Hindawi
Advances in Materials Science and Engineering
Volume 2022, Article ID 5697465, 7 pages
https://doi.org/10.1155/2022/5697465

Research Article

Enhanced Biodegradation of Battery-Contaminated Soil Using *Bacillus* sp. (*MZ959824*) and Its Phytotoxicity Study

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Received 27 May 2022; Revised 21 June 2022; Accepted 26 June 2022; Published 7 July 2022

Academic Editor: Samson Jerold Samuel Chelladurai

Batteries that have been used and thrown away are potential threats to the environment. The aim of the present study is to explore the bacterial bioremediation of the battery-contaminated soil. The battery contaminated soil sample was collected from the municipal compost yard, Vellalore, Coimbatore, Tamil Nadu, India. The *Bacillus* sp was isolated by the serial dilution method. The *Bacillus* strain was identified based on the colony morphology as well as the 16s ribosomal ribonucleic acid partial gene sequence and was designated the name HVRCBNR. It was deposited in the GenBank under the accession number *Bacillus* sp MZ959824. The bacterial growth was evaluated by measuring the optical density of the media (OD600), while the degradation was determined by FTIR analysis. The phytotoxic analysis was performed using *Trigonella foenum-graecum* to assess the toxicity of the battery waste before and after bacterial treatment. The spectroscopic study showed that the strain HVRCBNR achieved 83.6% degradation. The growth indexes of *Trigonella foenum-graecum* showed that the biodegraded soil was nonphytotoxic in comparison with the control. This study supports the degradability of the strain HVRCBNR, and this could pave a way for sustainable solution to battery contaminated soil treatment.

1. Introduction

Batteries are inevitable in this electronic era. They are employed in variety of applications such as digital clocks, watches, laptops, remote controllers, mobile phones, flash lights, and lot more. Concerns about the production and consumption of energy derived from fossil fuels have grown in recent years, in tandem with the rapid rise of the global population. As a result, there is a greater demand for the development of clean renewable energy sources in order to limit the usage of fossil fuels. In such a demand, batteries are now widely exploited right from military equipment to being the power source of electronic vehicles [1]. Lithium-ion batteries have dominated the global market due to its superiority. With a short life span of about 2 to 4 years, lithium batteries substantially contribute to the increasing problem of electronic waste. Every year, tons of waste batteries are produced globally [2]. The hazardous components such as cadmium, lead, zinc, chromium, cobalt, and mercury released from the waste batteries serve a potential threat to the environment and pose health risk to the humans. Cobalt, copper, nickel, thallium, and silver have an impact on the environment and also possess toxic effect on human beings [3]. More metals, particularly manganese and zinc may be

leached into the environment as a result of the growing number of batteries discarded in the landfills. These findings suggest that dumping of the spent household batteries directly into the municipal solid waste landfills can raise the heavy metal content of the landfill leachate [4]. These heavy metals infiltrating the soil alter the soil micro biome and affect the plant growth. They disrupt the development, morphology, and metabolism of microbes present in the soil, causing protein denaturation and destroying the integrity of cell membranes. Soil microbes are critical for the breakdown of organic materials in the soil; any reduction in microbial community may have an unfavourable effect on the nutrient uptake by crops or plants from the soil [5].

Heavy metal bioaccumulation in the food crops are of great concern as they have serious effects on the health of human beings. Intake of food crops contaminated with the heavy metals causes gastrointestinal cancer, weakened immune system, cardiovascular diseases, and other problems attributed to the liver, lungs and nervous system. Even some insects beneficial for agricultural purposes are affected by heavy metal accumulation in the soil [6]. With the current trends in technical breakthroughs and growth in demand, a more effective waste management approach is required. Soil flushing, soil washing, and stabilisation are some of the traditional remediation processes used to remove heavy metals from polluted areas. These techniques have some drawbacks, such as sluggish metal precipitation and high energy and reagent cost [7]. Microorganisms have the capability to sequestrate the metals. Bioremediation of metals is an effective approach that has gained popularity over the years [8]. Use of microorganisms for remediating the waste batteries will reduce the exposure to the hazardous compounds. This approach is economical and has minimal effect on the environment. In order to have a proper study of the treated soil, toxicological assays such as phytotoxicity analysis and chemical analysis can be performed [9]. This study assessed the biodegradation potential of the *Bacillus* species in the soil contaminated with the battery waste and in this experiment, *Trigonella foenum-graceum* was selected for the phytotoxicity analysis, considering their root and shoot length as the indicators of soil toxicity.

