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OPTIMIZING THE MANAGEMENT OF LEAD BIOACCUMULATION CAPACITY OF METAL-RESISTANT *PSEUDOMONAS STUTZERI* (IC29 MK965194) ISOLATED FROM THE E-WASTE CONTAMINATED SOIL SAMPLES

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ABSTRACT

E-waste is major environmental pollutants when present in soil and show potential toxic effects on growth and development in plants and animals. E-waste contains a number of toxic materials like lead, mercury, cadmium, hexavalent chromium and brominated flam. Most of the metal tolerant microbes as used for the remediation, this point of view, and the present investigations were carried out to isolate metal tolerant bacterial species from the E-waste contaminated sites on Trichy area. Totally 44 colonies were observed from 3 soil samples, among them 6 bacterial genera and 9 species were obtained. Among the 44 isolates, single isolate was tolerating up to 14mM concentration of lead. This isolate was confirmed as *Pseudomonas stutzeri* by using 16srRNA sequence. The bioaccumulation of started with media optimization and results showed maximum accumulation of heavy metal (lead) at 37°C temperature, pH 7.0, lactose and yeast extract as carbon and nitrogen source respectively. The results demonstrated that under such ideal condition bioaccumulation of Pb by *Pseudomonas stutzeri* was found to be at a maximum of 1144μg accounting to 39.4%.

Keywords: - E-waste, Metal, Lead, Bioaccumulation, *Pseudomonas stutzeri*

INTRODUCTION

The state of the environment is deteriorating, which is the result of population growth, industrialization and technological advancement. Further a long list of hazardous substances is published daily, which contributes to the global pollution burden. As part of this 'list', heavy metals are produced by industrial waste,

electronic waste, etc., which, if not properly mitigated, pose a serious threat to humans, animals and the environment.

The rapid growth in the production of electrical and electronic products represents the same rapid growth as the amount of electronic waste (e-waste), which is a major threat to India's environment. Environmental damage during e-waste recycling is mainly due to the entry of materials such as metal in e-waste into soil and water bodies [1].

In developing countries, the illegal e-waste sector is rising rapidly over in developed countries due to cheapest labor costs and week-long legislative structures. Contaminates have been reported to pass through the food chain via the root plant translocation mechanism to the human body, thus endangering human health [2]. Lead is a hazardous waste and is highly toxic to humans, plants and animals [3]. In humans chronic lead exposure produces neurotoxicity, anaemia and kidney damage and acute lead toxicity can be fatal [4].

Number of conventional methods is available for remediation of e-waste containing heavy metals from environment, however, those methods are associated with various disadvantages like high operational/maintenance cost, incomplete metal removal, low throughput, generation of

sludge, high reagent and energy requirements, aggregation of metal precipitates and fouling of the membranes [5].

Micro remediation is employed to transform toxic heavy metals into a less harmful state using microbes or its enzymes to clean-up a polluted environment. The technique is environmentally friendly and cost-effective in the revitalization of the environment [6]. There are 6 major principles in micro remediation of toxic metals, among them bioaccumulation are one of the technique with safe. Bioaccumulation and toxicity of Pb was also studied in some microbes. Microorganisms have great potential in heavy metals remediation and that bacteria possess a variety of mechanisms to deal with high concentrations of heavy metals and often are specific to one or a few metals [7]. This bioaccumulation is affected by various factors like media components, temperature and pH.

The present study aims to investigate the ability of natural inhabitant bacteria, isolated from the E-waste containing soil, in reducing and detoxifying Pb at privileged conditions, identification of those bacteria up to species level and determinate the bioaccumulation level on various media composition.

METHODOLOGY

Collection of soil samples

The soil samples were collected from 3 different points (Ariyamangalam, Trichy district TN, India), each separated by a distance of 200m using a sterile stainless steel spatula at 5 inches below the surface. The collected samples from all the points were collected in pre-sterilized, labeled, amber-colored glass bottles and the samples were transported to the laboratory in an ice-cold box maintained at 4°C. The enumeration of lead tolerance bacterial populations was estimated by following appropriate methodologies, described further.

Enumeration of lead (Pb) resistant bacteria

Lead resistant bacteria counts were enumerated by following the method of Rani *et al*. [8]. For this 0.1 ml of an aliquot of serially diluted soil suspensions were individually inoculated onto sterile Nutrient agar (NA) plates amended with 0.5mM of Pb in triplicates. All the plates were incubated at 37±2 °C for 48 hrs. After incubation, the plates having Pb resistant colonies were observed and counted.

