Bioremediation of Heavy Metal Using Consortia Developed from Municipal Wastewater Isolates

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Publication Info

Article history :
Received : 11th Nov. 2016
Accepted : 10th April 2017
DOI : 10.18090/samriddhi.v9i01.8339

Keywords :

Biodegradation, Toxic affects Chromium, Consortia, Lead and Zinc.

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Abstract

Nowadays heavy-metal pollution has become an environmental problem due to their toxic effects, and their invasion in to the food chain leads to serious environmental and health problems. Heavy Metal degradation through common physico-chemical techniques is very expensive and unsuitable in treating large contaminated area effectively. Bioremediation provides a promising means to reclaim such toxic substances in an economical and ecofriendly way. Bioremediation obtains microorganisms that capable to degrade toxic contaminants or have the ability to accumulate it in their cells. The present study was carried out to evaluate degradation capacity of consortia developed from municipal wastewater isolates. The activity of the isolates for hemolysis was studied on the Blood-Agar plates. The identification of isolates obtained through biochemical and morphological characteristics. Seven isolates and three defined consortia were tested for degradation of heavy metals (zinc, lead and chromium). Consortia 3 (R9 + S11 + T12) showed better degradation with 93.78% ability in reducing zinc when incubated for 72 hours and 86.16% when incubated for 24 hours. The lead reduction was found to be 84.33% by Consortia 1(A3 + B4) when incubated at 37°C for 72 hours incubation. The chromium was reduced by Consortia 2(C6 + D7) with 87.61% ability when incubated for 72 hours. The organisms had capacity to reduce the heavy metals depending on the factors like time and concentration of inoculum. As the time of incubation increases, more reduction was observed. This study gives us insight in to the inherent potential of the Consortia to bio remediate toxic heavy metals.

1. INTRODUCTION

Environmental pollution due to chemicals including heavy metals is a big problem because depositions of heavy metals have dramatically altered the biogeochemical cycles of atmosphere that may have negative consequences on environmental components. The levels of heavy metals in all environments, including air, water and soil are increasing in some cases to toxic levels, with contributions from wide variety of domestic and industrial sources.

Metal contaminated environments pose serious threat to human health and ecosystems.The most abundant pollutants in river water and in sewage are heavy metals [1, 12]. The heavy metals pollution comes from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge and the irresponsible

disposal of wastes by various industries [2, 21]. Some of the negative impacts of heavy metals on plants include decrease of lipid content and seed germination by cadmium, decrease enzyme activity and plant growth by chromium, the inhibition of photosynthesis by mercury and copper, and the reduction of seed germination by nickel and the reduction of plant growth and chlorophyll production by lead [10]. The impacts on animals include reduced development and growth, cancer, nervous system damage, organ damage and in extreme cases, death. Heavy metals contaminated drinking water reservoirs, and fresh water habitats can alter microbial communities. Chemical precipitation of heavy metals in water has been practiced as a prime treatment method to be disposed industrial waters for many years. Other chemical treatment techniques, such as ion exchange has been reported to be effective in heavy metal removal in polluted waters [5]. Adsorption is a widely used method for the industrial wastewater treatment containing color, heavy metals, and other inorganic impurities. These methods suffer from low adsorption capacities and in some cases complete removal is impossible and high adsorbent cost [24]. Chemical oxidation is a process in which industrial waste materials are removed by the help of chemical oxidation by the use of various chemicals mainly hydrogen peroxide is widely used for this purpose as reported [15]. Phytoremediation entails the use of plants to partially or substantially remediate selected substances in contaminated soil, sludge, sediment, groundwater, surface water and wastewater. It is referred to as green remediation, botano-remediation, agro-remediation or vegetative remediation [25]. All these methods have some limitations and the common problems that are associated with these methods make them expensive.

And they produce other waste disposal problems by own ways, which have limited their industrial applications [8]. Among the available treatment processes, the application of the microbiological treatment is gradually getting momentum due to the following reasons: Chemical's requirement for the whole treatment process is reduced by own cycles, Eco-friendly, Low operating cost and cost effective alternative of conventional techniques, efficient at any levels of contamination.

