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ISOLATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT AND PLANT GROWTH PROMOTING *STAPHYLOCOCCUS SP*. FROM FLY ASH DUMP SITE.

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Abstract

One of the largest known environmental pollutant, Fly ash (FA) is a byproduct of coal combustion. Fly ash dykes remain home to many microorganisms which are capable of heavy metal resistance. Due to the presence of some toxic heavy metals, like As, Cr, Zn & Pb etc. fly ash remains unused to quite an extent and its disposal remains a debatable issue. The present study is aimed at identifying some bacteria which have various heavy metal resistant and antibiotic resistant property as well as their potential in plant growth promotion. Biochemical and molecular characterization have identified the isolates belonging to Staphylococcus sp. Different plant growth promoting activities such as Ammonia production, IAA, Organic acid production and Phosphate solubilizing ability have identified the role of these bacteria in plant growth promotion. Thus, these isolates can prove to be desirable candidates in bioremediation of toxic fly ash into a soil ameliorant which can be beneficial in promoting agro-forestry.

Introduction:-

Fly ash (FA) is a major coal combustion remnant of thermal power plants stations and is comprised of solid particles of ash, dust, and many toxic heavy metals like lead, arsenic, chromium, copper, and mercury. FA dumps poses serious problems across the world due to leaching and or air disposal of particulates (Pandey and Singh 2012). Generally, the FA disposal is carried out by dry or wet disposal method. Dry disposal includes dumping the FA for construction of an embankment, but wet disposal involves mixing the FA with water to form slurry and transported and disposed of in impoundment called "ash pond". These ash ponds are sources of air, surface water and groundwater pollution (Mishra 2004).

The risks involved from these dumps are a matter of major concern and requires a phenomenon approach for remediation (Smith et al. 2006; Borm 1997). One of the most widely used management strategy can be revegetation which can be helpful for the stabilization of waste-dumps (Pandey and Singh 2014). Presence of plant's micro- and macro-nutrients, the phytomanagement of FA basins and self-sustainable FA ecosystem has been studied (Holl

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2002; Haynes 2009; Mustafa et al. 2012; Cheung et al. 2000). Revegetation process is a slow process due to certain limiting factors like alkaline soil pH, high concentrations of soluble salts, a low content of humus, phytotoxic levels of some elements (e.g., B), deficiencies of others (e.g., N and P) (Shaw 1992), multiple heavy metal stress and natural compaction and cemented layers of ash restricting root growth (Haynes 2009; Pavlovic et al. 2004). However, some microbes are known to bioaccumulate certain heavy metals thereby reducing the toxicity of heavy metal pollutants in the soil. Fly ash is a niche for many microbes which can resist the heavy metals present in it and help in promoting growth of certain plants. This phenomenon helps in ecorestoration of the fly ash dumping grounds and serves as a remedy for its disposal issue. The present study addresses on bioremediation aspect of heavy metal contaminated sites. This study has isolated and identified two heavy metal resistant microbes from contaminated fly ash site which not only had heavy metal resistance property but also possessed plant growth promotion property. Plant growth promoting activity of microbes play significant role in the formation of green cover thereby converting a dumpsite into a green land and they are the active ingredients of most biofertilizers.

Materials and Methods:-

Sample collection:-

The sample collection was done from coal fly ash (FA) dump site of Mejia Thermal Power Station of DVC located in Bankura, Durlovpur, West Bengal, India. The fly ash sample was collected in sterile containers and kept at 4° C.

Isolation of bacteria from ash samples:-

1gm of the ash sample was weighed and mixed with sterile milliQ water and kept on rotatory shaker for 24-48 hrs. Following serial dilution, the mixture was spread in LB agar plate for obtaining bacterial isolates. The individual colonies were selected and maintained on LB agar slants. Further, PVK media (Picovskaya 1948) plates consisting of glucose 10 g/l; tricalcium phosphate (TCP) 5 g/l; ammonium sulphate 0.5 g/l; sodium chloride 0.2 g/l; potassium chloride 0.2 g/l; magnesium sulphate 0.1 g/l; yeast extract 0.5 g/l; manganese sulphate trace; ferrous sulphate trace; agar 2%) was prepared with pH of 7.0 and individual colonies were spot inoculated to observe the phosphate solubilizing ability of the isolated bacteria.

