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# EFFECT OF HEAVY METAL ABSORPTION ON BACILLUS CEREUS MEMBRANE AND OPTIMIZE BIOSORPTION POTENTIAL

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**Abstract**
Worldwide, rising waste from industries and mines generates heavy metals increases serious environmental risks that can be threaten human being. The removal of heavy metals such as Pb, As and Hg from the contaminated site using bioremediation approaches is advantageous. The heavy metal biosorption ability of the bacterial strain Bacillus cereus was evaluated. Quantitative absorption ability of Bacillus cereus was found via ICP-MS. Environmental factors influencing activity were also investigated through optimization approaches. Lead, Arsenic and Mercury absorbed (87.6%, 70.6% and 87.5%) at 3% (v/v) metal ion was achieved by Bacillus cereus which was incubated with 10% Inoculum size (v/v), pH 8 and 10% inoculum at 35°C for 96 hours incubation. The Bacillus cereus was capable to resist heavy metal concentration up to 5.0%, after bacterial growth was decreased. This positive appearance of this strain makes for bioremediation agent of heavy metal contaminated sites. This is the first study that was investigated in Korba coal mine.

**Keywords—** Bacillus cereus, Heavy Metal, Biosorption, ICP-MS, Optimization.

## Introduction

Modern industrialization and rapid development continuously polluted the water, soil and air quality via leaching and high volume of waste discharge through industries. (Ruba et al., 2021) Environment constantly contaminated through various pollutants which are organic and inorganic compounds, radioactive isotopes at noticeable rate. Heavy metal is one of the rising contaminants in environment as toxic metal because of their higher atomic weight and high density. Heavy metal pollution poses major life threat of human being and become world environment problem. (K.K. Sodhi et al., 2022) Microorganisms have the capability to degrade human life threatening toxic metals which have been accumulate in nature through industry, pesticides and mining work. (Medfu Tarekegn et al., 2020) Mainly pesticides are organic & inorganic compounds contain Mercury, Arsenic, Zinc other HM which is not degraded naturally and affects plant growth. This is necessitate develop new treatment methodology using microbes to eliminate heavy metals from water and soil (Zheng X et al., 2022).
Lead has industrial application due to high atomic mass and stability but still it not has any biological function because of their toxicity effects on human being such as damage genetics and nervous system. (Parviz Heidari & Samaneh Sanaeizade., 2020) World wide range for lead is ranging up to 67mg/kg in surface soil. Lead concentration in soil reached up to 100 mg/kg through industries. High concentration of lead is highly toxic for animal, plant and live organism. (Low KS et al., 2000) Bacteria Brevibacillus brevis has been identified from heavy metal contaminated soil of Chhattisgarh and B. brevis can be able to tolerate Lead 1500 µg/ml determined via Minimum Inhibitory Concentration. (Sanjana Bhagat and Lakheshwar Thawait., 2018)

These days, arsenic contamination in drinking water refers a huge problem for human health in the world. High exposure of arsenic throughout drinking water for the long time effects lethal problem in human health. Arsenic concentration higher than 50µg/L in drinking water increases the risks cancer of lung and bladder. (Kour D et al., 2021) Due to high concentration of arsenic human faces gastrointestinal symptoms such as abnormal heart rhythm, severe vomiting, damage of nervous system and abdominal pain. (Syed Zaghum Abbas et al., 2014) In nature arsenic is present in very low amount but some commercial products such as processing of coal, wooden preservatives and pesticides contain arsenic increasing arsenic level in environment. High level of arsenic affects major health issues. WHO has recommended maximum limit of arsenic in drinking water is 10µg per liter. (Mehwish IQTEDAR et al., 2018)

In atmosphere mercury exists various from in nature with diverse forms include metallic, vapor and salts consequential showing lethal effects on environment and human health.( D. R. Kotwal et al., 2018) Mercury is most harmful heavy metals in the environment due to Human development increase in mercury concentrations in water and soil. Hg accumulated through food chain eaten by organisms. Mercury has a strong affinity for thiol group which can cause protein structure instability and affecting essential functions of the cells. (Martha M. Naguib et al., 2019)

There are some methods have been approaches such as physical as well as chemical to detoxify heavy metals from contaminated environments. (G. Akinci and D.E. Guven,. 2011) Bioremediation is eco-friendly process, low cost approach where using microorganism remediated the pollutants from the soil and water (J. Talley., 2005). The microbes are capable to resist heavy metals such as Lead, arsenic, Mercury, cadmium and copper. Researchers have been developed new bioremediation techniques based on type of pollutant. (W. Kanoksilapatham et al., 2015)

In the present study Bacillus cereus was used for bioremediation studies under the presence of Lead, arsenic and mercury. The assessment of ability regarding lead, arsenic and mercury removal from the coal mine soil was found out. Bacillus cereus can be a potential agent for decontamination of the metal polluted sites.

