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Molecular Characterization of Bacteria Isolated from E-waste Dumping Yards at Hyderabad, Telangana, India

Gayatri Y* and Shailaja Raj M

St. Francis College for Women, Begumpet, Hyderabad, Telangana, India

***Corresponding author:** Gayatri Y, St. Francis College for Women, Begumpet, Hyderabad, Telangana, India, Tel: +919704103888; E-mail: yeturigayatri82@gmail.com

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Abstract

Electronic waste or E-waste refers to electronic devices which are disposed into soil without being processed. These electronic goods are made of plenty of components which may contain toxic substances like heavy metals. Lead, one of the heavy metals present in many electronic goods gets accumulated in the soil and leaches into the surrounding environment if the goods are not processed properly. The metal is toxic at very low concentrations and may damage the flora and fauna. So the present study focuses on isolation and identification of bacteria from the samples collected from different E-waste dump yards located in Hyderabad, India. Based on cultural characteristics, biochemical tests and the ability of the organisms to adsorb lead present in the soil contaminated with the metal, the organisms were identified as *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus badius* 16SrRNA sequencing studies confirmed the identified strain as *Bacillus licheniformis* with an accession no. CP000002.3.

Keywords: E-waste; *Bacillus licheniformis* CP000002.3; Lead resistant bacteria; 16SrRNA gene sequencing

Introduction

The largest growing manufacturing industry is the electronic industry in the world [1]. The technological advancement is posing a new environment challenge in the form of “Electronic waste” or “E-waste”. The electronic devices are made up of plenty of components which may contain heavy metals like Lead, Cadmium, Beryllium, Zinc, Mercury, Copper etc. These components if not processed properly after their disposal leach into soil and pollute the environment [2]. Environmental pollution by any source has adverse effects on biological systems directly or indirectly. These heavy metals are toxic even at low concentrations [3]. The toxic materials which are used in the manufacture of electronic goods get accumulated in landfills because of informal processing [4]. These hazardous substances are present in different electronic devices such as Lead and Cadmium in circuit boards, Lead Oxide and Cadmium in Monitor, Cathode Ray Tubes (CRTs), Mercury in switches and flat screen monitors, Cadmium in computer batteries, Polychlorinated biphenyls (PCBs) in older capacitors and transformers, brominated flame retardants on printed circuit boards, plastic casings, cables and polyvinyl chloride (PVC) cable insulation [5].

Lead, an important component of different electronic goods can cause a wide range of adverse effects on biological systems depending on the concentration and duration of exposure. The effects include problems in kidneys, gastrointestinal tract, joints, reproductive system and chronic damage to CNS [6]. To minimize the effects of lead which is present in landfills of electronic waste different approaches have been followed like chemical oxidation, reduction, adsorption, precipitation, etc. A novel technique, biosorption which is efficient, eco-friendly and cost-effective proves to be a better alternative. Microorganisms play a prominent role in the removal of metals from the environment. Algae, bacteria, fungi and yeasts have been shown to be potential metal biosorbents. The negatively charged cell walls of bacteria adsorb positively charged cationic metals (eg: Pb) [7]. It has been reported that bacteria like *Pseudomonas* sp., *Chryseomonas luteola* and *Bacillus circulans* are involved in the removal of lead from wastewaters [8-10]. In this study, the biosorption ability and capacity of different bacteria isolated from E-waste soil for lead adsorption or bioaccumulation was investigated under various conditions.

Materials and Methods

Isolation of bacteria from the E-waste dump yards

The soil samples were collected from Maheshwaram and Shamshabad dumping yards in sterile vials separately. A stock solution was prepared by dispensing 1 g of soil sample into 100 ml sterile distilled water and 0.1 ml of the stock was inoculated onto nutrient agar media and the plates were incubated at 37°C for 24 h and were preserved on nutrient agar slants for further analysis.

