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# Bioremediation of Fly Ash Heavy Metals by Indigenous Microbes

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***Abstract*** **– In developing nations such as India coal based thermal power serves major source for power generation. In this practice a major product called fly ash is generated enormously that need to be owing to its properties and elemental composition. In this direction this study was done to presents the bioremediation potential of the indigenous microbes from fly ash for heavy metal remediation. 8 potential bacterial species namely *Bacillus endophyticus*, *Bacillus Aquimaris*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus marisflavi*, *Pseudomonas aeruginosa* were isolated from fly ash, lateron their bioremediation potential was analysed for metal remediation. Seven metals such as Zn, Cr, Pb, Fe, Cu, Cd, Ni were selected for study with different concentration. *Bacillus subtilis* showd best result for Ni, *Bacillus cereus* showed best result for Cr and Pb, *Bacillus endophyticus* showed best result for Fe, Cu, *Pseudomonas aeruginosa* showed best result for Zn, *Bacillus subtilis* and *Bacillus Aquimaris* showed best result for Cd in the highest concentration of metal.**

***Keywords*** **– Bioremediation, Heavy Metals, Indigenous Microbes.**

## I. INTRODUCTION

Bioremediation utilizes the biological interventions of biodiversity to mitigate the environmental pollutants. It can be acknowledged as novel, economical and eco friendly technology to protect and mitigate human impact on environment (Raghunandan et al., 2014, 2018; Singh and Gupta 2016). The term bioremediation has been introduced to describe the process of using biological agents to remove toxic waste from environment (Asha and Sandeep, 2013; McHughen, 2016). It utilizes yeast, fungi or bacteria as biological agents to clean up contaminated soil and water (Kumar et al., 2011). Bioremediation can be applied on the site of contamination (in situ) or away from contaminated sites (ex situ). This technology is more promising as compared to other available techniques for metal removal from contaminated surroundings. The bioremediation process can be divided into three phases or levels (Kaplan and Kitts, 2004). First, through natural attenuation, contaminants are reduced by native microorganisms without any human augmentation. In this microbe directly comes in contact of contaminants that are available and use them as substrate. Second, biostimulation is employed, where nutrients and oxygen are applied to the system to improve its effectiveness and to accelerate biodegradation. Finally, during bio augmentation, microorganisms are added to the system. These supplemental organisms should be more efficient. In biostimulation the activity of naturally occurring microbes is stimulated by circulating water based solutions through contaminated soils. Nutrients (nitrogen, phosphorus, carbon and others), oxygen or other amendments, may be used to enhance bioremediation and contaminant desorption from subsurface materials. Bioremediation involves use of indigenous organism where monitoring is least required or derived organism which needs proper monitoring (Santos et al., 2011; Asha and Sandeep, 2013).

Ledin (2000) explained the difference between microbial tolerance and resistance; he defines tolerance as the ability to cope with metal toxicity by means of intrinsic properties of the microorganisms, whereas resistance is the ability of microbes to detoxify heavy metals by being activated in direct response to the high heavy-metal concentrations.

In the present study efforts were made to analyse the ability of Microbial species of the fly ash for metal remediation. The objective of the study was to investigate potential microbes of ash that is by product, this may be helpful in making fly ash as resource material.

## II. MATERIAL METHODS

### *Sample Collection*

Fly ash samples were collected from Gandhinagar Thermal power station and its dumpsites. Microbial species were isolated from it later on different concentration of heavy metal solutions were prepared and microbes were added and analysis was done for metal remediation.

### *Identification of Isolated Microbes was Done in the Following Stages*

#### i. *Microbiological or Morphology Identification*

Pure isolated cultures are identified initially on the basis of morphology, colour, shape (rod, sphere, filaments etc.) of individual, arrangement (single, pairs, chain, cluster) of individual in colony, cell size, shape margin (smooth or serrated), etc.), and characteristics such as the secretion of watery or mucoid/gummy substances from colonies were seen.

#### ii. *Motility Test*

The test determines the weather bacteria are motile or non-motile.

Semisolid medium of agar was prepared in test tube. Bacteria were inoculated using needle single stab up to two third media and pulled back. The test tubes were incubates for 24-48 hr. at 37°C. Non-motile bacteria grew in the streak line with sharp defined margins leaving the surrounding medium transparent. While motile Bacteria grew diffusely spreading throughout media making media hazy.