## Materials and methods

### Sample Collection and Isolation of Bacteria

In summer, soil sample was collected from 2cm depth surrounding of opencast mine of Gevra coal mine Korba (22.336312, 82.545748) (Fig. 1). Soil sample was carefully stored in polythene zipper bag and transport to Laboratory. The soil sample was homogenized and sieved as well as soil sample was stored in 720ml Whirl-Pak sampling bag (Sigma-Aldrich). Soil sample was diluted with 0.89% saline water, this method was called $10^{-1}$ dilution. In this method 1 milliliters of the $10^{-1}$ dilution was aliquot to test tube containing 9 ml of 0.89% autoclaved saline water and this process was repetitive various times until 10−7 dilutions were made and plated on agar media (Himedia, India) to isolate heavy metal resistant bacteria. Spread agar media was incubated at 37oC for 24 hours. (A.S. Downey et al., 2012)

### Heavy Metal Analysis of Soil

Soil sample was dried on Hot Air Oven and sieved by 2 mm mesh sieve. The soil sample was digested with 15ml aqua regia acid solution (35%HCL and 65% HNO3). After that cooled the solution and filtered using whatman filter paper. Filtered solution was diluted with 50 ml deionized water and sample solution was analyzed for Pd, As and Hg using an Atomic Absorption Spectrophotometer (Agilent, 240FS AA) and result was analyzed by Agilent SpectrAA Software. (Senthamilselvi P et al., 2021)

### Screening of Multiple Metal Resistance Bacteria

Bacillus cereus isolated from soil sample of gevra coal mine Korba (Unpublished data but sequence submitted to GenBank). The preliminary test of B. cereus was performed on Luria Bertani agar (Himedia, India) supplemented with 100µg/ml analytical grade Lead nitrate (Himedia).Streaked agar plate was incubated at 37oC for 24 hours. After incubation developed colony was tested against multiple heavy metal LB Broth media (Himedia). Loop full bacteria streaked on LB agar media suspended with NaAsO, Pb(NO3)2 and HgCl2. Bacterial growth was observed after 24 hours of incubation. (R. Kumar et al., 2013)

### Visualization of B.cereus cell structure by SEM

B.cereus culture were fixed using 2.5% glutaraldehyde for 2 hours, washed several times in the phosphate buffer, dehydrated via acetone in increasing concentration (30% to 100%) and dried. Dried cells were stained on metal as well as covered with gold. Using Scanning electron microscope (JSM-IT200, Joel) images was generated.

### Heavy Metal Biosorption Assay

Bacillus cereus supernatant was prepared in LB broth medium in shake flask, pH 7.0 and temperature 37oC were maintained and incubated at 150rpm for an hour in rotary shaker (Remi, Model RS-12 Plus) to get 0.6 optical density at 600nm for equal enzyme activity. After then 300 ppm of freshly prepared heavy metal (NaAsO, Pb(NO3)2 and HgCl2) was added separately in culture flask, maintained pH 7.0 and incubated at 37oC for 24 hours and the same medium used as control without bacterial supernatant. After 24 hours culture was centrifuged at 5000 rpm for 15 min. The supernatants were collected and mixed two volume of concentrated Nitric Acid. Then the complete acid digestion, mixture was heated on a Hot Plate at 100oC until the volume decrease to early volume of supernatant. (Lolo Wal Marzan et al., 2017) The extract was filtered by Whatman 42 filter paper and collected into a volumetric flask. Total heavy metal reduction of extract was assessed by Inductively Couple Plasma Mass Spectrophotometer (Agilent, 7800 ICP-MS). (Debajit Kalita and S.R. Joshi, 2017) The percentage of heavy metal degradation capacity (%) calculated as follows:

$$\% \text{ of heavy metal utilized} = \frac{\text{Heavy metal utilized (ppm)}}{\text{Heavy metal added to the LB broth (ppm)}} \times 100$$

Heavy metal utilized (ppm) = Heavy metal added to the LB broth (ppm) - Heavy metal at the end of culture (ppm)