Screening for lead resistant bacteria

Lead resistant bacteria were detected by inoculating the cultures isolated from E-waste dump yards onto nutrient agar and minimal agar media containing 0.1% Lead acetate and the plates were incubated at 37°C for 24 h.

Characterization of the organisms

The isolated microorganisms were identified by Gram Staining Method and by biochemical tests 6 and 16 S r RNA typing [11,12].

16S r RNA typing

The 16S rRNA gene is used as the standard for classification and identification of microbes, as it is present in most microbes and shows proper changes. Type strains of 16S rRNA gene sequences for most bacteria and archaea are available on public databases. In the present study, DNA was isolated and evaluated on 1.0% Agarose Gel. A single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

Effect of culture media on the microbial growth

0.1 ml of the sample from pure culture was inoculated into 50 ml of Nutrient broth and 50 ml of minimal broth.

Effect of static and shaker conditions

0.1 ml of the sample from pure culture was inoculated into flasks containing 50 ml of Nutrient broth and 50 ml of minimal broth and incubated under static conditions at 37°C for 72 h and in orbital shaker (REMI) at 120 rpm, 37°C for 72 h.

Effect of substrate on microbial growth

0.1 ml of the cultures was inoculated into flasks containing minimal broth (100 ml) with different concentrations of lead acetate (0.05%, 0.1%, 0.3% and 0.5%) and was incubated at 37°C for 5 days.

Results

Characterization of Lead resistant bacteria

The samples analysed showed colony morphology and microscopic observation similar to that of the standard culture obtained and procured. The colonies were with regular margins, opaque, mucoid, creamy colonies and on gram staining, Gram-positive bacilli were observed as seen in **Figure 1.** The Cultures labelled as EWS1, EWS2 and EWS3 were Gram-positive bacilli, aerobic and spore formers, which belong to genus Bacillus. All the 3 cultures hydrolysed starch while EWS 1 and EWS 2 was VP test positive EWS3 was VP negative. The cell diameter for EWS1 and EWS 2 is less than 1 mm whereas for EWS2 it is greater than 1 mm. Citrate test confirmed that EWS3 is *Bacillus badius* based on Bergy's Manual of Bacterial identification. Growth in 6.5% NaCl and at a temperature 55°C could differentiate EWS1 and EWS2 as *Bacillus licheniformis* CP0000023 and *Bacillus subtilis*. *Bacillus licheniformis* CP000002.3 (EWS1) grows at 55°C but EWS 2 does not grow at that temperature as seen in **Table 1**. EWS1 culture was analyzed by 16S rRNA typing and the result was similar to the biochemical analysis. EWS1 culture was identified as *Bacillus licheniformis*; YDGW3; EU334004 as the S_ab score is 0.913 and the Unique common oligomers were 1246. The 16S r DNA gene sequence was used to carry out BLAST with the database of NCBI genbank database **(Table 2).** Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 7 **(Figure 2).**

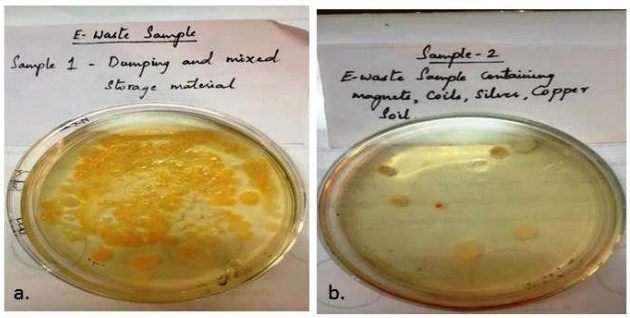

Figure 1 Isolation of Lead resistant bacteria from soil samples collected from different E-waste dump yards in Hyderabad, Telangana, India; (a) The soil sample collected from Maheshwaram Mandal dumping yard, (b) The soil sample collected from Shameerpet dumping yard.

