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Full Length Research Paper

Isolation and biochemical chracterization of heavy metal resistant bacteria from pyrochemical contaminated soil

Srinidhi Thanga Thirupathi[1], Indiraleka Muthaiah[1]*, Sugumari Vallinayagam[1], Premnath Dhanaraj[2]

[1]Department of Biotechnology, Mepco Schlenk Engineering College, Sivakasi, Tamilnadu, India.
[2]Department of Biotechnology, School of Agriculture and Biosciences, Karunya Institute of Technology and science (Deemed to be University), Coimbatore 641114, Tamilnadu, India.

Corresponding author email: indirajith1812000@gmail.com; Tel. 7402389414.

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ABSTRACT

Soil pollution because of metal wastes and a suitable way to mitigate the effects is a big concern nowadays. Because of industrialization, metal and heavy metal contamination has soared up in the past few decades. This has given rise to a plethora of resistant microbial population, which may be quite helpful in the process of bioremediation. In the present study, microbes were isolated from normal soil and pyrochemical contaminated soil from Sivakasi and their tolerance towards various metals like lead, mercury, cadmium, chromium, aluminium, zinc and copper was checked. Isolates from normal soil did not show much tolerance, whereas most of the isolates from pyrochemical contaminated soil were tolerant. Isolate 9, which was predominant in pyrochemical contaminated soil was tolerant to lead, mercury, aluminium, chromium and copper. Colony morphology and biochemical tests reveal that the isolate maybe *Actinomycetes Sp.* Isolate number 9 was found to remove chromium and lead through spectrophotometric and induction coupled plasma analysis respectively. The isolate removed lead drastically and it had an MIC of 1400µg/ml. Role of actinomycetes in remediation of heavy metal contamination has been reported in many studies. However this is the first study that reports these actinocyetes are predominant population present in pyrochemical contaminated site. From this study, it can be concluded that the isolate number 9, an Actinomycete can be considered as a prospective candidate for bioremediation of pyrochemical contaminated soil.

Keywords: Fireworks, Metal tolerance, Actinomycetes, Bioremediation

INTRODUCTION

Fireworks are used in most of the celebrations and events to add up aesthetics. But, they impart a huge impact on not only the environment but also the health of human and other living organisms. India is the second largest manufacturer of fireworks. Sivakasi has been the top manufacturer of fireworks and it single handedly makes India the second largest manufacturer of fireworks (Roysam Varsha, 2016). It produces about 90% of India's total firework. Reports say that there are 450 firework factories in this industrial town, which gives employment

to around 40,000 people. Pollutants released from fireworks include lead, manganese, magnesium, arsenic, beryllium, cadmium, chromium, nickel etc. (Licudine *et al.,* 2012; Satarug, 2018; Gad, 1989). Heavy metals cause several health effects; for example, aluminum used as a major component in firework causes pulmonary aluminosis, encephalopathy, Alzheimer's disease and breast cancer too (Klotz *et al.,* 2017). Lead causes problems like Attention Deficit Hyperactivity Disorder (ADHD) and mental retardation in children, lung cancer and it can affect the central nervous system too (Rajam *et al.,* 2015; Smolkova *et al.,* 2014). Perchlorate is a highly water soluble molecule. It even contaminates the ground water through percolation. It exists in the water for a very long period of time and causes various problems. In Sivakasi the concentration of percolate was between 0.005 and 7,690 µg/L in groundwater. It was between 0.005 and 30.2 µg/L in surface water and between 0.06 to 0.393 µg/L in tap water. The perchlorate in the ground water level in the firework factory areas was comparatively higher than the level of perchlorate in normal area and this proves that firework factories are major contributors of perchlorate contamination. (Tomohiko Isobe *et al.,* 2014)

In Sivakasi town, the presence of heavy metals like cadmium, lead, copper, magnesium, zinc, sodium, magnesium and potassium were found in a concentration higher than the prescribed limit (Stella Muthu Rajam *et al.,* 2015). So, it is the need of the hour to find a proper method to reduce the pollution and bioremediation would be a good method to mitigate pollution. Bioremediation is a permanent solution that can end with degradation or transformation of environmental contaminants into harmless or less toxic forms (Zouboulis *et al*., 2014). Soil microbiomes are natural carriers of bioremediation. Different microbes like *Flavobacterium sp, Achromobacter sp, Bacillus sp. Pseudomonas sp, Corynebacterium sp, Micrococcus sp* isolated have been reported for their heavy metal degradation (Dhasarathan *et al.,* 2010; Aizawa *et al.,* 2010; Hussain *et al*., 2013; Cui *et al.,* 2014; Chatterjee *et al.,* 2012). Hence our aim was to isolate metal tolerant organisms from the pyrochemical contaminated site and identify potent microbial candidate for ~~Bioremediation~~ bioremediation.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from the burn bed (the area where the pyrochemical wastes are dumped and burnt) area of the firework industry and stored in fresh resealable plastic covers. Soil samples were collected from different heights (Level 1- 5cm deep; Level 2- 10cm deep; Level 3- 15cm deep; Level 4 – 20 to 25cm deep). The soil samples were stored at 4^{o}C.