### Optimization of Heavy Metal Biosorption using One Factor at a Time

To optimize the efficiency of Bacillus cereus against Lead, Mercury and Arsenic, five environmental factors pH, time, temperature, inoculums concentration and metal ion concentration were assessed using UV-VIS Spectrophotometer (P. Meenambigai et al., 2017). The LB broth media used to optimize the biosorption of NaAsO, Pb(NO3)2 and HgCl2 concentration varies from 1% to 10% (v/v). The pH was maintained via digital pH meter and pH range from 2 to 10 pH. The incubation temperature was maintained from 35oC to 45oC and incubation time was varied from 24 hours to 96 hours. After every 24 hours of incubation 2 ml media was extracted and centrifuged at 5000rpm for 15 minutes, collected supernatant mixed with two volume of Concentrated HNO3 and heated at 100oC until solution decrease early amount of supernatant. The heavy metal biosorption was measured by UV-VIS Spectrophotometer. (Ghangale Sharmila S et al., 2017)

$$\text{Percentage of Degradation} = \frac{Ci - Cf}{Ci} \times 100$$

Where, Ci initial concentration, Cf Final Concentration.

## Results and discussion

### Isolation and Screening of Isolates

Bacillus cereus identified through 16s rRNA sequencing (GenBank accession No. OQ691647) and isolated from soil sample of Gevra coal mine Korba. B.cereus tested against 100µg/ml Pd for primary screening and able to developed colony in lead nitrate supplemented LB agar media. B.cereus also tested for multiple heavy metal includes NaAsO, Pb(NO3)2 and HgCl2. After incubation B.cereus developed growth against NaAsO, Pb(NO3)2 and HgCl2. (Fig. 2)

### Heavy Metal Analysis in soil

Heavy metal analyzed through AAS against Pd, As and Hg. The lead concentration 236 ppm/kg, Arsenic 13 ppm/kg and Mercury 56 ppm/kg was assessed in soil sample of Gevra coal mine (Fig. 3).

The nutritional limit of heavy metal metals in soil is very important and permissible limits of the heavy metals in agricultural soils have been reported by WHO as Hg-0–0.3 µg g-1, As - 1–30 µg g-1, and Lead -300 µg g-1. (Hamid Shirkhanloo et al., 2015)