Identification of the bacterial strains:-

Morphological identification was carried out using biochemical and molecular methods. The microscopic identification was carried out by Gram's staining method to study the morphology following biochemical tests for the isolates as per the methods in Bergey's Manual of Systemic Bacteriology (Krieg and Holt 1984). The 16S rDNA PCR was used to amplify 16S rRNA genes using primers f27 (5'-AGAGTTTGATCMTGGCTCAGTAC-3') and r1492 (5'-GGYTACCTTGTTACGACTT-3'). The PCR products were purified using the Quaigen gel extraction kit and then sent for sequencing using Sanger Sequencing.

Qualitative determination of phosphate solubilization by the isolates:-

Tri-calcium phosphate (TCP) solubilizing activity on PKV plates was tested by spot inoculating on the center of the agar plate aseptically. The plates were incubated at 30 ± 2°C for 7-10 days. Appearance of a clear zone around a growing colony was marked and measured as phosphate solubilization index (PSI). PSI was calculated as the ratio of the total diameter (colony + halo
zone) to the colony diameter. All the observations were recorded in triplicates.

Quantitative measurements of phosphate solubilization:-

Solubilization of TCP in liquid medium was further analyzed using Phospho-molybdate blue color method (Murphy and Riley 1962). Pikovskaya's broth (100ml) (adjusted to pH 7) with sucrose and tricalcium phosphate (0.15g /50ml) was dispensed in different 100ml flasks and autoclaved. Log phase cultures were inoculated and the flask was kept for 5 days in a rotatory shaker. Later the culture broth was centrifuged and the supernatant was spectrophotometrically studied for phosphate solubilization and reduction in pH at 660 nm, against a standard KH_2PO_4 curve.

Screening for different plant growth promoting activities:-

Detection of and quantification of indole acetic acid:-

Indole Acetic Acid (IAA) production by the isolates was done by method of Loper and Scroth (1986) with modifications. The strains were inoculated in three test tubes containing 10ml LB broth with L-Tryptophan (200 mg/L) and incubated at 30°C. 5 ml each of the bacterial culture was removed after 6 days and centrifuged for 10 minutes @ 10,000 rpm. Later, 2ml of supernatant was treated with few drops of orthophosphoric acid and 4ml of

Salkowski's reagent consisting 1ml (0.5M) of FeCl3 and 35% of perchloric acid making a final volume of 50 ml. The supernatant and reagent mixture was incubated in dark at room temperature for 15 min to 30 minutes until the development of pink colour which was analyzed spectrophotometrically at 530nm and calculated using a standard of IAA (Sarwart et al. 1992)

Detection and quantification of ammonia production:-
Fresh (24hrs) bacterial cultures were inoculated in 10 ml peptone broth and incubated at 30°C for 48-72 hour in BOD shaker. After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of yellow to dark brown color indicated the production of ammonia (Cappuccino and Sherman with modifications 1992). Quantification of ammonia production was done using Nesslerization spectrophotometric method in which 24 hour-old bacterial cultures were inoculated in 10 ml peptone broth and kept at 30°C for 24-48 hours with constant shaking. Later, 2ml of bacterial culture was taken in an Eppendorf tube and centrifuged at 10,000 rpm for 5 min. with addition of, 40ul each of Na-K-tartarate (25g/50ml) and Nessler's reagent was added to the supernatant and the absorbance was measured at 450 nm. NH3-N standard curve was prepared using Nesslerization spectrophotometric method. The concentration of ammonia was estimated using the standard curve of ammonium sulphate in the range of 0.1–1 μml−1.