Isolation of microbes from normal and pyrochemical contaminated soil:

Leuria Bertani agar was prepared by suspending 4g of Leuria Bertani agar in 100ml of distilled water and autoclaving. The lukewarm agar was poured into autoclaved petri plates and allowed to solidify. L rod (glass) was used to make spread plate. The L rod was dipped in ethanol and exposed to the flame of the Bunsen burner to make it sterile. The L rod was then allowed to cool down. After solidification of the agar, 100µl of the sample solution (serial dilution) was pipetted out using a micro pipette and spread using the sterile L rod (Ji *et al.,* 2016). The plates were incubated at a temperature of 37^{o}C for 48 hours and then observed.

Primary screening of heavy metal resistant bacteria

For screening of heavy metal resistant bacteria, the bacterial culture was separately cultured in 300 µg/mL of different heavy metals (aluminum, cadmium, chromium, copper, lead, mercury, zinc) incorporated into LB (Luria Bertani) agar plates and plates were incubated at 37 °C. After 24 h of incubation the plates were observed for any kind of development on the culture medium. After preliminary screening, the desired bacteria were isolated by serial dilution method (Azad et al, 2013; Saini *et al*., 2013). Control plates were also prepared with LB media without including any heavy metal for comparison. Colonies differing in morphological characteristics were selected, picked and then preserved on different plates for further studies.

Determination of minimum inhibitory concentration (MIC)

To determine MIC, selected isolates were grown on gently increasing concentration of heavy metal incorporated LB agar media against respective heavy metal (aluminum, cadmium, chromium, copper, lead, mercury, zinc). The concentration of heavy metal was increased until the isolates failed to give colonies on the petri plate. The culture growing on the final concentration was transferred to the higher concentration each time by streaking on the agar plate. When the isolates failed to grow on petri plate, MIC was assessed according to standard protocol of European food safety authority (EFSA) (Batta *et al*., 2013).

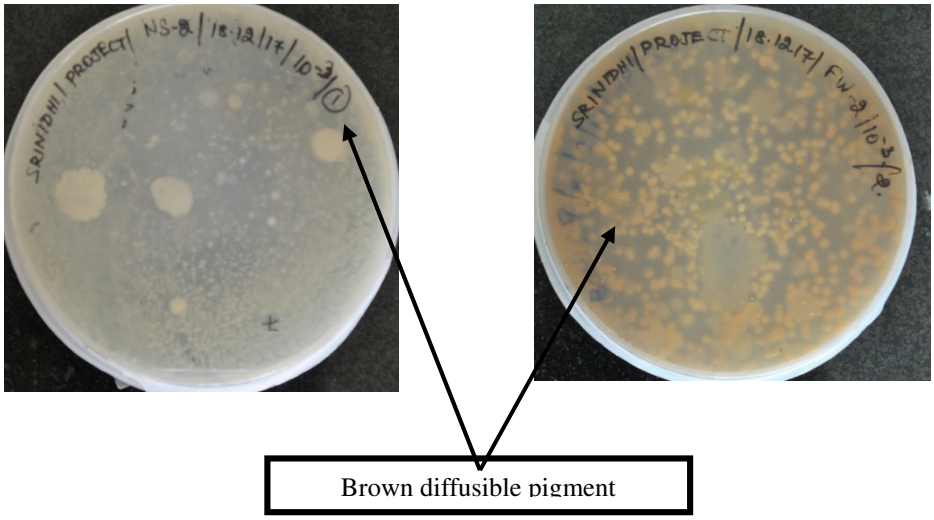


Figure 1. Comparison of microbes isolated from A) normal soil and B) Pyrochemical contaminated soil

Heavy metal biodegradability assay

Bacterial isolates were inoculated into the shake flask containing LB broth medium for one hour in a rotary shaker at 150 rpm and the temperature was maintained at 37 °C. After the culture was reached at 0.6 optical density it was inoculated in the media containing 100 ppm of sterilized heavy metal (aluminum, cadmium, chromium, copper, lead, mercury, zinc) separately in every culture flask and again incubated for 24 h at the same condition. The culture was then centrifuged at 5000 rpm for 15 min and the supernatant was isolated. It was mixed with the double volume of concentrated HNO_3 and heated at 100 °C on a hotplate stirrer to accomplish acid digestion until the final volume decrease and down to initial supernatant volume. The extract was filtered using Whatman filter paper to remove any insoluble material and collected into a volumetric flask and then diluted. Then the heavy metal reduction was analyzed by Atomic Absorption Spectrophotometer and the result was compared with control to calculate heavy metal degradation capacity (%).