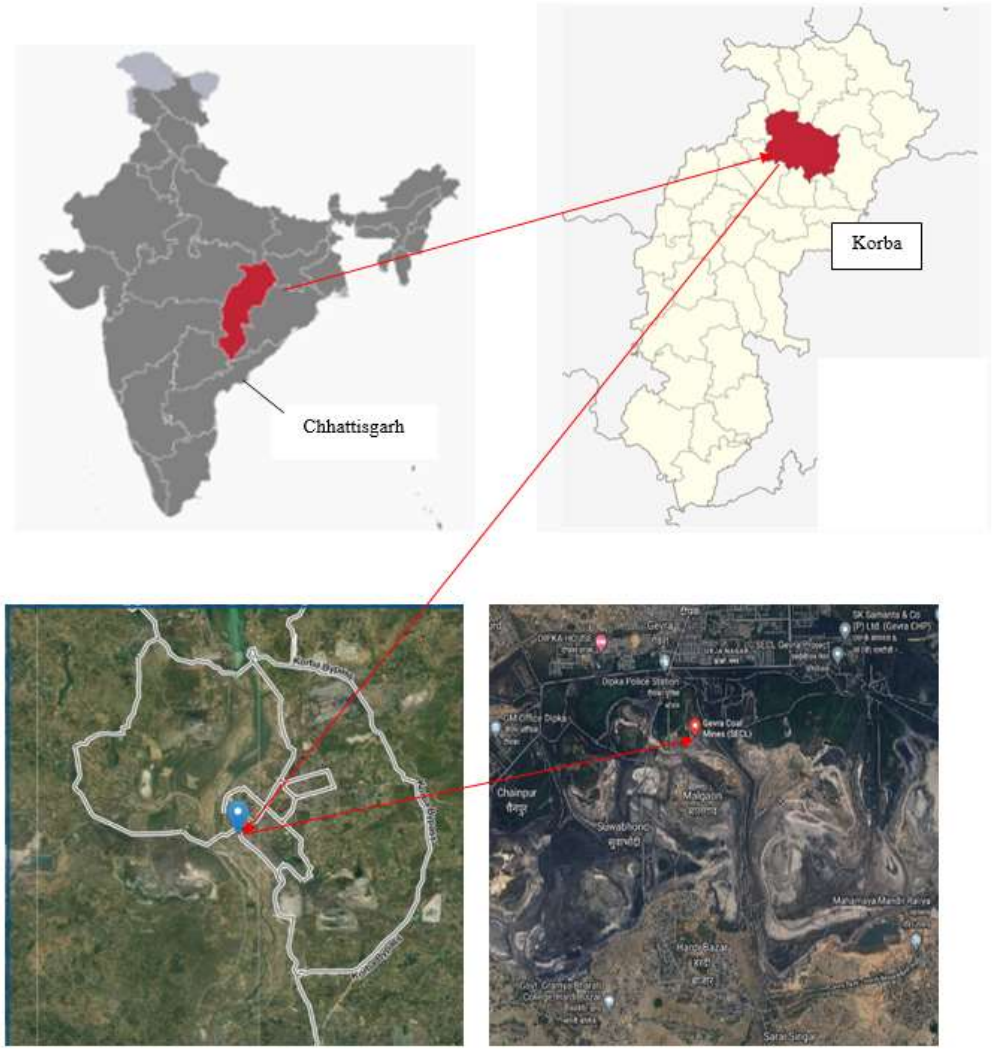


Fig. 1 Map view of Bacillus cereus Isolation site Gevra coal mine Korba, Chhattisgarh.

Fig. 2 Multiple Heavy Metal Resistant analysis (A) Arsenic (B) Lead (C) Mercury

## Heavy Metal Analysis in soil

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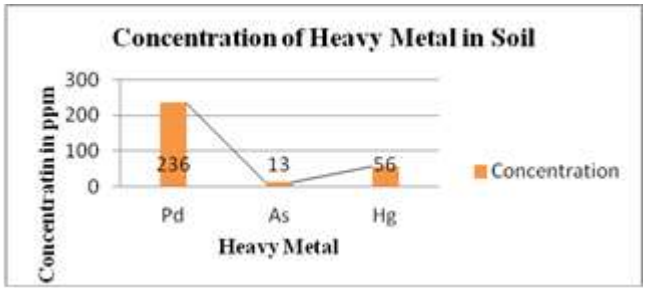


Fig 3: Heavy Metal Concentration in Soil analyzed via AAS.

## Visualization o the cell structure

Scanning Electron microscope was used to determine morphological structure of Bacillus cereus. Before heavy metal adsorption, obtained the cells image were short rod-shaped with smooth surface. B.cereus treated with 300 ppm Pb(II) and Hg(II) was analyzed that large particles adhered to the surface of cell showed granular form. Similarly testes were made in B.cereus treated with As(II). Arsenic treated B.cereus decrease surface area and shrinking comparison with untreated cells (Fig 4).