Identification of Pb tolerance bacterial population

The enumerated Pb tolerance bacteria (44 isolates) were first isolated based on the differences of their morphological character and individually stored in NA slants at 4°C for further identification. These isolates were identified up to the genus level, by a combination of information from primary and secondary identification. Primary identification was performed based on colony morphology and by Gram staining. Representative colonies that observed on agar plates were checked for purity through microscopy and the pure isolates were carefully picked aseptically and streaked on NA slants and stored at 4°C until secondary identification. Secondary identification was carried out by performing a series of biochemical tests. Both primary and secondary identifications were carried out on the physical characterization and the biochemical tests outlined in Bergy's Manual of Determinative Bacteriology [9].

Screening for Pb tolerance efficiency - MIC

Lead resistance efficiency was carried out by analyzing the MIC (Minimum Inhibitory Concentration) of heavy metal (Lead) for those Pb tolerance bacterial strains through the agar plate method. The MIC was determined by the plate dilution method against metal by gradually increasing its concentration in nutrient agar. The initial concentration used was 1mM/20ml with a

gradual increase until the strains failed to give colonies on the plate, which was 15mM/20ml. The lowest concentration of Pb that prevented bacteria to grow was considered as the MIC for that particular isolate.

Molecular characterization

The Pb resistant strain that demonstrated the maximum resistivity among the 3 sampling sites was identified up to species level by 16s rRNA sequencing and phylogenetic analysis.

Optimization of Pb bioaccumulation

The optimum growth conditions such as different pH, temperature, carbon and nitrogen sources were tested for the highly tolerant bacterial isolates. The medium was inoculated with *Pseudomonas stutzeri* at the concentration of 10^8 cells/ml and incubated at 37°C for 72 hrs at 150 rpm in a rotary shaker. Samples were collected at 72 hrs and culture fluid was centrifuged at 5500 rpm for 15 min and the concentration of lead present was estimated using inductively coupled plasma - optical emission spectrometry (ICP-OES). The uninoculated medium was used as the blank. After optimization process, best parameters were selected from above mentioned process and subjected to determination of bioaccumulation level by ICP-OES. Biological removal efficiency (BRE) was calculated as shown in the following formula:

BRE % = Primary lead concentration (non inoculated)

Residual lead concentration inoculated / Primary lead concentration (non inoculated) X100 [10]

Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and was analyzed using a student's "t" test and Two-way ANOVA test at a 5% level of significance using computer software SAS 9.4 (Statistical Analytical System, North Carolina, and USA).

RESULT AND DISCUSSION

The Pb resistant bacteria population was isolated from 3 different sites of the E-waste contaminated soil. A totally 44 morphologically dissimilar colonies were observed from all soil samples. Among them, 7 of them were isolated from site 1, 2 of the isolates were from site 2 and 35 isolates from site 3. The isolates were identified up to species level and found to belong to 9 species. Among them, 23 (52.2%) of were gram-positive and 21 (48%) were gram-negative. The gram-positive isolates included *Bacillus subtilis*, *Bacillus cereus Micrococcus luteus* and *Bacillus megaterium* accounting for 15.91% (n =7), 20.45% (n =9), 9.09% (n =4) and 6.82% (n =3) respectively. The gram-negative isolates

included *Serratia marcescens, Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli* and *Pseudomonas stutzeri* accounting to 6.82% (n =3), 11.36% (n =5), 18.18% (n =8), 9.09% (n =4), and 2.27% (n =1) respectively.

The earlier study of Sanusi *et al.,* [11] observed the various types of bacterial genera from e-waste dump site soil samples. Gayatri *et al.,* [12] have isolated *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus badius* as Pb resistance isolates from the E-waste dumping sites in Hyderabad, Telangana, India. In 2017, Anjanapriya and Lalitha [13] also identify the heavy metal resistant organisms from solid waste dumpsite in Madurai, belonged to identify as *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* and *Bacillus thuringiensis.*

Among the 44 isolates, *Pseudomonas stutzeri* (IC29) was resistance uptp 14mM concentration of metal and which was belonging to site 3 accounting to 2.27%. Similar to our study Haroun *et al.,* [14] findings recorded 10mM of tolerance by *Pseudomonas aeruginosa* isolated from Makera-Kakuri industrial drain in Kaduna, Nigeria. The study by Verma *et al.,* [15] demonstrated a lead tolerance of 5mM by