2. Materials and Methods

2.1. Chemicals. All the chemicals used in the present study were obtained from Hi Media, India.

2.2. Sample Collection. The battery-contaminated soil sample was collected from the municipal compost yard, Vellalore, (battery-dumped soil) Coimbatore, Tamil Nadu, India with the co-ordinates of 10°57′28.1″N 77°00′01.3″E. This soil sample was collected in a sterile container.

2.3. Isolation and Screening of the Native Heterotrophic Bacteria. Bacteria from the battery-contaminated soil sample were isolated using the serial dilution technique. One gram of soil sample was mixed with 9 ml of sterile distilled water and was serially diluted up to 10^{-3} fold dilution. 0.2 ml of each dilution was inoculated in the nutrient agar medium (Hi Media 28.0 g/L–NAM; pH 7.0) with using the pour plate method, and the agar plates were incubated at 37°C for 48 hours [10]. Based on the morphological characteristics, the colonies were subcultured in nutrient broth containing battery components and maintained at 37°C [11]. The isolate that exhibited maximum growth in the nutrient broth containing battery components was selected for phenotypic characterization.

2.4. Phenotypic Characterization of Isolate. An aliquot of 0.1 ml of the subculture was inoculated on *Bacillus*-selective agar base using the single streak plate technique to obtain a pure culture of the bacterial isolate followed by incubation at 37°C for 48 hours. After incubation, the bacterial colonies appeared irregular, pink, large, and undulated [12].

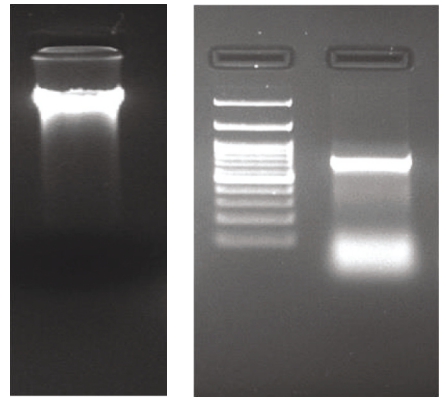
2.5. Biochemical Characterization. Gram's staining, spore staining, citrate utilization test, catalase test, urease test, oxidase test, and gelatin test were performed to analyse its biochemical characteristics [13].

2.6. Molecular Identification of Isolate. The molecular identification of the biodegrading bacterial isolates was carried out using 16s rRNA sequencing. This involved the following steps.

2.6.1. DNA Isolation and PCR Gene Amplification. The bacterial DNA was isolated using the phenol:chloroform: isoamyl alcohol method and the presence of the DNA was confirmed using agarose gel electrophoresis [14]. The extracted DNA was taken for 16s rRNA gene amplification (Figure 1(a)). The polymerase chain reaction (PCR) was carried out in 42 μl reaction mixture containing 5 μl of template DNA, 10 μl primers (Eurofins), 16 μl PCR reaction buffer, and 12 μl of PCR water (Figure 1(b)). The following universal bacterial forward and reverse primers were used: 16sr RNA for (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16sr RNA rev (5′-ACGGCTACCTTGTTACGACTT-3′). The PCR conditions were 30 cycles of 1 minute at 95°C, 15 seconds at 55°C, 30 seconds at 72°C, and final extension at 72°C for 5 minutes [15].

2.6.2. DNA Sequencing. The amplified product was subjected to partial 16s rRNA gene sequencing that was carried out using genetic analyser [16]. The sequenced data were further analysed by Basic Local Alignment Search Tool (BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database for phylogenetic relationship [17].