#### iii. *Staining Reactions*

Staining test was done to determine the gram positive and gram negative bacteria under phase contrast microscopy. For this slide with smear of bacteria is stained step by step using staining kit. Finally colored slide was observed under microscope. Gram-positive bacteria appeared Violet/Purple while gram-negative bacteria appear - Red/Pink.

### *Cultural Characteristics*

Isolated individuals were tested for their cultural behavior using agar plates or broth in this various traits observed were growth (absent, slight, moderate or abundant), Form (circular, irregular), Colour, consistency (opaque, translucent). Growth on broth: surface growth (ring pellicle none), clouding (slight, heavy none), sediment (abundant, scanty, none, granular, flaky or flocculent).

### *16s RNA for Identification of Isolated Bacteria*

Isolated individual bacteria were submitted to GSBTM (Gujarat State Biotechnology Mission) for 16sRNA for confirm identification and authentication of unknown bacterial cultures and for sequence submission to NCBI (National Centre for Biotechnology Information).

*Construction of Phylogenetic Tree*

Phylogenetic tree was constructed using neighbour-joining relationships are estimated with MEGA4 software (Tamura et al., 2004).

## III. RESULTS

*Bioremediation of Heavy Metals*

Bioremediation study was conducted in several steps such as i) Isolation of indigenous bacteria. ii) Screening of potential bacteria for heavy metal remediation. iii) Heavy metal remediation by potential organism. iv) Heavy metal remediation by microbial consortium.

I) *Isolation of Indigenous Bacteria*

Bioremediation study was conducted by isolating indigenous bacteria from fly ash, root zone of potential plant (here *Prosopis juliflora*). Total 17 indigenous bacteria were isolated for the study. These organisms can be considered to be resistant to the fly ash heavy metals at varying concentrations. Sequences for the isolated indigenous bacteria are presented in annurex. Phylogenetic analysis using 16S rDNA indicated that the 17 bacteria strains belong to genus *Bacillus, Pseudomonas, Agromyces sp.* Figure 1 shows phylogenetic tree of isolated individuals.

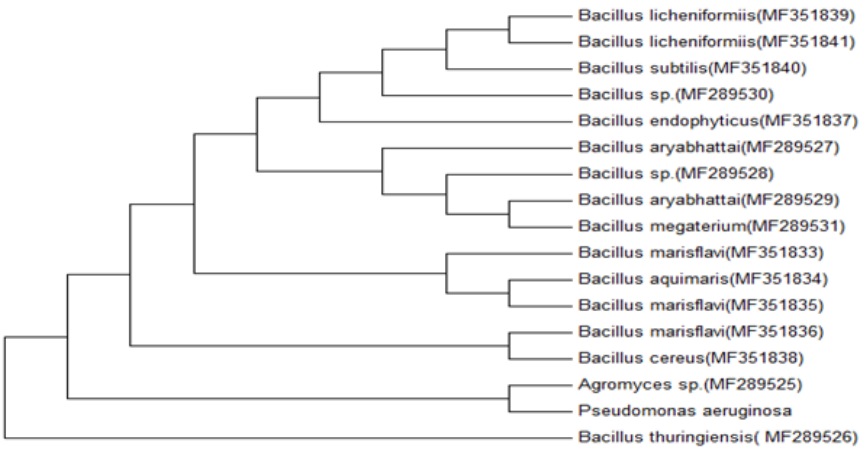



Fig. 1. Phylogentic tree for isolated organisms.

*Evolutionary Relationships of Taxa*

The evolutionary history was inferred using the Neighbor-Joining method [Saitou and Nei M. (1987)]. The optimal tree with the sum of branch length=1.63747408 is shown. The evolutionary distance was computed using the Maximum Composite Likelihood method [Tamura K et al (2004)] and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotides sequences. Codon positions included $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 882 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [Kumar S, et al., (2016)].

II) *Screening of Potential Bacteria for Heavy Metal Remediation*

For Determining potential bacteria for heavy metal remediation bacteria were cultured with selected heavy metals for 24hrs. Potential isolates were isolated and used for further remediation study. Phylogentic tree for

potential organism is shown in Figure 2. These isolated individuals were able to grow at higher concentrations of the heavy metals. Maximum tolerance concentration for different metals was found different and the values were found from 500ppm to 75ppm for different metals.