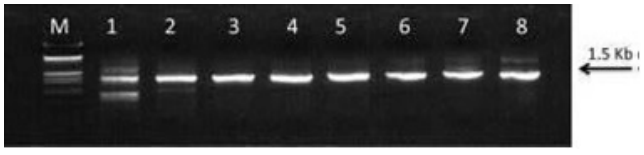

Figure 2 16S rDNA PCR amplicon carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Standard Marker (M), Lane 1- 8 indicating increasing amplification of PCR product.

Effect of substrate concentration

0.1 ml of *Bacillus licheniformis* CP000002.3 (EWS1) culture when inoculated into minimal broth with different lead concentrations like 0.05%, 0.1%, 0.3% and 0.5% and incubated at 37°C there was a coagulant observed at the bottom of the tubes at different time intervals. On the third day of inoculation coagulation was observed in tubes with 0.05% and 0.1% Pb, and

partial coagulation was observed in tubes with 0.3% and 0.5% Pb. On fifth day coagulation was observed in 0.3% Pb and partial coagulation in tube with 0.5% Pb and on seventh day coagulation was observed in tube containing 0.5% of Pb as seen in **Figure 3**.

Table 1 Biochemical tests for identification of isolated bacteria

Identification	Starch Hydrolysis	VP Test	Cell diameter>1mm	Citrate	6.5% NaCl growth
EWS1	+	+	-	+	+
EWS2	+	+	+	+	-
EWS3	+	-	-	-	-

Table 2 Blast report of Baillus licheniformis CP000002.3 (EWS1)

Short ID	S_ab Score	Unique common oligomers	Sequence full name
S000721095	0.912	1152	*Bacillus licheniformis*; TY7; AB250304
S000980352	0.910	1205	*Bacillus licheniformis*; 3EC4A3; EU304928
S000980364	0.913	1212	*Bacillus licheniformis*; 3EC4A15; EU304940
S000980815	0.913	1246	*Bacillus licheniformis*; YDGW3; EU334004
S002918124	0.908	1253	*Bacillus licheniformis*; NM18; JN409995
S002965183	0.911	1290	*Bacillus licheniformis*; SCK 121059; JQ030983
S003756091	0.911	1252	*Bacillus licheniformis*; RGS23; KC469614

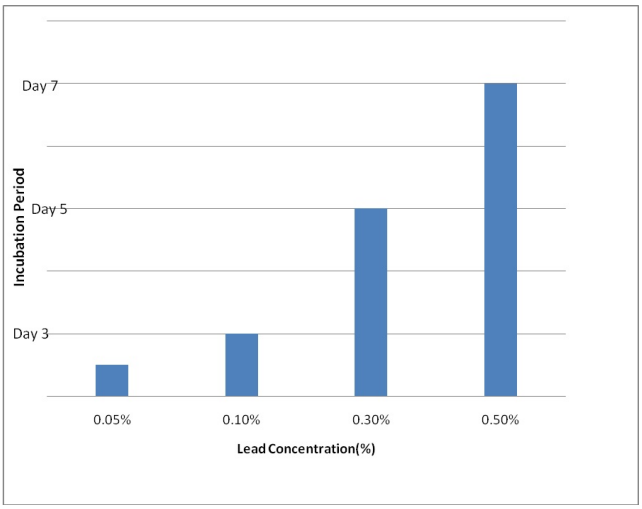

Figure 3 Effect of substrate concentration on coagulant formation with respect to incubation period. The time taken for the coagulant formation increased with increase in substrate concentration.