Pseudomonas stutzeri heavy metal contaminated sites of Udaisagar Lake and Gadwa pond, Udaipur, Rajasthan, India. The greater resistance of 14mM by *Pseudomonas stutzeri*, when compared to the other microbes isolated in our study, shall be attributed to their difference in their resistance mechanism [16] which shall be accomplished by biosorption and/or bioaccumulation mechanism [17]. This isolate was confirmed with 16srRNA sequence and deposited in to NCBI and get accession number (IC29 MK965194) **(Figure 1)**.

Optimization studies by *P. stutzeri* on bioaccumulation of Pb, revealed that pH 7, temperature 37°C, lactose and yeast extract both at 1% concentration demonstrated maximum bioaccumulation activity. The bacterial tolerance to heavy metals is not only affected by the membrane properties of the bacterium but also on abiotic factors like pH, temperature and substrate concentrations [18]. Similar effects of lead bioaccumulation have also been observed for bacteria such as *Sphaerotilus natans* [19], *Pseudomonas* sp. [20], *B. pumilus* [21] and *Achrombacter* sp. TL-3 [22]. As the pH increased from 7 in our study the uptake of lead decreased which shall be

accounted to the fact that there was a decrease in solubility lead [23] (Figure 2).

In the present study maximum bioaccumulation of lead (28%) was recorded at 37°C. A similar study reported by Shruti *et al.,* [24] demonstrated that the maximum uptake of Pb by *B .cereus* was at 37°C again substantiates our findings. Temperature effects are always limited to metabolism-dependent metal accumulation [25]. Kamsonlian *et al.,* [26] reported that the uptake of heavy metal ions was dependent on temperature and there was a maximum uptake of metal ions from 25-40°C.

Optimization of various carbon sources on bioaccumulation of Pb by *P. stutzeri* revealed that lactose as the chief carbon source demonstrating 17.3% of bioaccumulation activity. Qu *et al.,* [27] reported different carbon sources to have a crucial role in the activities and metabolisms of microbes. The study reported by Maleej *et al.,* [28] on optimization of *Pseudomonas stutzeri* AS 22 for its heavy metal binding capacity had revealed starch as the optimized carbon source. Whereas in various nitrogen sources on bioaccumulation of Pb by *P. stutzeri* revealed that yeast extract as the chief yeast extract source demonstrating 31.2% of bioaccumulation activity. Different nitrogen sources were used by Kumar and

Riyazudin [29] and found the increase in the biosorption and bioaccumulation process. They used a mixture of peptone and beef extract as organic nitrogen sources in medium containing different heavy metals (Co, Cd Ni and Pb) for biosorption by *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp. and *A. niger* (Figure 3).

Statistical variation of different parameters on bioaccumulation of Pb

The statistical Two-way ANOVA test revealed that the variation between pH on bioaccumulation of Pb by *Pseudomonas stutzeri* was significant (F = 15.8284; P< 0.01). Similarly, the variation between the temperature on Pb bioaccumulation was also statistically significant (F= 34.2086; P< 0.01). Concerning the variation between carbon sources on bioaccumulation of Pb by *Pseudomonas stutzeri* demonstrated an F- the value of 48.0849 and P< 0.001 which is statistically significant. Also nitrogen sources variation on exhibited statistically significant value of F = 41.9818 and P< 0.001 (Table 1).