2.7. Biodegradation of Heavy Metal Using Bacillus sp. (MZ959824). Mineral salt media (MSM) containing 10.00 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 10 g/L dextrose anhydrate was prepared for 100 ml and was autoclaved at 121.1°C at 15 psi for 15 minutes after which it was allowed to



(a) (b)

Figure 1: (a) Isolation of DNA and (b) PCR amplification. Lane 1: 100 bp DNA ladder; lane 2: PCR product (842 bp).

cool [18]. Equal amounts of prepared MSM were taken in two different conical flasks, and each was inoculated with 0.5 ml of bacterial isolate. The battery components were added to one of the conical flasks and were labelled as the test sample. The other sample was taken as control. The samples were placed in the shaker at 90 rpm for 1 hour followed by incubation at 37°C for 12 days, and the optimum day was found to be 8^{th} day.

2.7.1. Analytical Evaluation of Biodegradation of Heavy Metals Using UV Spectroscopy. 1 ml of the test sample was centrifuged at 8000 rpm for 10 minutes and the cell-free supernatant was collected. The UV absorption spectra of the test supernatant was recorded using a UV-vis spectrophotometer (Labotronics LT291) against distilled water as a blank, and the absorbance was read at 600 nm [19]. The percentage of degradation of battery waste by the bacterial isolate was determined using the equation,

$$\%\text{degradation} = \left[\text{Initial } OD - \frac{\text{Final } OD}{\text{Initial } OD}\right] \times 100, \quad (1)$$

where initial *OD* corresponds to the absorbance of the supernatant before incubation and final *OD* corresponds to the absorbance of the supernatant after growth. The absorbance was noted at a regular interval of 4 days until a maximum percentage of degradation was observed.

2.7.2. Solvent Extraction of Degraded Compound. Equal volumes (50 : 50) of ethyl acetate were added to the cell-free supernatants of the control and the treated sample and were manually shaken until a clear aqueous layer was formed. The aqueous layer was carefully collected and was refrigerated.

2.7.3. TLC. Thin layer chromatographic (TLC) analyses of extracts of control and test were done using precoated silica gel plates (specifications). Increasingly polar mobile phase was made with the mixture of ethyl acetate, chloroform, methanol, acetic acid, and water in a ratio of 5 : 2 : 2 : 2 : 1. The sample spots were made with a pencil and the extracts of the control and the treated were applied on the same. The prepared mobile phase was poured into the TLC chamber. The plates were placed in the TLC chamber such that the sample spots were well above the level of the mobile phase and were closed with a lid. Once the spots were developed, the plates were taken out and dried. The spots were visualised by exposure of plates to iodine vapour and the corresponding Rf values were determined using the equation [20, 21],

Rf = Distance travelled by solute/Distance travelled by solvent.

2.7.4. FTIR. Fourier transform infrared (FTIR) analysis was performed (SHIMADZU) in a scan range from 400 to 4000 cm^{-1}. The peak values of control and test extracts were recorded [22, 23].

2.8. Phytotoxicity Study. The phytotoxicity study was performed using *Trigonella foenum-graecum* seeds procured from Tamil Nadu Agricultural University, Coimbatore. The healthy seeds were selected and rinsed with distilled water. Then, the seeds were soaked in three different conditions, i.e., tap water as control (A), cell-free supernatant of untreated sample (B), and cell free supernatant of *Bacillus* sp.-treated sample(C) for 2 hours at room temperature, for pregermination treatment. 10 seeds were collected from the three different experimental set ups and were sowed in the respective pots labelled A, B, and C. For irrigation, tap water was used for the pot labelled A and the supernatants were added to the respective labelled pots. At the end of the study, parameters such as shoot and root lengths were recorded. The percentage of toxicity was determined using the equation [24], % toxicity = [(radicle length of A − radicle length of C)/radicle length of A] × 100.

Furthermore, the comparative study of control and test samples was performed by estimating the carbohydrates and protein contents of the same.