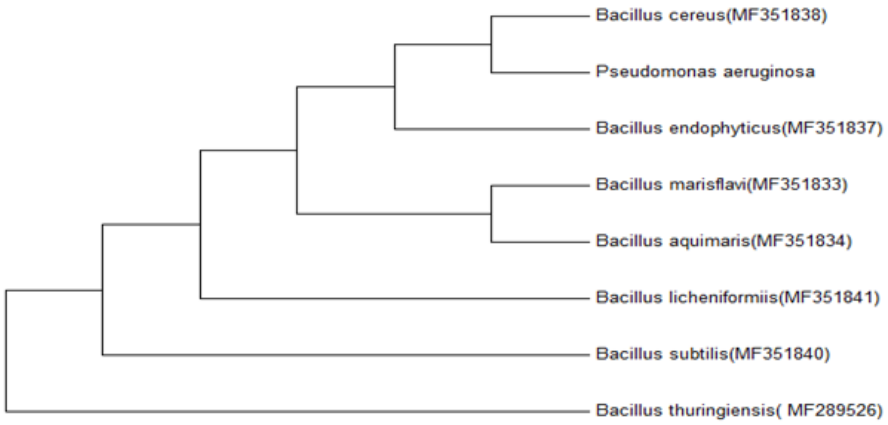



Fig. 2. Phylogentic tree of heavy metal resistant bacteria.

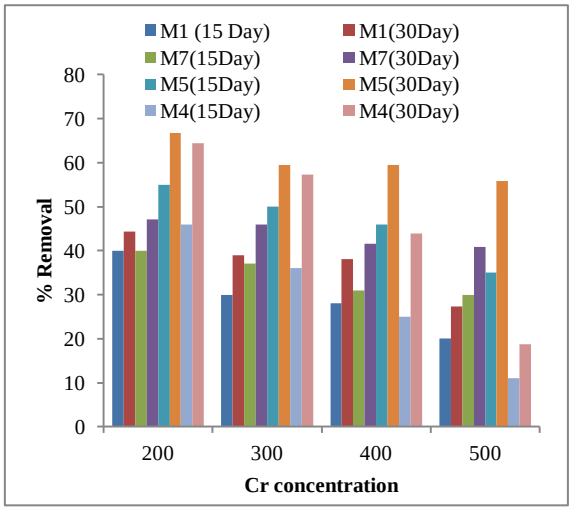
*Evolutionary Relationships of Taxa*

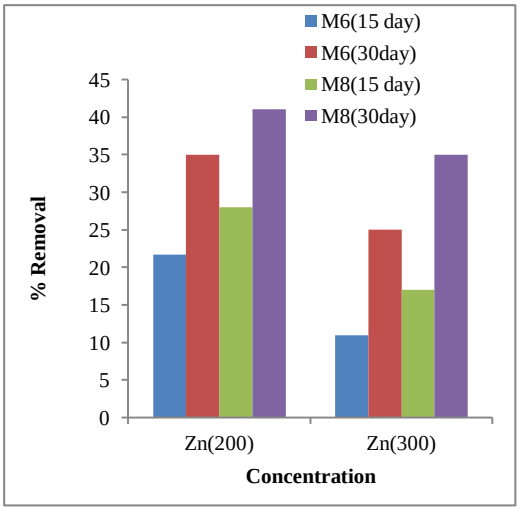
The evolutionary history was inferred using the Neighbor-Joining method [Saitou and Nei M., 1987]. The optimal tree with the sum of branch length = 1.41448252 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method [Tamura K et al., (2004)] and are in the units of the number of base substitutions per site. The analysis involved 8 nucleotides sequences. Codon positions included $1^{st}$ + $2^{nd}$ + $3^{rd}$ + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1108 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [Kumar S, et al., 2016].