The statistical student's t-test was conducted on the results of bioaccumulation revealed that the values are statistically significant with t- value = 3.9547 to 6.9343 whereas P-value ranged from P< 0.01 to P< 0.05 at varied pH, temperature, carbon and nitrogen sources (Table 2).
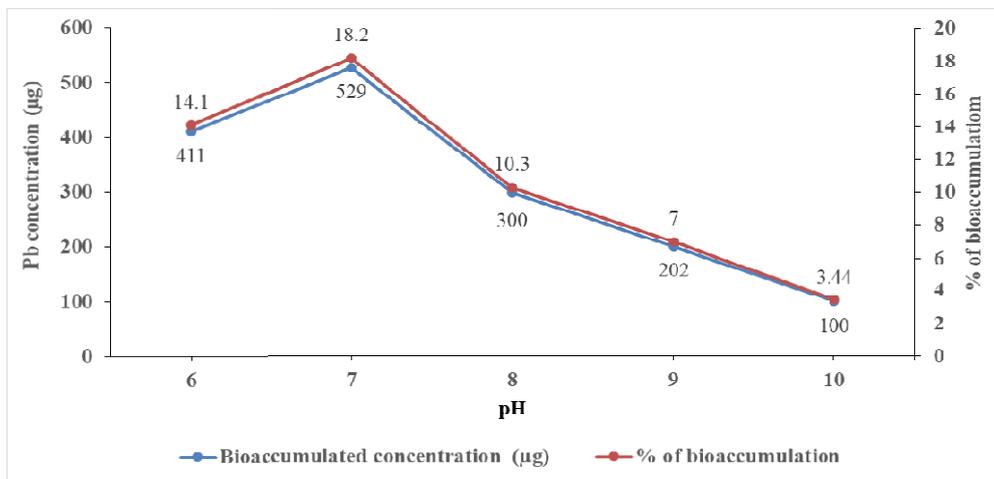
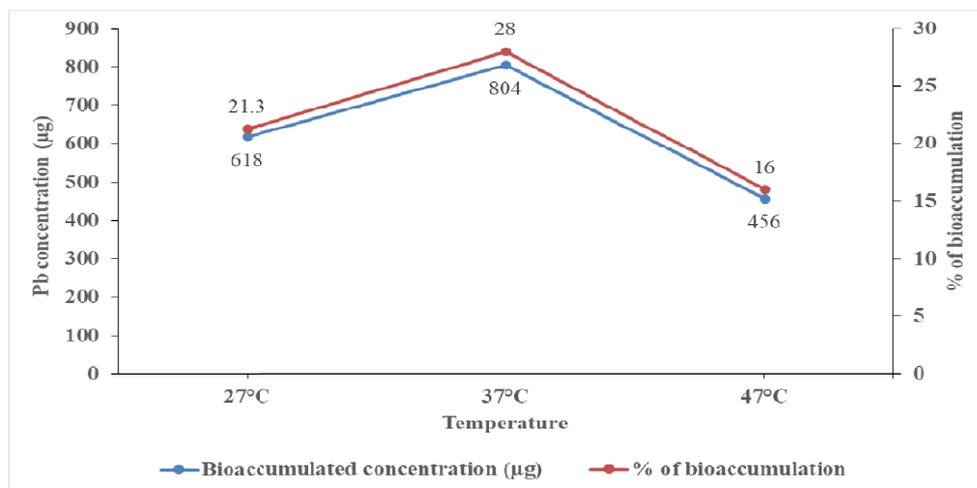**Figure 1: Bioaccumulation of Pb at different pH by *Pseudomonas stutzeri***