2.8.1. Preparations of Plant Extracts. 1 g of each sample was taken without the roots and washed under running tap water

to remove soil particles. The samples were ground in mortar and pestle using 10 ml of distilled water. It was filtered using filter papers, and the filtrates were collected. The extracts were used to estimate carbohydrates and proteins.

2.8.2. Carbohydrate Estimation. To 0.5 ml of the extracts, 2.5 ml of anthrone reagent was added and incubated in water bath at 40°C for 15 minutes along with the blank (0.5 ml distilled water with 2.5 ml anthrone reagent). The absorbances of untreated, treated, and tap water were read at 620 nm using a colorimeter (ELICO SL 159 UV-Vis spectrophotometer).

2.8.3. Protein Estimation. For protein estimation, a reagent was prepared by mixing 2% sodium bicarbonate, 0.1 N NaOH, and 0.5% CuSO4 in a ratio of 50 : 1. To 0.5 ml of extracts, 2.5 ml of the prepared reagent was added and incubated at room temperature for 10 minutes along with the blank (0.5 ml distilled water with 2.5 ml prepared reagent). Then, 0.2 ml of Folin's phenol reagent was added. The absorbances were read at 660 nm using a colorimeter.

3. Results and Discussion

3.1. Screening and Identification of Bacteria. Four different bacterial cultures (36.8 CFU/ML) were isolated from the collected soil sample and were labelled 1, 2, 3, and 4. The four isolates were then subcultured in the nutrient broth containing battery components. The isolate labelled 3 showed maximum growth and was selected for phenotypic characterization and molecular identification. The bacterial isolate was identified by 16S rRNA gene based partial sequencing which was carried out at the Centre for Bioscience and Nanoscience Research, India. The results of the biochemical tests are shown in Table 1. The phenotypic characterization using *Bacillus* agar (Figure 2) showed the isolate belonged to *Bacillus sp.*, and the molecular identification confirmed the same. It was assigned the name HVRCBNR and deposited to the NCBI GenBank under the accession number *Bacillus sp*. MZ959824. A phylogenetic tree was built using the MEGA software (Figure 3). The phylogenetic analysis showed that the strain *Bacillus sp*. HVRCBNR showed maximum similarity with *Bacillus cereus* (99%). Lata et al. [25] also isolated and identified two potential degrading strains, one of which was found to be *Bacillus salmalaya.* Jiang et al. [26] isolated a different genus of bacteria, *Burkholderia* sp. that was resistant to heavy metals, especially Pb and Cd that are some of the major constituents of battery.

3.2. TLC Analysis. The TLC plate, when placed in the iodine chamber, a brown band was observed indicating the presence of degraded compounds in the test samples (Figure 4). The Rf value of the control was 0.87, and the Rf values of the test samples were 0.70 and 0.77.

3.3. Biodegradation Analysis. The biodegradation capacity of the *Bacillus* sp. strain HVRCBNR was evaluated by incubating the strain in MSM with battery contaminants (Figure 5). The UV-spectrometric analysis showed that strain *Bacillus sp*. HVRCBNR had 86.3% degradation potential (after 8th day). Nrior et al. [27] performed similar study of biodegradation of laptop batteries using *Pseudomonas* sp. and *Bacillus* sp. The degradation potential of the individual strain of *Bacillus* species was 12.49% and that of the individual strain of *Pseudomonas* sp. was 18.65% for Li-Dell battery while the consortium of *Bacillus* species and *Pseudomonas* species showed a higher degradation potential of 37.63% for the same, concluding that the consortium was able to degrade the batteries better than the individual strains. However, this was contradicted in the study made by Jing et al. [28]. Bourzama et al. [29] used fungi for

Table 1: Biochemical characterisation of *Bacillus* sp. (*MZ959824*).

