. is resistant to the concentration of 1660 mg/l.

Conclusion

When the lead concentration is high, the total bacterial count is low; when the lead concentration is low, the total bacterial count is high or inversely related. In liquid media, strain *B.thuringiensis* MN01 was able to reduce the lead concentration up to 46% within 1 month. Consortium bacteria have been determined in laboratory conditions to be active in reducing the one mM lead concentration to 0 mM after 1 month.

Acknowledgement

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Author Contributions

All authors contributed to the study conception and design. Khishigsuren Gombojav, Bayarbayasgalan Erdenebayar, and Tugsjargal Batbaatar participated in the research activities. Bayarchimeg Bayarsaikhan provided reagents, materials, and analytical equipment. Altantsetseg Khajidsuren and Khorloo Yundendorj contributed to research methodology development and provided scientific advice. Enkhjargal Gombojav supported the research through financial assistance and advisory input. Tumenjargal Davaasuren initiated and designed the research project and provided laboratory facilities essential for the study.

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