Phenotypic and biochemical characterization of bacterial isolates

The bacterial isolates were characterized based on cultural, morphological and biochemical characteristics as described in the Cowan and Steel's Manual for the identification of Medical Bacteria (Barrow and Feltham, 1993). For the activities of oxidase, catalase, methyl red, indole production, citrate utilization and carbohydrate (Glucose, Sucrose, Maltose, Xylose and Lactose) utilization, isolates were biochemically analyzed (Barrow and Feltham, 1993). According to Bergey's Manual of systemic Bacteriology the isolates were provisionally identified up to genus level (Claus and Berkeley, 1986) (Islam *et al.,* 2014).

RESULTS AND DISCUSSION

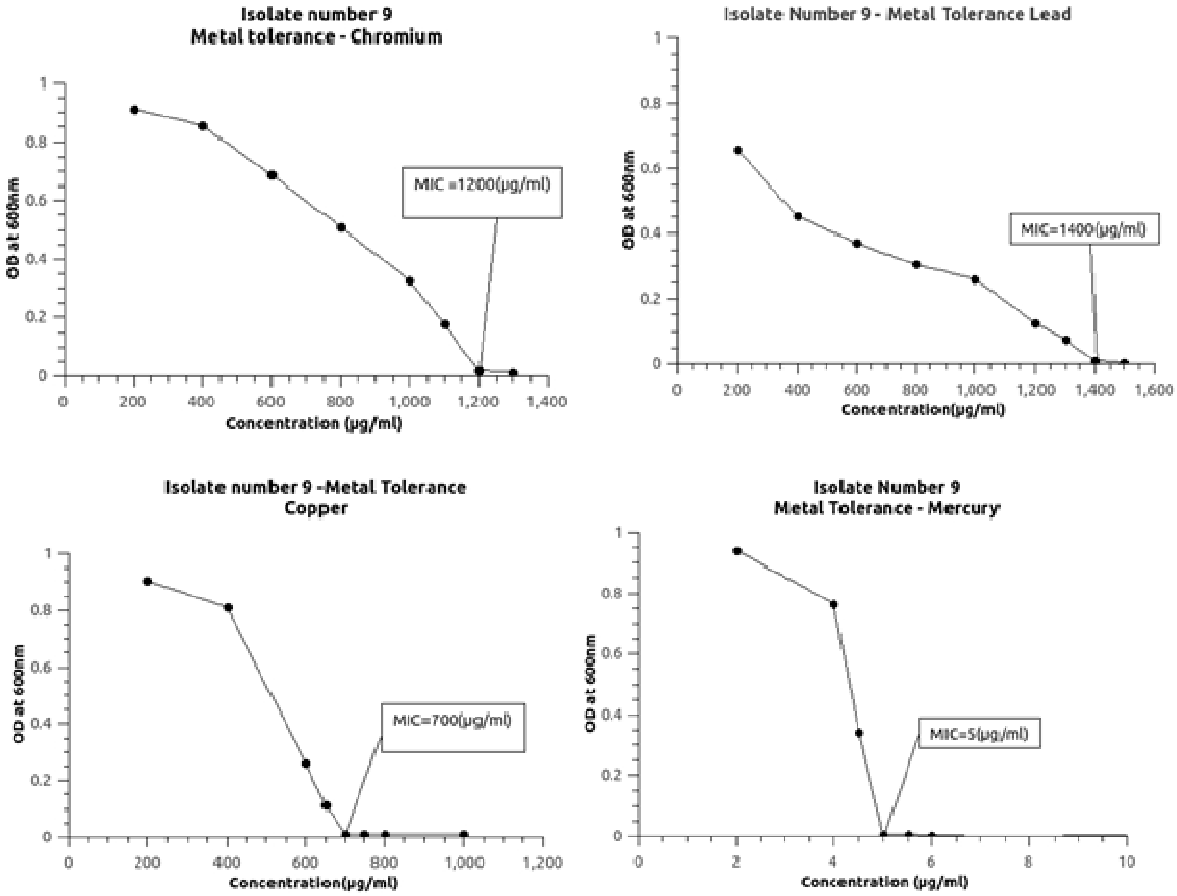
Microbes were isolated from the different level of soil sample collected from the pyrochemical contaminated site and normal site. Very few microbial populations were isolated from Level 1 and 3 samples. Eighteen colonies were isolated, 10 from pyrochemical contaminated soil and 8 from normal soil. These isolates were designated as isolates 1 to 8 for normal soil and 9 to 18 for pyrochemical contaminated soil (SF.1&SF.2). In the pyrochemical contaminated soil, white colonies which produced brown diffusible pigments were predominantly found (isolate number 9), followed by bright yellow colonies. The same yellow colonies were found in the normal soil also, but the number was reduced. A few brown pigment producing colonies similar to isolate 9 were seen in normal soil (Figure 1 above). The colony morphology of the isolates was studied (S. Table1).