Bioremediation is the use of microorganisms or their enzymes to break down and thereby detoxify toxic and hazardous chemicals in the environment [14, 23]. It plays a major role in making the environment clean from carcinogenic pollutants and heavy metal contamination [5]. Microbial activity is thought to play a key role in the detoxification of heavy metals in water. In heavy metal remediation (or biodegradation), bio surfactant play a most important role including wetting, contact of bio surfactant to the surface of the sediments and detachment of the metals from the sediments. Bioremediation is an economical, eco-friendly and requires less expensive techniques for water pollution. But the correct microbe should be utilized in the appropriate place with the precise environmental factors. Therefore, the main goal of present study was to examine the ability of bacterial consortia isolated from municipal wastewater for bioremediation of toxic heavy metals.

2. MATERIALS AND METHODS

2.1 Sample Collection

Heavy metal contaminated Water samples were collected from river around Allahabad district. The water and sediment samples collected from different locations were kept in clean glass jars and transported to the laboratory in the cold black box. The samples were stored in the freezer till analysis. Heavy metals (zinc, lead and chromium) in the sediment were determined by digesting 1g sediment sample with concentrated HNO3 made

S-JPSET : Vol. 9, Issue 1, ISSN : 2229-7111 (Print) & ISSN : 2454-5767 (Online)

up to 50 ml volume. The sediment elutriates were prepared by shaking sediment in water at 1:4 ratio for 24h. The supernatant was separated by centrifuging at 6,000 rpm for 60 min at 4 ºC. Elutriate was stored at 4 ºC until analysis. Elutriate were acidified and directly used for the estimation of trace metals by Differential Pulse Anodic Striping Voltammetry (**DPASV**) method [20].

2.2 Isolation and Identification of Municipal Wastewater Bacterial Isolates

Municipal wastewater sample was collected from railway pulliya MNNIT Campus Allahabad district, India, in pre-sterilized bottle according to standard procedures from American Public Health Association (APHA, 2005) and transferred immediately to the laboratory. Municipal wastewater sample was serially diluted and inoculated on the Nutrient agar medium separately. Morphologically different colonies were isolated and maintained at 4^0C on nutrient agar slants. The purified isolates were identified by morphological and biochemical characteristics based on Bergey's Manual of Determinative Bacteriology [17, 18].

2.3 Analytical Analysis

These water samples were subjected to various analyses like COD, BOD, TDS, metal analysis. The physical-chemical parameters including calcium content and Microbial count were estimated using standard methods [3, 19].

2.4 Metal Analysis

Complex metric titrations are commonly known as EDTA titrations since EDTA acts as a most versatile complexation agent. A wide range of metal ions including Pb, Zn & Cr removed as complexes with least difficulty using modified method [26].

2.5 Construction of Bacterial Consortia

The Seven different isolates that demonstrated good growth were chosen to construct consortia of heavy metal degraders. In total, seven different bacteria were constructed three consortia and tested for in this study to confirm their metal biodegradation capabilities.

2.6 Preparation of Consortia Inoculum

The isolates were grown separately in NB and processed to yield separate suspensions with an absorbance reading of 0.5 at 550 nM. Specific aliquots of the bacterial inoculum were then separately added into normal saline solution to give a final combined inoculum concentration of 10% (v/w) [2, 20].

2.7 Screening for Bio Surfactant Producing Capacity

The Seven different isolates and three consortia were obtained in pure cultures and tested for their bio surfactant production by the following methods.

2.7.1 Oil Spreading Assay

In oil spreading assay 10 ìl of crude oil was added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 ìl of culture or culture supernatant were gently placed on the Centre of the oil layer. The presence of bio surfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30 seconds, which correlates to the surfactant activity, also known as oil displacement activity [22].

2.7.2 Blood Hemolysis Test

The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated for 48 to 72 hours at 37°C. The plates were then observed and the presence of clear zone around the colonies indicated the presence of bio surfactant producing organisms. Results were recorded based on the type of clear zone observed [4, 28].

2.7.3 Blue Agar plate (Bap) Method

Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic bio surfactant. 30µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic bio surfactant production [28, 29].

2.7.4 Emulsification test (E24)

Several colonies of pure culture were suspended in test tubes containing 2 mL of mineral salt medium after 48 h of incubation, 2 mL hydrocarbon (oil) was added to each tube. Then, the mixture was vortexed at high speed for 1 min and allowed to stand for 24 hours. It is expressed as percentage [6, 13].