Detection of hydrogen cyanide (HCN) production:-
Bakker and Schippers (1987) method was used for detecting Hydrogen cyanide production. Briefly King's B medium plates were amended with 0.4% of L-Glycine was used for the purpose. The isolates were streaked on the media plates and Whatman No. 1 filter paper strips soaked in a 0.5% picric acid+2% Na2CO3 solution were placed on the lids and tighly wrapped with a parafilm and incubated. A change in colour of the filter paper was monitored and noted from brown to dark reddish brown. The results were denoted by **+, ++, +++** for colour intensities.

Identification of Siderophore production:-
For Siderophore production the isolates RR1 and RR2 were tested on the Chrome Azurole S agar plates as per the method suggested by Clark and Bavoil (1994). CAS agar plates were prepared, spot inoculated with test organisms and incubated at 30°C ± 2 for 3 days. Developments of yellow – orange halo around the colonies were considered as positive for Siderophore production.

Multiple heavy metals and antibiotic resistance ability of the isolates:-
Different heavy metal stock solutions in millimolar (mM) were prepared with sterile milliQ water for determination of heavy metal tolerance activity of the strains. The heavy metals solutions were sterilized by filtration through 0.22-p.m-pore-size membrane filters (Roychowdhury et al. 2015). The MIC of the heavy metals for the strains was determined by the plate dilution method as adopted by Summers and Silver (1972) and Aleem et al. (2003). The lowest concentration that prevented bacterial growth towards arsenate (AsV), arsenite (AsIII), chromium (Cr), lead (Pb), and copper (Cu), was considered as the MIC. 25 ml of melted LB agar containing different concentrations of metal were poured onto sterile petri plates which was streaked with the strains RR1 and RR2 and incubated for 24-48 hours. Similarly, antibiotic resistance was tested using LB agar plates containing kanamycin (100 μg ml-1), streptomycin (200 μg ml1), ampicillin (500 μg ml-1), and tetracycline (50 μg ml-1), which were added aseptically to the medium after autoclaving. The agar plates without antibiotics were used as controls. The experiments were carried out in triplicates. Culture plates were incubated at 30° C ± 2 for 24 – 48 hrs until growth was observed.

Results and Discussion:-

Isolation of phosphate solubilizing bacteria:-
Ten distinct bacterial isolates showed phosphate solubilizing zones on Pikovskaya's agar plates
after 7 days of incubation. However, two of the ten isolates that showed highest zones of phosphate solubilization on PVK media plates were further chosen for PVK broth assay for phosphate solubilization and pH reduction.

Identification of the PGPR strains:-
Physiological, morphological and biochemical characterization of selected strains performed are presented in Table 1. The two strains named as RR1 And RR2 respectively were identified as *Staphylococcus pasteuri*, and *Staphylococcus sp.* based on the 16 S rRNA gene sequence analysis.

Determination of phosphate solubilization index:-

RR1 (*Staphylococcus pasteuri)* formed the highest halo zone followed by RR2 (*Staphylococcus* sp.strain). Table 2 shows the phosphate solubilization index (PSI) values of the three strains. PSI = (Colony diameter + Halo zone diameter)/Colony diameter.

Phosphate solubilization kinetics:-
The 2 strains showed solubilization of TCP in liquid media with a drop in pH from a neutral 7 to acidic pH of 4.6 by *Staphylococcus pasteuri* and 4.3 by *Staphylococcus species*. A control containing media showed no drop-in pH, after 5 days of incubation. However, on 5th day of incubation the maximum P solubilization was recorded by *Staphylococcus species* strain RR2 (680 µg ml-1) with a maximum drop in the pH to 4.3 followed by *Staphylococcus pasteuri* (567µgml^{-1}) with a pH drop of 4.6. Fig. 2 shows their phosphate solubilization efficiency, growth curves and the concomitant decrease of pH in the PVK medium.