The isolates were checked for their metal tolerance against different salts aluminum sulphate, cadmium chloride, potassium dichromate, copper sulphate, zinc sulphate, and lead acetate. Concentration varied from 10 to 1500µg/ml. *E.coli* was used as the negative control (Abas *et al*., 2014; Joutey *et al.,* 2003; Rathi *et al.,* 2011; François *et al.,* 2011; Limcharoensuk *et al.,* 2015).

The isolates 9 and 17 were found to show a good tolerance against aluminum sulphate up to 1260 µg/ml (S. Table 2). The isolate 11 showed good tolerance up to a cadmium chloride concentration of 100µg/ml (S. Table 3). The isolates 9 and 18 showed good tolerance and grew well in plates with the salt concentration of 100µg/ml

Table 1. MIC of heavy metal on microbial growth

Si.No	Metal	Minimum Inhibitory Concentration (µg/ml.)
1	Chromium	1200
2	Lead	1400
3	Mercury	5
4	Aluminium	1260
5	Copper	700



Fiure 2. MIC of heavy metals on microbial growth

(S.Table 4). The isolates 9,10,12,14,17 and 18 showed good growth in the concentration 100 µg/ml of copper sulphate (S. Table 5) . The zinc salt concentration was 650 µg/ml and none of the isolates were tolerant towards it. The isolates 9, 12, 17 and 18 showed good tolerance to lead acetate up to a concentration of 1000 µg/ml (S. Table 6).

Metal tolerant test showed that the isolate number 9 which is predominantly found in pyrochemical contaminated soil was having metal tolerance to most of the metals (S. Table 7). Hence isolate **9** was selected for finding out the MIC for the five metals (Al, Cu, Cr, Pb, Hg), checking the metal removal property and further characterization. MIC of heavy metal on microbial growth results showed that that isolate 9 can effectively tolerates all the metals except mercury (Table 1 and Figure 2).

To evaluate the heavy metal biodegradability of isolate no 9 against potassium dichromate, the cells were inoculated in the metal containing media (Gibb *et al.,* 1989). After overnight incubation the supernatant was collected and absorbance was measured at 440 nm. Uninoculated broth containing metal salt was used as the