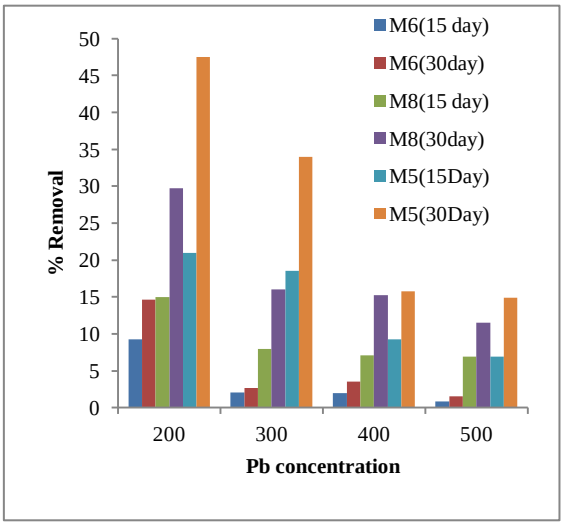
III) *Remediation of Selected Fly Ash Heavy Metals by Potential Bacteria*

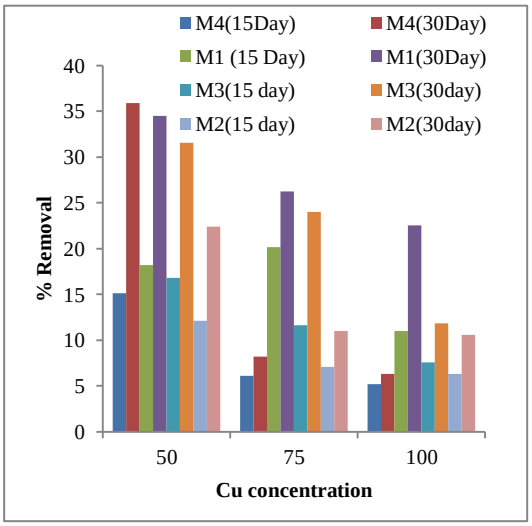
Isolated organisms were tested for potential survivability in different metals at different concentrations. Potential for metal removal by bacteria was determing by analyzing percent removal of metal at two intervals for one month. 8 potential indigenous bacteria showed different survivability in selected heavy metals. *Bacillus thuringiensis* showed maximum metal removal in Cu followed by *Bacillus endophyticus*, *Bacillus subtilis*, *Bacillus Aquimaris* with maximum removal percent 35.8%, 34.4%, 31.5% and 22.3% respectively. Maximum survival ppm for copper was found 100ppm. In Fe microbes survived upto 500 ppm maximum metal removal was observed by *Bacillus endophyticus* followed by *Bacillus subtilis* and *Bacillus licheniformis* with maximum removal percent 98.4%, 97.1% and 95.9% respectively. In Cd potential survivors were *Bacillus Aquimaris*, *Bacillus subtilis* metal removal was maximum by *Bacillus subtilis* (11.4%) at 75 ppm followed by *Bacillus Aquimaris* (9.2%). In Zn potential bacteria were *Bacillus licheniformis*, *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* (41%) showed maximum metal removal percent followed by *Bacillus licheniformis* (35%). In Cr *Bacillus cereus* (66%) showed maximum metal removal followed by *Bacillus thuringiensis* (64%), *Bacillus marisflavi* (47%) and *Bacillus endophyticus* (44%). In Pb maximum metal removal was observed by *Bacillus cereus* (41%) followed by *Pseudomonas aeruginosa* (29.7%) and *Bacillus licheniformis* (14.6%). *Bacillus subtilis* (95%) showed maximum metal removal for Ni followed by *Bacillus endophyticus* (93%) and *Pseudomonas aeruginosa* (83%) Figure 3 shows metal removal percentage by potential indigenous bacteria. Maximum percentage removal of metal found the order Fe>Ni>Cr>Pb>Cu>Zn>Cd. High removal percent may

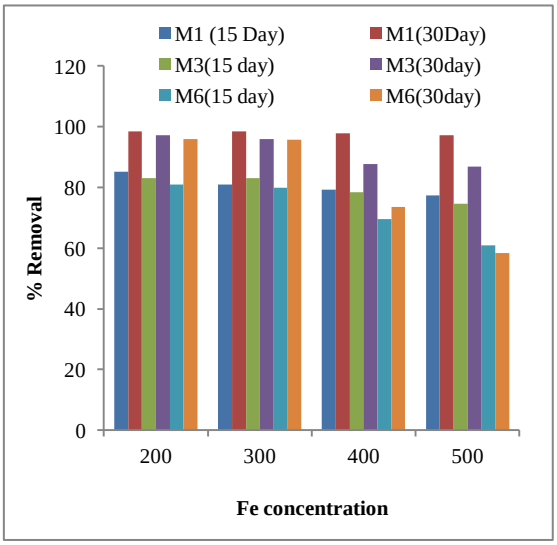
be due to ease mobility of metal while immobile nature of metal may cause less removal percentage of metal, Cd was found to be least mobile metal but showed increased percentage removal with increasing concentration and time, Similar trend was observed for percent removal of Ni. While other metals showed increase percent removal with time but negative relation with concentration i.e. with increase metal concentration metal removal percentage declined.