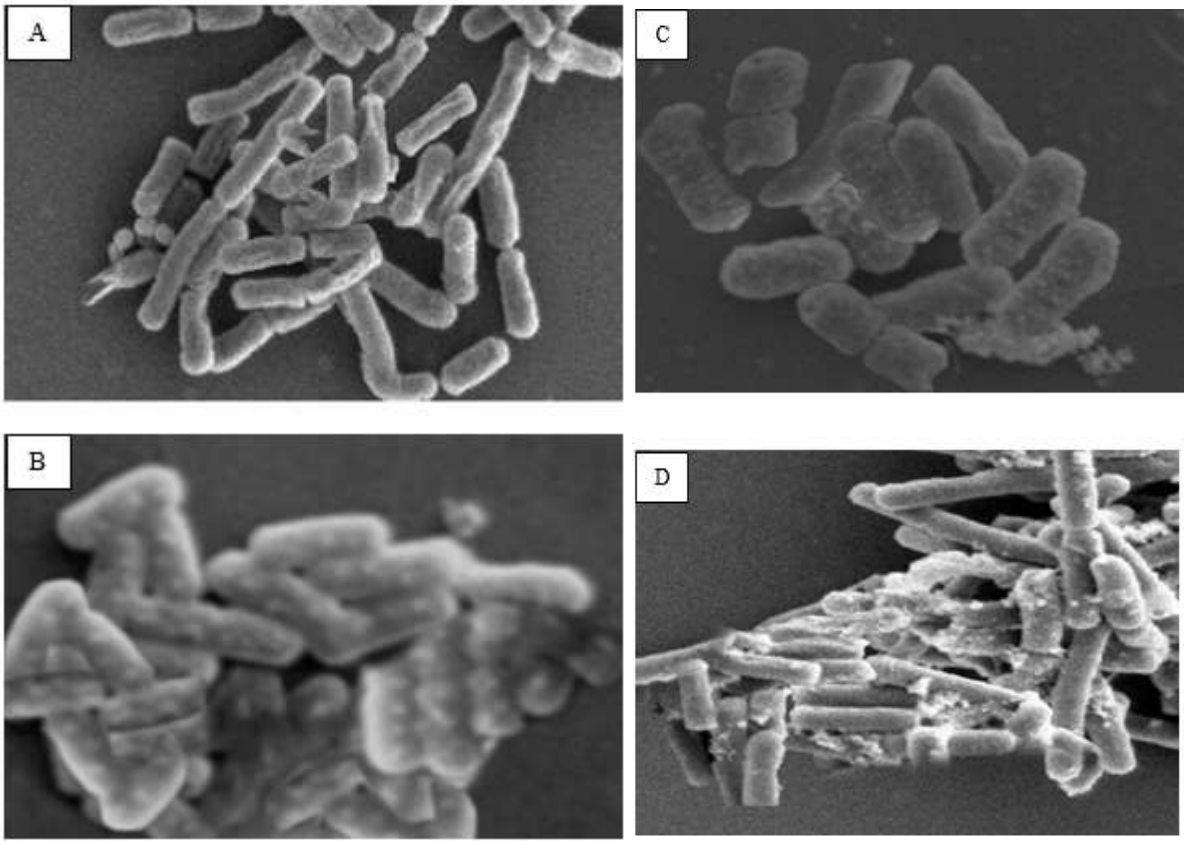


Fig 4. Scanning Electron microscope image of B.cereus culture (a) without treated (b) NaAsO (c) $HgCl_2$ (d) Pb $(NO_3)^2$.

## Assessment Biosorption of Heavy Metal

Inductively Coupled Plasma Mass Spectrometry assessment presented that Bacillus cereus capable to decrease concentration of Lead, Mercury and Arsenic after incubation compared with control. Bacillus cereus absorbed 82% lead, which compared with Babita Sharma and Pratyoosh Shukla (2021) obtained result 79.26 % remediated against lead at initial concentration at 100ppm, 67% of Arsenic, which was compared with Uttiya Deya et al., (2016) obtained result 51.45% at 100ppm after 72 hours of incubation and 89% of Mercury, Aatif Amin et al., (2022) similarly reported remediated 85% of Hg at initially 30µg/ml concentration after 72 hours of incubation.

Table 1: Heavy Metal Biosorption by Bacillus cereus analyzed through ICP-MS.

| **Heavy Metal** | **Initial Concentration (µg/L)** | **Final Metal Concentration (µg/L)** | **Metal Absorbed (µg/L)** | **% Biosorption** |
|---|---|---|---|---|
| NaAsO | 300 | 99 | 201 | 67 |
| $Pb(NO_3)^2$ | 300 | 54 | 246 | 82 |
| $HgCl_2$ | 300 | 33 | 267 | 89 |

## Optimization via One Factor at a Time to determine biosorption and Bacterial growth

The efficient degradation of Lead, Arsenic and Mercury was performed with low to higher concentration pH, time, temperature, inoculums concentration and metal ion concentration.

Effect of pH

The pH is the most significant physical parameter. pH can be able to influence heavy metal hydrolysis, organic or inorganic ligands complication , precipitations, metal ions competition and functional group activity in the biomass.(Friis et.al.,1998). The pH range was taken from 2 to 10. Highest absorption was indicated at pH 8 while below range of pH 7 shows minimum degradation of about 52.2%, 32%, and 26.5% respectively. Albeit at pH 10 absorption were presented at 69.7%, 40% and 30.6% respectively. P. Meenambigai et al., (2017) studies have been established that appropriate pH range for biosorption of heavy metal for Bacillus thuringiensis under controlled over a range 2 to 10. Albeit that the pH 8 is best supported for bioremediation and growth of bacteria.

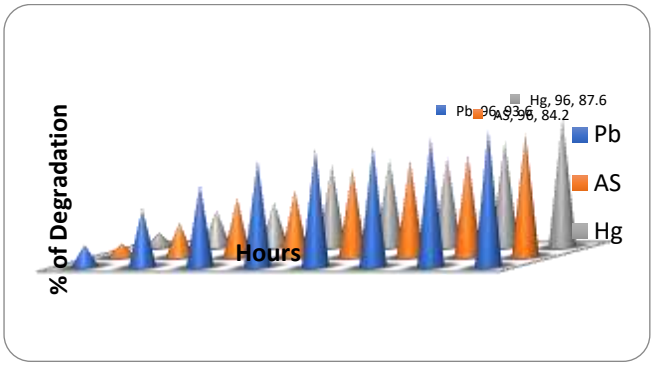


Fig 5: The effect of initial pH on bacterial biosorption by Bacillus cereus at 8pH, 300 ppm (Pb(II), As(II) and Hg(II)), Temperature $37^{O}$C and 10% (v/v) inoculum size after 96 hours incubation.