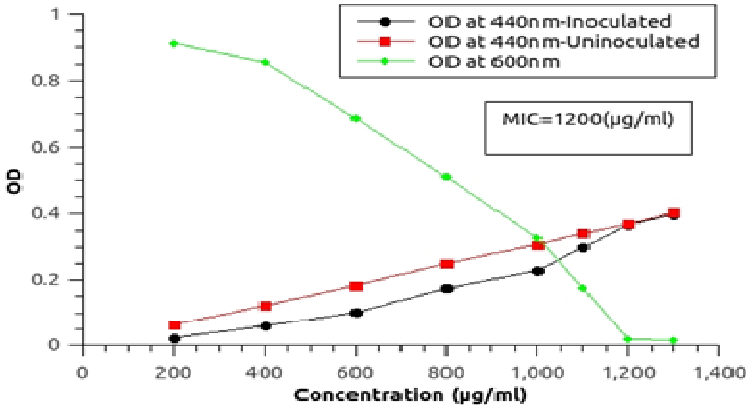


Figure 3. Biodegradability of Isolate 9 on Chromium

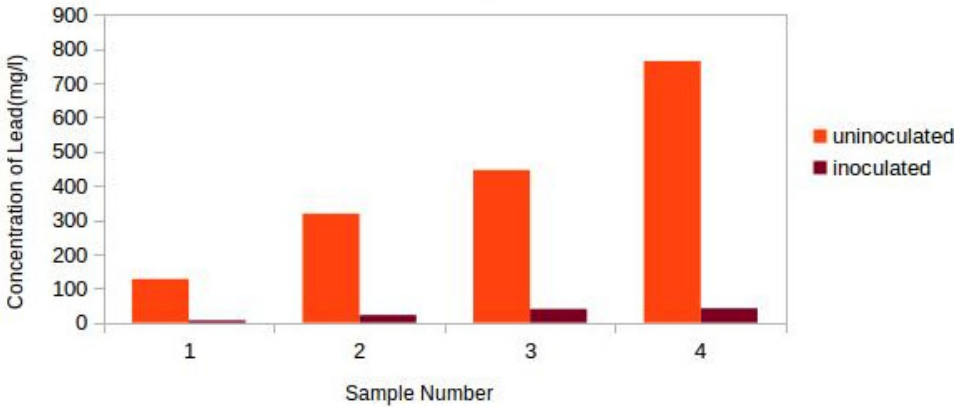


Figure 4. Biodegradability of Isolate 9 on Lead

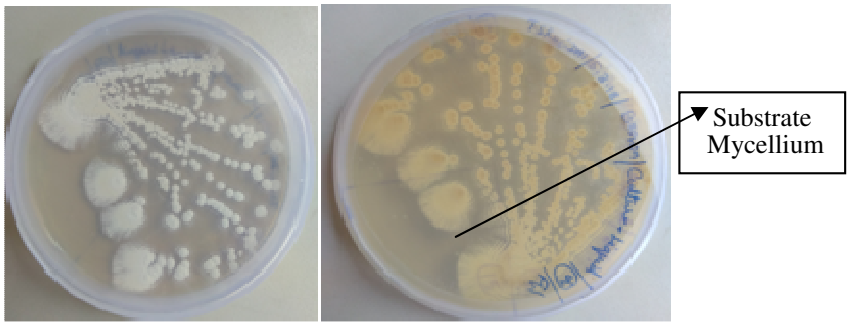


Figure 5. Spore production of Isolate 9 in Soy agar

control. Results show that there is the significant reduction in absorbance in inoculated sample (Figure 3 below).

Biodegradability of isolate no 9 against lead was evaluated using induction coupled plasma. Lead produced a characteristic flame with a maximum wavelength of 200nm. It was found that the concentration of lead had decreased drastically after overnight incubation (Figure 4).

The isolate 9 was considered for biochemical characterization because it was predominant in pyrochemical contaminated soil and it showed metal tolerance too. This isolate produced both aerial and substrate mycelium in soy flour agar, which is a characteristic of actinomycetes (Figure 5).

Production of gelatinase is a characteristics of actinomycetes.

The ability of bacteria to produce gelatinases can be checked using gelatin hydrolysis test. The uninoculated gelatin is used as negative control. If gelatinases are produced by the microorganism, then, it hydrolyzes gelatin and liquefies it. The liquefaction can be checked by cooling the tubes. Isolate 9 showed positive result to gelatin hydrolysis test. It was found that the gelatin in the inoculated tube liquefied. When the negative control tube solidified at 25°C, the sample (inoculated tube) still remained a liquid (SF.3)

Esculin decomposition by isolate no 9 was tested by inoculating the organism in esculin containing media (Islam *et al.,* 2014). The microorganism will produce the enzyme esculinase which can hydrolyze esculin to esculetin. Glucose will be digested by the microorganism, while esculin reacts with the ferric citrate in the medium to produce a dark colour. Isolate 9 showed positive result to esculin hydrolysis test and a dark colouration was produced around the inoculated area (SF. 4). Isolate 9 showed negative result to citrate test. This also confirmed that the isolate no 9 would be actinomycets.

CONCLUSION

Heavy metal soil pollution has received more attention nowadays. Sivakasi is populated with large number of firework industry and the soil is polluted with heavy metal beyond the prescribed concentration. Such heavy metals are deleterious to human health and aquatic life. These metals cannot be completely degraded however it may reduce to nontoxic form. Microbes are the natural tool for heavy metal degradation. Those microbes can transform the heavy metal complex form to simple which can survive in the polluted site. Hence in this present work the heavy metal resistant organisms are isolated from pychemical contaminated site in Sivakasi area and metal resistant was compared with the microbes isolated from normal soil. The results found that among all 18 isolates actinomycetes could resist different heavy metal effectively. Biodegradability test confirmed that the ability of microbes in heavy metal degradation. Hence this study concludes that actinomycetes are potential candidate for bioremediation of pyrochemical contaminated soil.

Conflict of interest statement

The authors declare no conflict of Interests

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