Detection and quantification of indole acetic acid:-
IAA concentration was after 24 hrs and 48 hrs for each of the strains as within 30-120 minutes the colour intensity was feeble. Hence the OD was measured after 24 hrs and then after 48 hrs of incubation. The strains did not report colour intensity after 48 hrs. Fig. 3 shows the IAA production by the two strains. Strain RR1 was weak in IAA production after 48 hrs 14.361µg ml^{-}1 compared to RR2 with 23.569 µgml^{-}1.

Detection of ammonia production:-
Production of ammonia was weak in RR2 compared to RR1 with 23.871µg/L whereas in RR1 it was found to be 39.479 µg/L.
Fig. 2 depicts the ammonia production by the selected strains at 24 hours and 48 hours of incubation.

Detection of hydrogen cyanide production:-
Both the strains were positive for hydrogen cyanide production. Highest production of HCN was achieved by *Staphylococcus* sp. strain RR2 and was recorded as +++, while RR1 was ++, after 48 hrs of incubation. Table 3 shows HCN production by the two strains.

Siderophore production:-
Both the strains were tested for Siderophore production; however, the orange halo zone was better in RR1strain than in RR2. The results were noted after 72 hrs of incubation. The halo zone was 1.8 cm ± 0.5 in RR1, but in strain RR2 the zone was feeble and measured 0.9cm.

Minimum inhibitory concentration (MIC) of heavy metals and antibiotic resistance:-
All the bacterial strains showed different degrees of resistance towards the different heavy metals and antibiotics. The MIC of the heavy metals as detected by the plate based assays has been presented in Fig. 4. Strain RR1 exhibited antibiotic resistance characteristics to kanamycin (100 µg ml-1), and streptomycin (200 µg ml-1) while RR2 was resistant to ampicillin (500µg ml-1).

The present study shows presence of potential strains which may aid in phytorestoration of fly ash dump sites. This study characterized two such microbes belonging to Staphylococcus genera that have both phosphate solubilizing ability as well as plant growth promoting abilities including indole acetic acid production, siderophore, HCN, and ammonia production. These two strains RR1 and RR2 also showed multiple heavy metal resistance as well as resistance to some antibiotics. These strains can serve in as bio inoculants in the ash dykes which would not only help reduce heavy metal toxicity but can help in promoting plant growth to generate a green cover on abandoned fly ash dykes. The ideology of ecorestoration by green capping is to minimize leaching of heavy metals from fly ash dump sites into underground water reservoirs.

Table 1:- Morphological and biochemical characteristics of the RR1 & RR2 isolated from fly ash samples collected.

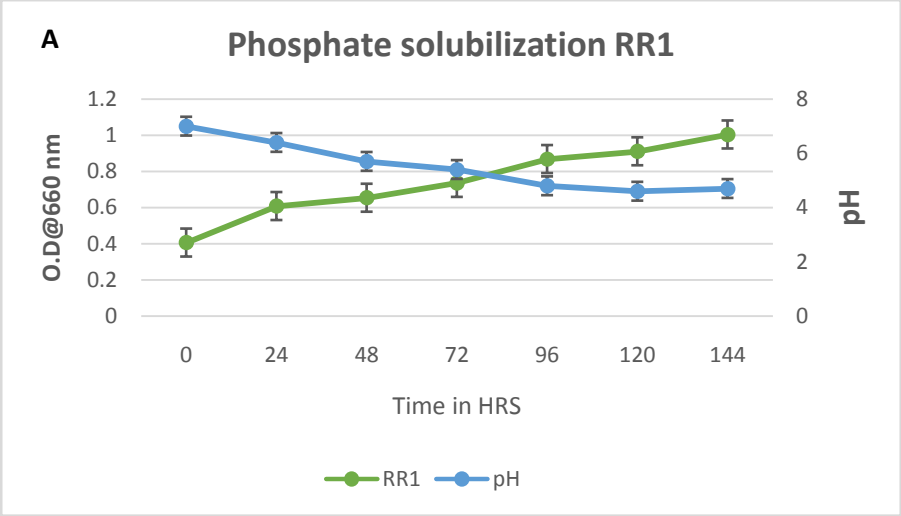
Tests	*Staphylococcus pasteuri RR1*	*Staphylococcus sp. RR2*
Morphology	**cocci**	**cocci**
Colony in LB agar plate	**Creamish white**	**Shiny white**
Gram reaction	**Positive**	**Positive**
Spore formation	**ND**	**ND**
Nitrate reduction	**Negative**	**Negative**
Citrate utilization	**Positive**	**Positive**
MRVP	**Negative**	**Negative**
Catalase	**Positive**	**Positive**
Starch Hydrolysis	**Positive**	**Positive**
Casein Hydrolysis	**Positive**	**Positive**
Urease Production	**Positive**	**Positive**