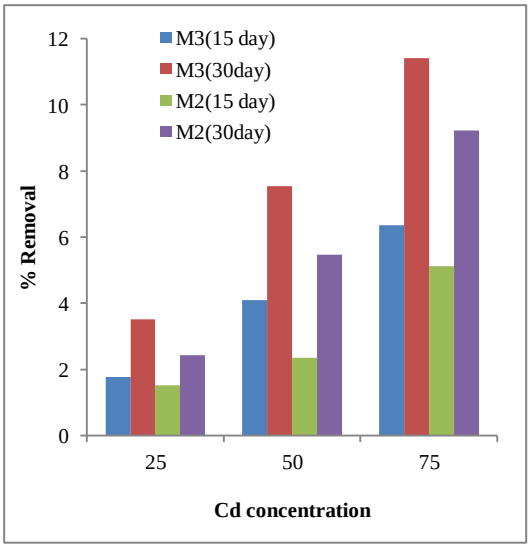


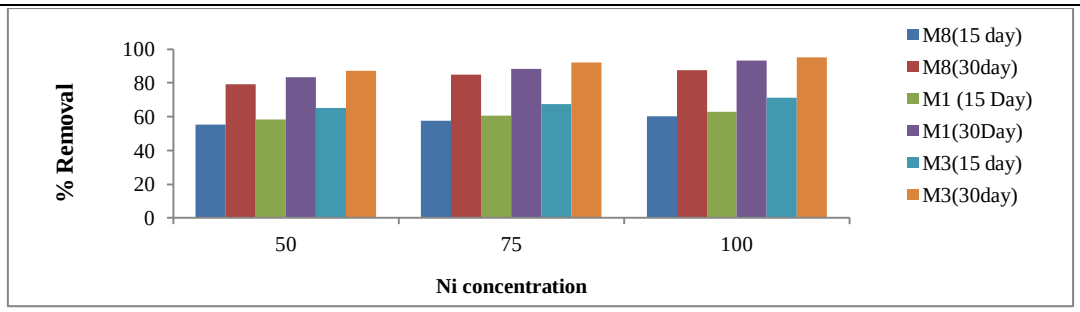



Fig. 3. % Removal by potential indigenous bacteria (Id's:-M1: *Bacillus endophyticus*, M2: *Bacillus Aquimaris*, M3: *Bacillus subtilis*, M4: *Bacillus thuringiensis*, M5: *Bacillus cereus*, M6: *Bacillus licheniformis*, M7: *Bacillus marisflavi*, M8: *Pseudomonas aeruginosa*).

### 5.11. *Bacteria Growth Analysis*

For the further study of metal removal form fly ash leachates with different amendments of fly ash CFU count study was done to determine the survivality rate of bacteria in the media for one month. CFU values shown in figure 4 depicts that isolated bacterial strains growth was very fast in starting seven days of incubation of bacteria in various amendments of fly ash, this can be due to additional carbon source from the nutrient broth this trend continues upto 15 days but rate of growth slows down. However at 30 days growth was found minimal this might be due to depletion of organic carbon in the medium. Results reveal the survival of bacteria in the NB media for the month though growth retarded containing 100% fly ash.