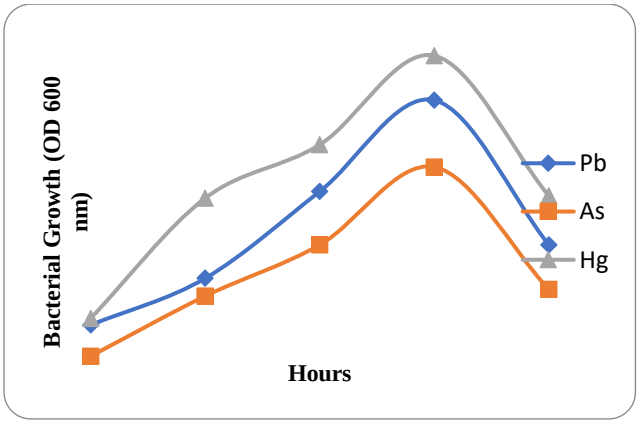


Fig 6: Growth curve of Bacillus Cereus at 8pH with Heavy metal stress.

Effect of Temperature

Temperature is major environmental factor to influence metabolic pathways of microorganism. Highest biosorption was observed at 35oC, however at various temperatures 25, 30, 40 and 450C shows

minimum absorption. 40.5%, 25.2% and 10.2% absorption of Lead, Arsenic and Mercury was recorded at below 300C respectively. On other hand, at above 400C Lead, Arsenic and Mercury were observed 60.6%, 35% and 23.8% absorption respectively. D. R. Kotwal et al., (2018) also investigated an optimum temperature 35°C for degradation via Pseudomonas aeruginosa.

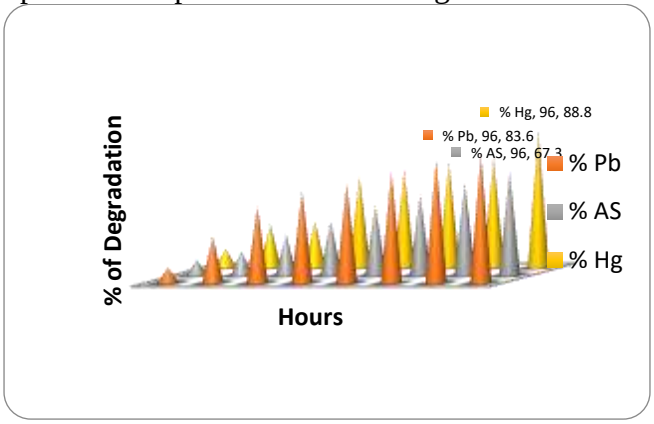


Fig 7: The effect of initial Temperature on bacterial biosorption by Bacillus cereus at 7 pH, 300 ppm (Pb, AS, Hg), Temperature 35OC and 10% (v/v) inoculum size after 96 hours incubation.

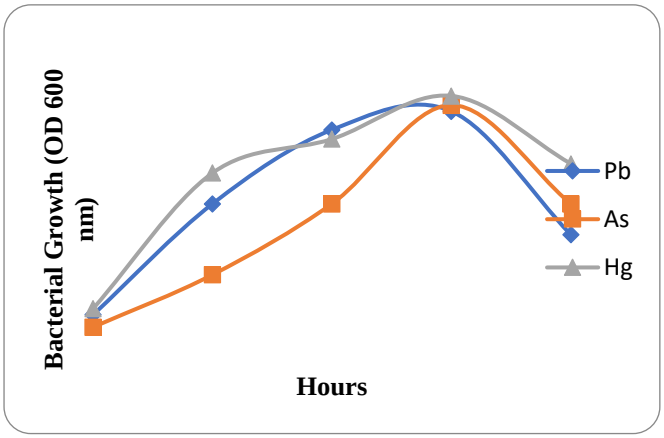


Fig 8: Growth curve of Bacillus Cereus at 35oC with Heavy metal stress.

Effect of Incubation Time

Incubation time plays very important role for microorganism growth to equally distribution of microbe's activity. Initially at 24 hours of incubation biosorption ability was very low but after increasing of the incubation time absorption was increased. At 96 hours of incubation time highest absorption was observed. After 96 hours microbial activity was decreased.