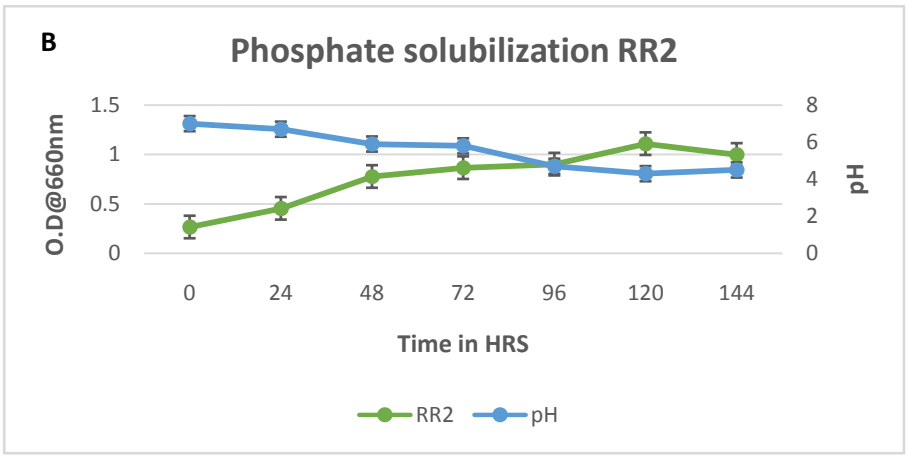
Table 2:- Phosphate solubilization index values of the two strains.

Isolate	Colony diameter (cm)	Halo zone diameter (cm)	PSI (Colony diameter + Halozone diameter)/Colony diameter
Staphylococcus pasteuri strain RR1	0.76	0.87	2.14
Staphylococcus sp. strain RR2	0.63	0.91	2.44

Table 3:- HCN production by the two strains.

Strain	**24 hrs**	**48 hrs**
Staphylococcus pasteuri strain RR1	+	++
Staphylococcus sp. strain RR2	++	+++