As the concentration of lead increased the time for coagulation also increased which indicates the ability of the organism *Bacillus licheniformis* CP0000023 (EWS1) to sustain high metal concentrations in correlation with the studies carried out by Ray et al., on lead resistance by *Bacillus cereus* the adsorption increased with an increase in initial lead concentration ranging from 25-150 mg/l [13]. The studies also revealed that *Bacillus licheniformis* could sustain upto 100 ppm of Lead but here we are reporting the strain of *Bacillus licheniformis* that can sustain upto 0.5% of Lead concentration [14]. Microscopic observation of coagulant under Phase contrast revealed that the bacteria were found enmeshed with lead in the coagulant as seen in **Figure 4.**

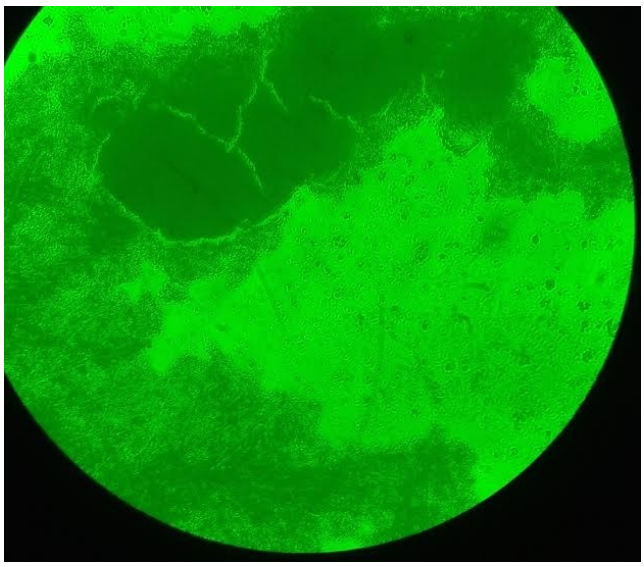

Figure 4 Phase contrast microscopic view of lead adsorbed onto Bacillus licheniformis CP000002.3 (EWS1).

Effect of culture media on the microbial growth

To determine the effect of only the metal on the growth of the organism 0.5 ml of *Bacillus licheniformis* CP000002.3 (EWS1) was inoculated into 50 ml nutrient broth and 50 ml of minimal broth and incubated at 37°C for 48 h. Growth was luxuriant in both the media.

Both can be used for culturing purposes. The growth in minimal media proves that the organism can grow effectively in presence of metal ion with minimal amounts of nutrients available in. So it was used as culture media for the growth of EWS1.

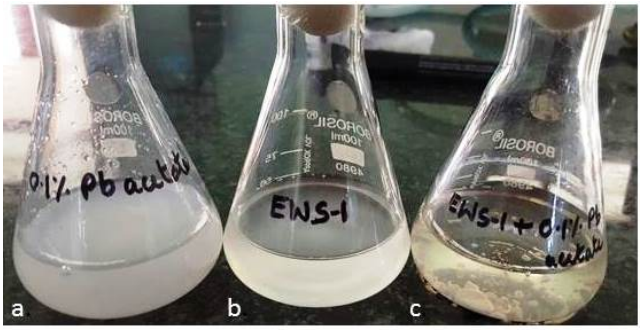

Figure 5 (a) Flasks incubated in the orbital shaker with 0.1% metal as control, (b) Innoculated with Baillus licheniformis CP000002.3(EWS1) without metal and inoculated with Baillus licheniformis CP000002.3 (EWS1), (c) 0.1% metal concentration where the coagulant is observed.

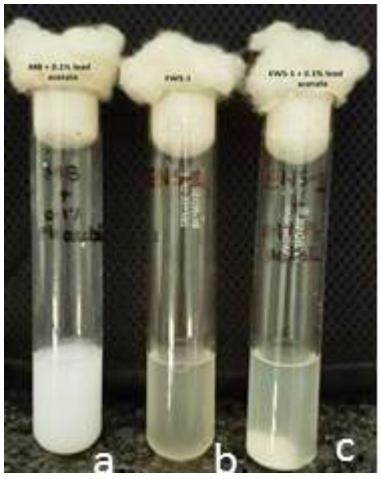

Figure 6 (a) Tubes incubated under static conditions with 0.1% metal (control), (b) with Baillus licheniformis CP000002.3 (EWS1) without metal and with EWS1 and (c) 0.1% metal concentration where the coagulant is observed.