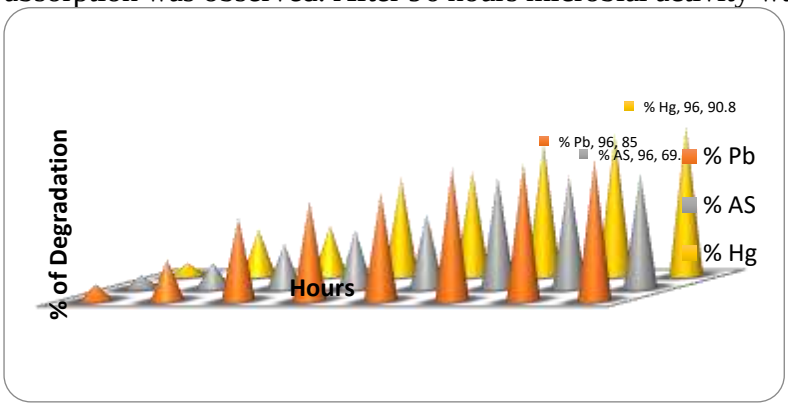


Fig 9: The effect of initial Incubation time on bacterial biosorption by Bacillus cereus at 7 pH, 300 ppm (Pb(II), As(II) and Hg(II)), Temperature 35OC and 10% (v/v) inoculum size after 96 hours incubation.

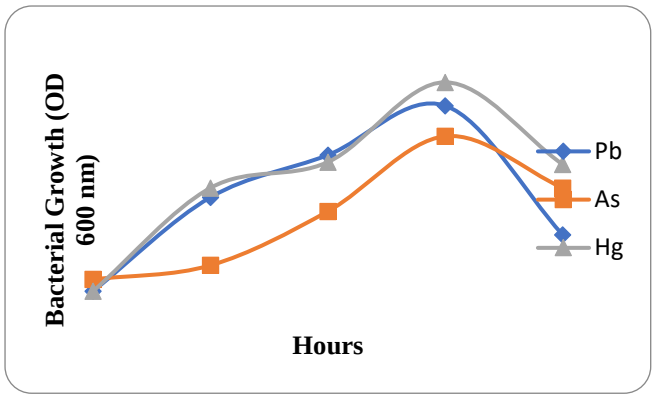


Fig 10: Growth curve of Bacillus Cereus at 96 hours of incubation time with Heavy metal stress.

Inoculum Concentration

The size of bacterial inoculum affects the synthesized enzyme levels to serve cell metabolism. The effect of inoculum concentration over a range 0.5 – 20% (v/v) was performed. Inoculum size was optimized of 8–10% (v/v), after that bacterial growth was a decline. Lead, Arsenic and Mercury at 15% and 20% inoculum size reduced the effectiveness of enzyme to 40.%, 30.32% and 22.12% absorption respectively. Inoculums size 8-10% increases biosorption ability, likely insufficient nutrient and dissolved oxygen for larger inoculums size deplete the growth and absorption ability. Sharma K and Rathore M (2010) reported a 6% inoculums size rapid increase in enzyme production as well as with above inoculums size decreases the enzyme production result of insufficient nutrient.

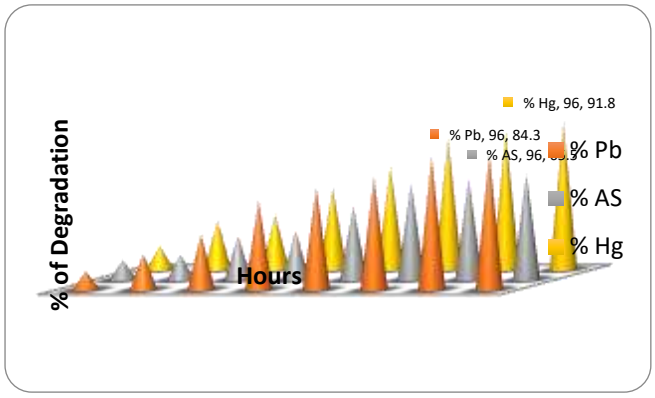


Fig 11: The effect of initial Inoculum concentration on bacterial biosorption by Bacillus cereus at 7 pH, 300 ppm (Pb, AS, Hg), Temperature 35OC and 8-10% (v/v) inoculum size after 96 hours incubation.

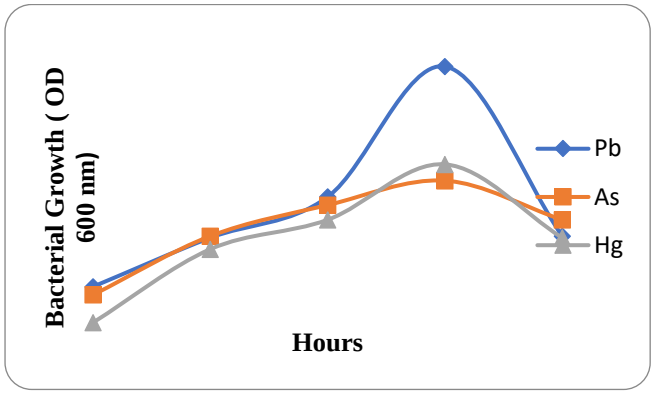


Fig 12: Growth curve of Bacillus Cereus at 8-10% of inoculums size with Heavy metal stress.