Figure 2: Bioaccumulation of Pb at different temperature by *Pseudomonas stutzeri*
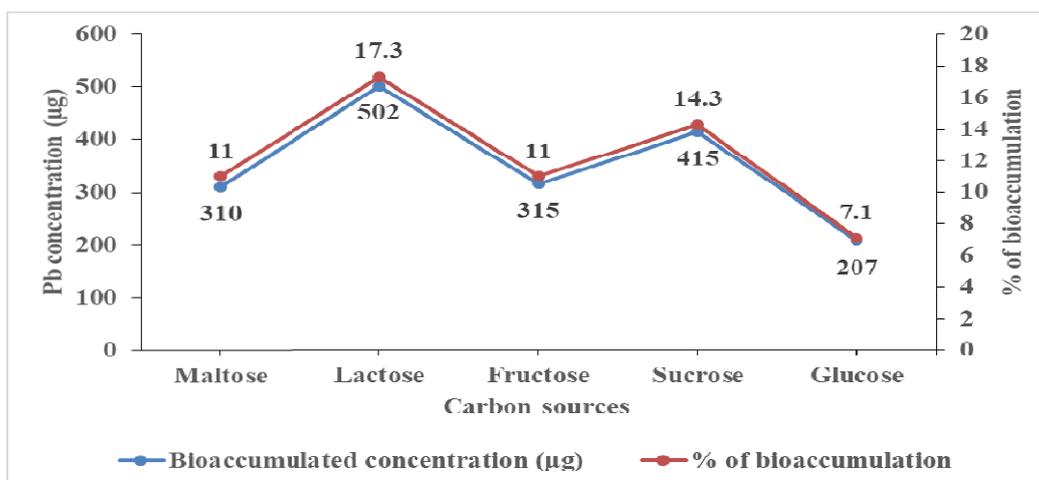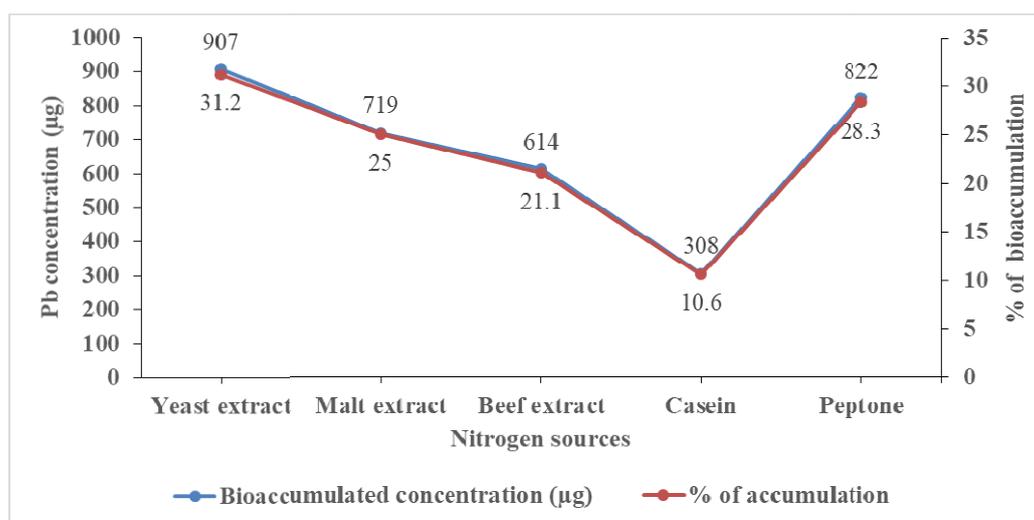

Figure 3: Bioaccumulation of Pb at different carbon sources by *Pseudomonas stutzeri*

Figure 4: Bioaccumulation of Pb at different nitrogen sources by *Pseudomonas stutzeri*

Table 1: Two-way ANOVA for the data on bioaccumulation of Pb by *Pseudomonas stutzeri* as a function of variation between pH, temperature, carbon and nitrogen sources

Source of variance	Sum of Square (SS)	Degrees of Freedom (DF)	Mean Square (MS)	F-value	P-value
pH	225600.4	4	56400	15.8284	P< 0.01
Temperature	520381.5	2	260190.75	34.2086	P< 0.01
Carbon	304153.6	4	76038.4	48.0849	P< 0.001
Nitrogen	1132323	4	283080.75	41.9818	P< 0.001

P< 0.01 and P< 0.001 are statistically significant

Table 2: Summary table of student't' test on bioaccumulation of Pb by *Pseudomonas stutzeri* at different pH, temperature, carbon and nitrogen sources

Parameters	t-value	P-value
pH	3.9547	P< 0.01
Temperature	5.6991	P< 0.05
Carbon	6.9343	P< 0.01
Nitrogen	6.4793	P< 0.01

The present data revealed that under optimized culture conditions of pH 7, temperature 37°C with 1.0% lactose and yeast extract bioaccumulation of Pb by *Pseudomonas stutzeri* was found to at a maximum of 1144µg accounting to 39.4%. The results of the present study are in agreement with the study conducted by Das and Kumari [30]. They found that *Enterobacter* sp. and *Klebsiella* sp. isolated from industrial effluents significantly reduced Pb to 36%. The study by Gawali *et al* [31] illustrated that *E. coli* was able to remove Cu and Pb with removal percentage of 62% and 45% respectively.

This study has revealed and concluded that *P. stutzeri* MK965194 isolated from E-waste contaminated soil, has got the efficient bioaccumulation ability of heavy metals concerning Pb. The study proves that *P. stutzeri* MK965194 can be utilized as a potential candidate for bioremediation of heavy metal contaminated sites. However, still, we need further understanding of the microbial physiology of *P. stutzeri* MK965194, identify the exact mechanisms involved in the uptake, ability of the strain to continually take in heavy metals, the ability to bioaccumulate other heavy metals and complete genome sequencing which will enable in utilizing the candidate for efficient industrial applications. Thus this studies demonstrated possibilities for the development of eco-friendly means of 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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