Effect of static and shaker conditions

Minimal broth (50 ml) with 0.1% Lead acetate when inoculated with *Bacillus licheniformis* CP000002.3 (EWS1) incubated at 37°C, 120 rpm in orbital shaker incubator (Remi) the coagulant formation was observed after 14 days in comparison with minimal broth (10 ml) with same concentration of lead acetate and *Bacillus licheniformis* CP000002.3 (EWS1) the coagulant formation was observed after 7 days under static conditions at 37°C **(Figures 5 and 6).** Control was maintained with the same concentration of Lead acetate without the organism for both the conditions. There was no coagulant formation in the control which was maintained under the same conditions as that of test samples. There is a reduction in the time for the adsorption under static conditions.

Discussion and Conclusion

Electronic waste is the growing waste in the world. The waste is getting accumulated in the soil as the processing techniques employed are not proper. Earlier studies reveal that bacteria like *Thiobacilli.* and fungi like *A. nige*, *P. simplicissimum* are involved in mobilization of metals from electronic and electrical wastes [15]. *Bacillus subtilis* and *Bacillus cereus* were used to have better lead adsorption capacity from aqueous solutions [13,16]. The present study focused on the isolation of the bacteria resistant to lead from the Electronic waste dumping yards soil. Gram-positive bacilli were isolated from the samples and were identified as *Baillus licheniformis* CP000002.3, *Bacillus subtilis* and *Bacillus badius* based on the biochemical tests. 16SrRNA sequencing results also confirmed that EWS1 as *Bacillus licheniformis* YDGW3; EU334004 as the the S_ab score was 0.913 and the Unique common oligomers were 1246 whereas for the other strain *Bacillus licheniformis*; 3EC4A15; EU304940 though the s_ab score was same as the previous the unique common oligomers were 1212. So the strain with maximum similarity was considered. The three cultures were inoculated into minimal broth (100 ml) with different concentrations of lead acetate ranging from 0.05% to 0.5% there was a coagulant formation in the broth with *Baillus licheniformis* CP000002.3(EWS1) but not in the broth with EWS2 and EWS3 cultures. The coagulant may be formed due to adherence of the metal onto the surface of the cell which could conclude that *Bacillus licheniformis* CP000002.3 (EWS1) has the capability to adsorb lead which makes a way to use this organism as an agent to remove lead from metal polluted areas. The maximum tolerance level of *Bacillus licheniformis* CP000002.3 for lead was 0.5% which is significantly higher than most reported metal tolerance levels. It is proved that *B.licheniformis* has 65-70% biosorption efficiency for lead metal ions at 10 and 50 ppm [17]. It was also observed that as the metal concentration increased the adsorption also increased which may be because of enhanced electrostatic interactions of the cell with metal ions [18,19]. *Bacillus licheniformis* was naturally tolerant up to 50 ppm of Lead concentration [14]. The cells might uptake the metal ions and deposit them on their surface or intracellularly by a process referred to as Bioaccumulation and has been reported for many metals including lead [17]. The lead resistant bacteria employ different resistance mechanisms like efflux mechanism, extracellular sequestration, biosorption, precipitation, alteration in cell morphology, enhanced siderophore production and intracellular lead bioaccumulation [2]. The microorganisms like bacteria, fungi, yeast and algae reduce toxic metal ions to non-toxic state either by adsorbing onto the cell surface or accumulate and complex inside the cell [20]. Aeration is an important parameter for the growth of aerobes but the growth of *Baillus licheniformis* CP000002.3 (EWS1) was good even at static incubation condition which emphasizes that for the adsorption of the metal in the metal polluted areas the available aeration is sufficient for the adsorption. This study shows possibilities for development of eco-friendly and effective technologies for the removal of heavy

metals from metal contaminated areas. Complete genome sequencing of the above strains could be further investigated during the future study.

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