Effect of Metal ion

Microorganisms not able to survive at high concentration of metal ions can be toxic. Bacillus cereus has the potential to survive in as well as degrade high concentration of metal ions. The effects of metal

ions over a range 1–7% (v/v) on bacterial growth rate were studied. Optimum biosorption was obtained at 3% concentration of metal ions, whereas, Lead 87.6%, Arsenic 70.6% and Mercury 87.50% after 96 hours of incubation. Biosorption and bacterial growth decreases rapidly at above 4% concentration metal ion. The obtained data signify that at above 4% concentrations metal ion is toxic to Bacillus cereus, with reduced growth and absorption.

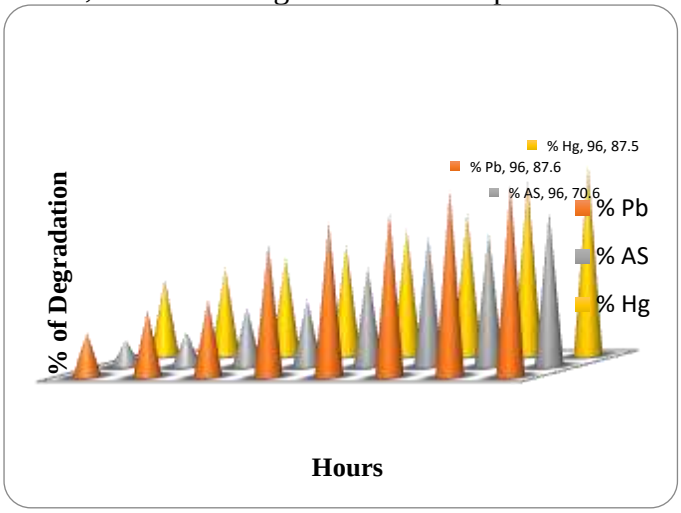


Fig 13: The effect of initial metal ion concentration on bacterial biosorption by Bacillus cereus at 7 pH, 3% Pb, AS, Hg, Temperature 35OC and 10% (v/v) inoculum size after 96 hours incubation.

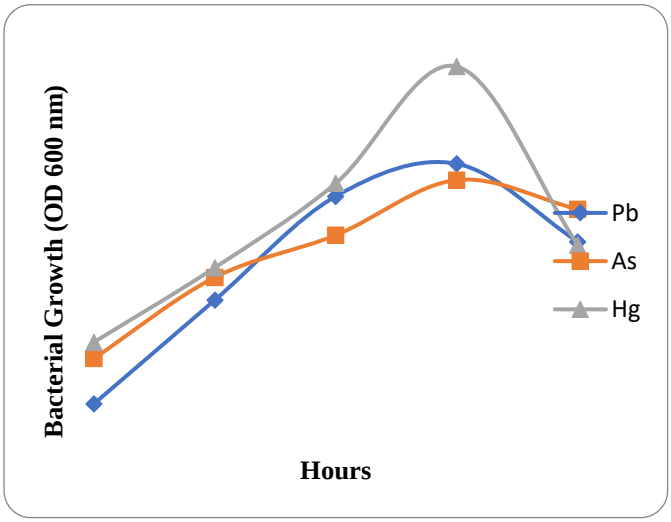


Fig 14: Growth curve of Bacillus Cereus at 3% of Metal ion.

## Conclusion

This current study confirmed that Bacillus cereus capable to remediate NaAsO, Pb(NO3)2 and HgCl2. Firstly isolated from Gevra coal mine, Korba, Chhattisgarh. The Bacillus cereus able to grow in the presence of Pb(II), As(II) and Hg(II) would be greatly useful for the bioremediation of heavy metal contaminated sites. In this study, analyzed quantitative biosorption of heavy metals was obtained through ICPMS and optimized the biosorption as well. The optimum biosorption rate via Bacillus cereus were achieved at initially 3% metal ion concentration, a temperature 35oC, pH 8, Inoculum size 8-10% and incubation time 96 hours. The current results, the biosorption method could be an alternative valuable method to decontamination of heavy metal contamination environment.

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