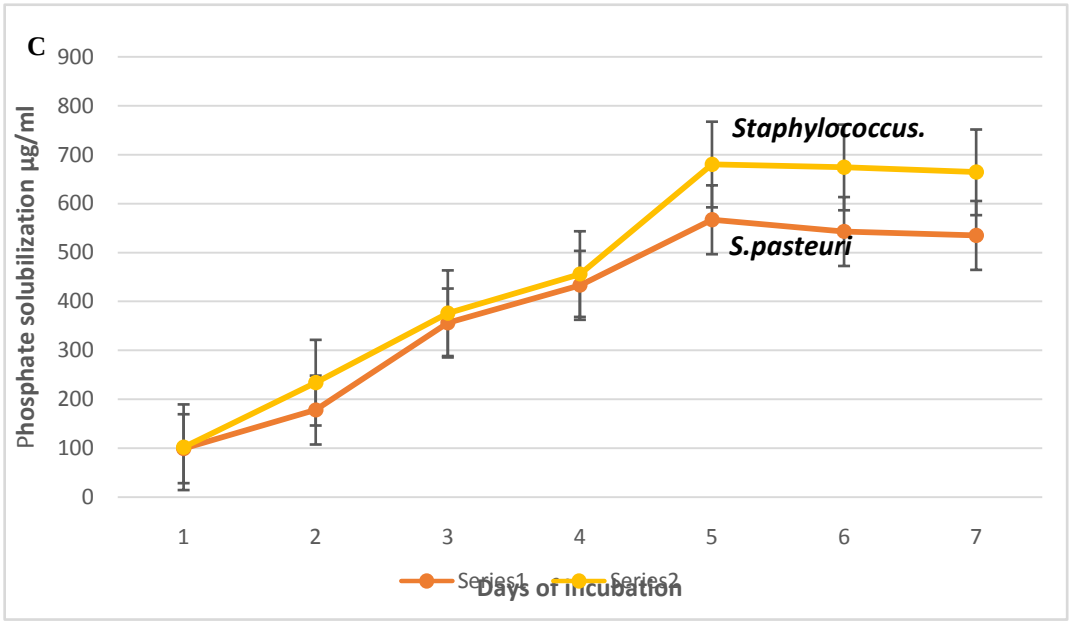


Fig. 1:- Phosphate solubilization efficiency of the two isolates along with decrease in pH. (A) & (B)shows the concomitant decrease of pH in the PVK medium and the growth curves of the isolates RR1 and RR2. (C) shows the production of soluble P µg ml-1. O.D values at 660nm.

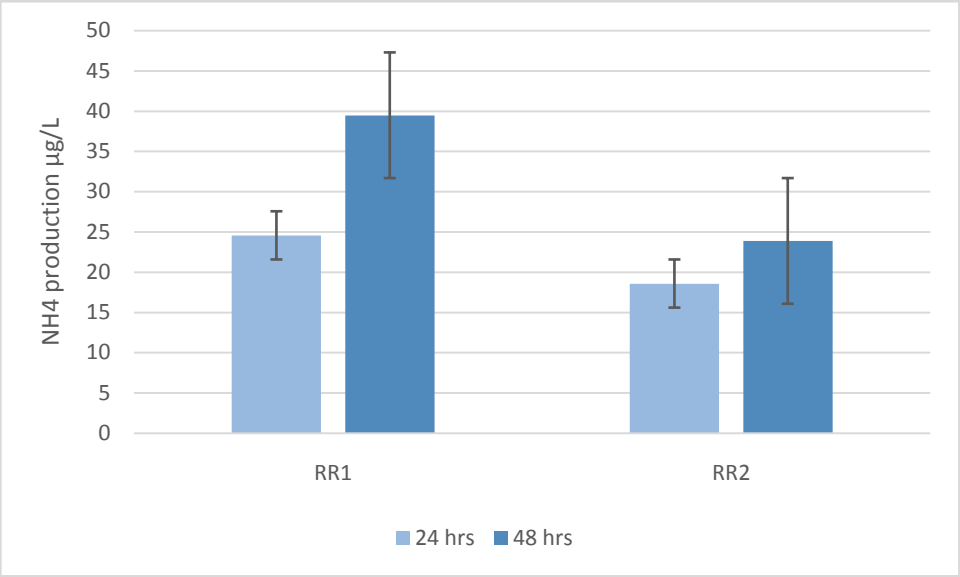


Fig. 2:- Quantitative estimation of ammonia production by RR1 & RR2 isolates in peptone water using Nesslerization reaction.