Biochemical test	Result
Gram's staining	+
Spore's staining	+
Citrate utilization test	+
Catalase test	+
Urease test	+
Oxidase test	+
Gelatin test	+

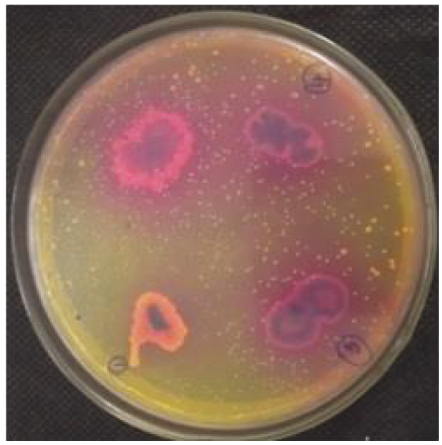


Figure 2: Phenotypic characterization of the isolate using selective *Bacillus* agar.

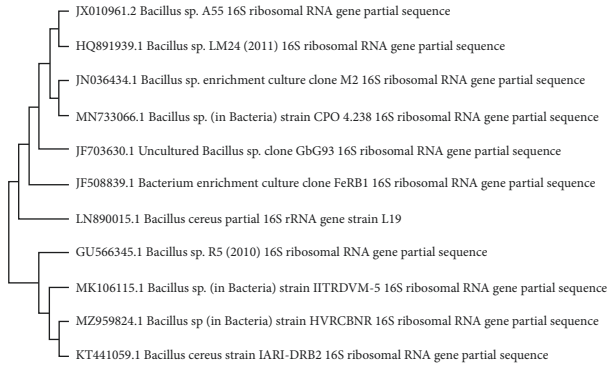


Figure 3: Phylogenetic tree indicating the taxonomic position of *Bacillus* sp. strain HVRCBNR.

Figure 4: TLC analysis for the presence of degraded compound in the treated sample.

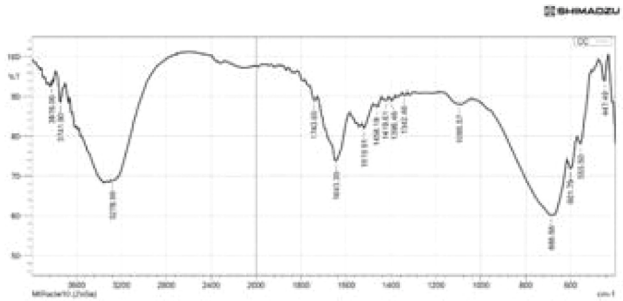


Figure 5: Growth of strain HVRCBNR in MSM-containing battery contaminated soil and its degradation potential.

biodegradation of batteries. The results showed that *Geotrichum candidum* was capable of degrading battery with a rate of 23% while *Rhizopus stolonifer* was capable of degrading battery with a rate of 7%. Of all the mentioned studies, *Bacillus* sp. strain HVRCBNR exhibited the highest degradation potential. The microbial biodegradation depends on many factors like nutrient and substrate bioavailability, oxygen availability, electron acceptors, temperature, pH, salinity, and pressure [30]. The FTIR analysis of the control and the treated showed variations in functional groups indicating degradation of battery waste (Figures 6(a) and 6(b)). The analysis of control sample showed peaks in the regions, 3741.90 cm^{-1}, 3278.99 cm^{-1}, 1743.65 cm^{-1}, 1643.35 cm^{-1}, 1519.91 cm^{-1}, 1458.18 cm^{-1}, 1419.61 cm^{-1}, 1396.46 cm^{-1}, 1343.46 cm^{-1}, 1095.57 cm^{-1}, 686.66 cm^{-1}, and 601.79 cm^{-1}. The FTIR spectrum of the treated sample showed the disappearance of peaks at 3278.99 cm^{-1} (Alcoholic OH stretching vibration) and 1643.35 cm^{-1} (C=C A.

3.4. Phytotoxicity Analysis. The phytotoxicity study was performed using *Trigonella foenum-graecum* and parameters such as root and shoot heights and percentage of toxicity were measured. The average root and shoot heights of the control were 2.6 and 2.1, respectively, and that of treated were 2.2 and 2.3, respectively. The percentage of the toxicity was found to be 11.54% indicating that the metabolites produced after degradation of battery waste are less phytotoxic when compared to the untreated sample. Similar study was performed by Branzini and Zubillaga [31] on *Sesbania virgata* which showed similar results but showed germination seed delay.