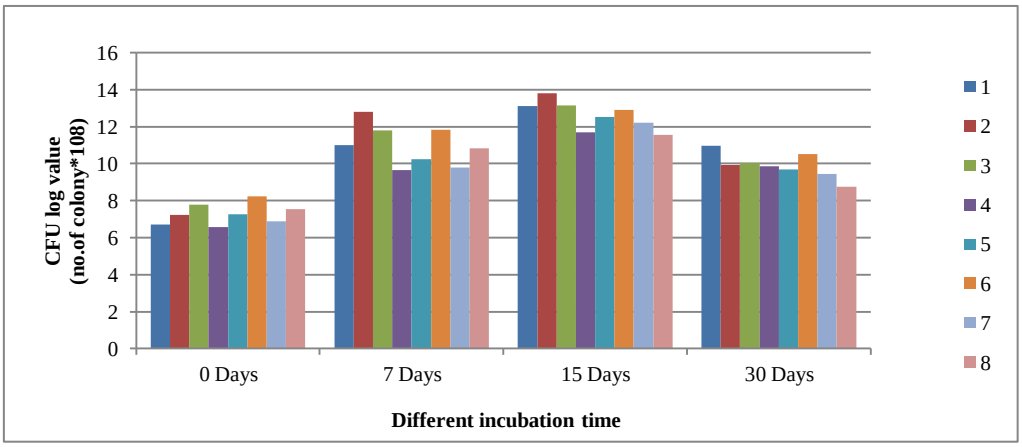



Fig. 4. Survivality of potential bacteria in media with fly ash (Id's:-1: *Bacillus endophyticus*, 2: *Bacillus Aquimaris*, 3: *Bacillus subtilis*, 4: *Bacillus thuringiensis*, 5: *Bacillus cereus*, 6: *Bacillus licheniformis*, 7: *Bacillus marisflavi*, 8: *Pseudomonas aeruginosa*).

## IV. Discussion

On earth surface we faces serious concern in terms of substances that are produced as waste or by products that are generated because of industrial activities or by accidental processes such as spillages etc. This leads to environmental pollution and accumulation or release of elements beyond the permissible limits in the surroundings where they are disposed or stored (Verma and Kuila 2019). Of these metals released some metal may be beneficial to living being when present in small quantity, but their excess may cause toxicity or mutations (Valko et al., 2016). While there are also some metals that are hazardous even in small amount (Iram et al., 2013). Metals with specific density more than 5 g/cm3 are categorised as heavy metals. Their small concentration is toxic to living forms, thus they form an unfavourable condition, as they are disease causing, inhibits the normal functioning of organs in living organisms, creating environment unfit for organism to

survive in heavy metal polluted area (Jaishankar et al., 2014). Thus it is required to manage the concentration of heavy metals in environment for living species to survive (Toth et al., 2016).

Currently remediation using microbes has driven focus as it could be implemented both ways in situ and ex situ remediation methods also they take up theses metals as nutrients for their growth and thus clean the environment. Thus it appears as sustainable method for remediation heavy metals contaminated sites. (Raghunandan et al., 2014, 2018; Kumar et al., 2016). The microorganisms have the ability to accumulate metallic elements and this has been studied at first from toxicological point of view (Volesky, 1990). The microbes survived in polluted soil are prone to show higher tolerance to heavy metals as compared to the strains from non-contaminated sites Zouboulis et al., 2004. Microbial populations in metal polluted environments adapt to different concentrations of heavy metals and become metal resistant (Prasenjit and Sumathi 2005). Srinath et al., 2002 reported the bioaccumulation capacity of *Bacillus circulans* and *Bacillus megaterium.*

Phylogenetic analysis using 16S rDNA indicated that the 17 bacteria strains belong to genus *Bacillus, Pseudomonas, Agromyces sp.* Out of those 8 potential bacteria survived for individual metal remediation. For different metals different organism showed better remediation potential. Klose et al., (2004) documented that after ash application, bacteria dominate over fungi. In his study he found *Pseudomonas aeruginosa* survived in concentrations of thorium and uranium, similarly *Bacillus megaterium* was found to better survived in high aluminum (Al) concentrations. Rathnayak et al. (2010) investigated the effect of different concentrations of lead on the growth of *Bacillus thuringeinsis* and *Paenibacillus* bacteria. Various studies prove Genus Bacillus and Pseudomonas to be the powerful bioaccumulator of heavy metals from the contaminated sites.

## V. CONCLUSION

Heavy metal pollution is the prime pollutant of our environment, its persistent nature makes it more important for its remediation from the released environment. This research is useful in terms of investigating indigenous microbes of fly ash with potential of heavy metal treatment. 8 potential bacterial species namely *Bacillus endophyticus, Bacillus Aquimaris, Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus, Bacillus lichenifor mis, Bacillus marisflavi, Pseudomonas aeruginosa* isolated from fly ash showed potential treatment ability for various metals in varying concentration, thus we can conclude beneficial usage of the microbes for remediation techniques.

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