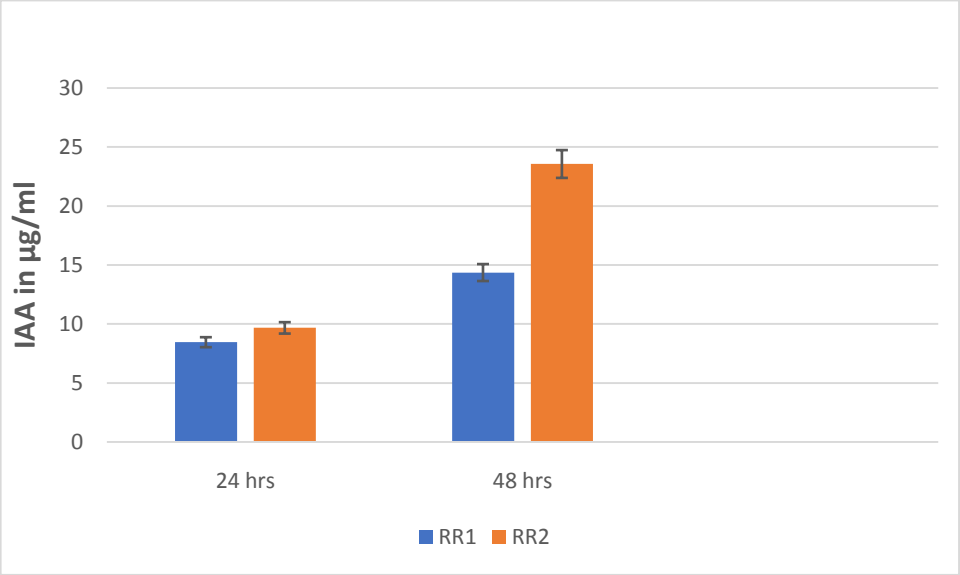


Fig. 3:- Indole acetic acid production of the two isolates, RR1 & RR2.

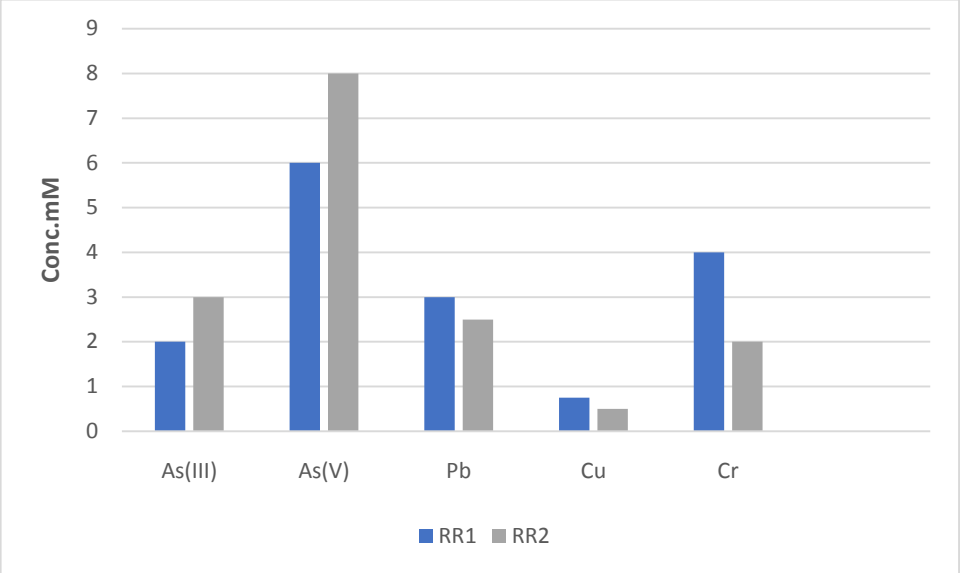


Fig. 4:- MIC values of the 5 heavy metals tested on the two strains in mM. concentration.

Conclusion:-

The present study concludes that strains RR1 and RR2 isolated from ash dumps belonging to Staphylococcus sp., can make serve as ideal agents for application as bioinoculants in eco- restoration of vegetation in fly ash dump and mining dump sites. These two strains characterized for heavy metal resistance properties and plant growth promoting activities such as Siderophore production, IAA production and phosphate solubilizing ability can be studied further in pot based assays for their abilities for plant growth promotion.

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