3.5. Chemical Analysis. The protein and carbohydrates contents were estimated using Lowry's and anthrone methods, respectively. The protein content of the treated showed an absorbance of 0.41 which is closer to the control with the value of 0.36 and the carbohydrate content of the treated showed an absorbance of 0.028 which is closer to the control with the value of 0.024 indicating that the hazardous components were almost degraded by the strain and the metabolites produced did not affect the growth of the plant in any aspect (Figure 7).

4. Conclusion

A novel *Bacillus* strain was isolated from the battery-contaminated soil and was characterized phenotypically and genotypically. It was deposited in the GenBank under the accession number MZ959824 and was designated as

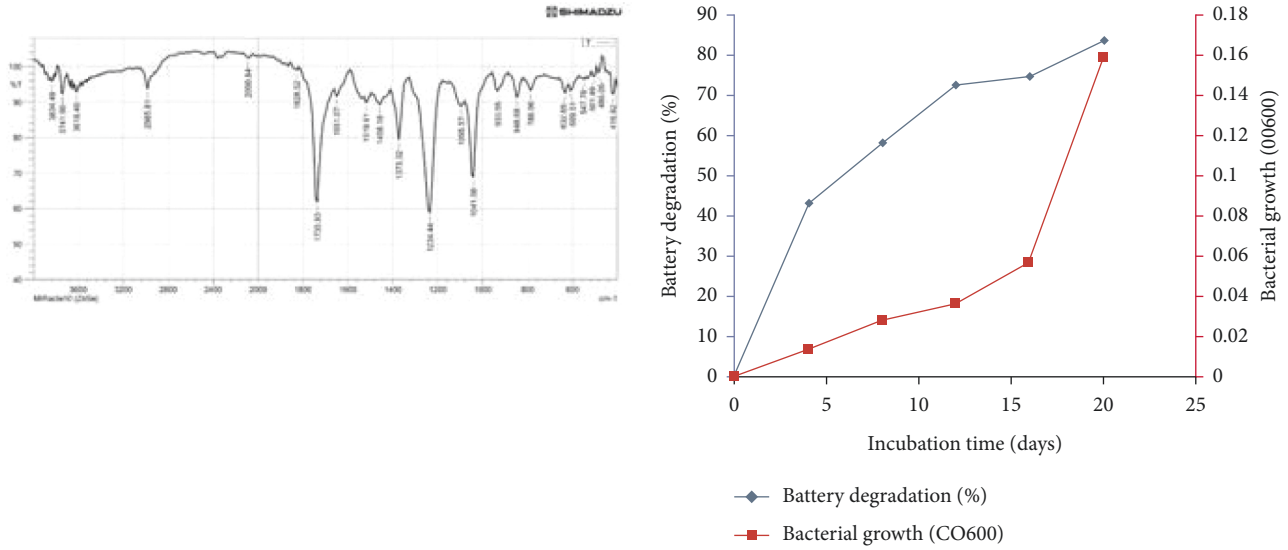


Figure 6: (a) FTIR analysis of control and (b) FTIR analysis of treated sample.

Figure 7: Comparison of growth of seedlings (tap water, treated with HVRCBNR strain, and untreated containing the battery contaminants).

HVRCBNR. The results interpreted from this study showed a degradation potential of 83.6% by *Bacillus* sp. strain HVRCBNR. The treated soil sample analysed using *Trigonella foenum-graecum* seeds by the phytotoxic and chemical analysis method revealed that they have less toxic effects on the plants and are found to be safe. There is a necessity to deal with the battery waste generated in tons annually across the global. Bioremediation is found to be safe and an economical approach to deal with this problem. Further studies are needed to understand degradation mechanism of battery-contaminated soil under natural conditions by *Bacillus* sp. strain HVRCBNR.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors are thankful to the Department of Biotechnology, Centre for Bioscience and Nanoscience Research, for providing all the necessary facilities required to carry out the research work.

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