2.8 Bio surfactant Production

Isolates were grown in 500ml Erlenmeyer flasks containing 100ml mineral salt medium adjusted to pH 7.0 was used as culture medium. The flasks were incubated at 37 °C on a shaker incubator for 7 days. To extract the bio surfactant, the bacteria were removed by centrifugation and the remaining supernatant liquid was collected. Bio surfactant was obtained by adjusting the supernatant pH 2.0 using 6N HCl and keeping it at 4°C overnight. The precipitate thus obtained was pelleted by centrifugation for 20min, dried and weighed [16, 30].For further purification the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 60°C. The dry product was extracted with Chloroform: Methanol (65:15) filtered and the solvent evaporated.

2.9 Determination of Bio surfactant

Chemical composition of the bio surfactant was analyzed and Carbohydrate content of the bio surfactant was determined by DNS method, absorbance was were taken at 620 nm [7, 31].The bio surfactant from the sample was estimated for glycolipids using orcinol assay method. The orcinol assay was used for the direct assessment of the amount of glycolipids in the sample. To 100 µL of each sample, 900 µL of a solution containing 0.19% orcinol (in 53% H2SO4) was added. After heating for 30 min at 80ºC, the samples were cooled at room temperature and the OD at 421nm was measured. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg mL^{-1}) [28]. Protein content of the bio surfactant was determined by using biuret method, which forms purple colored complex by reacting with peptide bond present in the proteins. Bovine serum albumin was used as a standard and OD readings were taken at 540 nm [11, 13].

3. REMOVAL OF HEAVY METALS FROM WATER SAMPLES

Degradation of heavy metals experiments were carried out in 250 ml of separate flasks containing 100 ml of metal contaminated water samples. The pH was adjusted to 7 using NaOH and H_2SO_4. Then, the flasks were autoclaved at 121ºC for 15 minutes. The autoclaved flasks were inoculated with 0.2 ml of bacterial inoculum of each isolates. The flasks were kept in mechanical shaker and incubated at 37ºC for 24 hours and 72 hrs. The sterilized water sample without microorganism served as control [2, 27].

4. HEAVY METAL ANALYSIS

After 24 hours 50 ml of the treated sample was taken and centrifuged at 10,000rpm for 10 min to remove the cell suspension. The cell free broth is now taken and analyzed for the reduction in Zinc, Lead and chromium using the procedure as mentioned above. The remaining 50 ml of the sample is further incubated for 72 hours and the same procedure was followed [5, 16].

S-JPSET : Vol. 9, Issue 1, ISSN : 2229-7111 (Print) & ISSN : 2454-5767 (Online)

5. RESULT AND DISCUSSION

5.1 Constituents of all the Water Samples

Physical chemical characteristics of effluent waste water were analyzed using standard methods and are tabulated in Table 1.

Table-1: Physical & Chemical Components of Water Samples

PARAMETERS	CON.	S1	S2	S3	S4
Color	Color less	Color less	Colorless	Colorless	Slightly turbid
pH	7 ± 0.1	7.3 ± 0.2	8.5 ± 0.3	6.5 ± 0.2	8.2 ± 0.3
BOD(mg/l)	0.64 ± 0.09	2.72 ± 0.43	1.6 ± 0.15	2.24 ± 0.31	0.96 ± 0.23
COD(mg/l)	12 ± 1.2	17.6 ± 1.5	40 ± 3.5	32 ± 2.1	54 ± 2.9
TDS(mg/l)	238 ± 3.4	216 8 ± 4.7	3658 ±7.3	3216 ± 8.6	4312 ± 4.8
Ca(mg/l)	10 ± 0.98	30 ± 1.5	20 ± 2.6	40 ± 3.9	20 ± 1.87
Zn(mg/l)	0.6 ± 0.04	7.6 ± 0.81	9.8 ± 0.56	8.8 ± 0.74	9.5 ± 0.16
Pb(mg/l)	-	4.75 ± 0.25	5.17 ± 0.89	3.72 ± 1.78	6.003 ± 0.015
Cr(mg/l)	-	7.2 ± 0.52	10.5 ± 0.34	4.85 ± 1.32	17.5 ±1.3

Control - Tap water, Sample 1- Baluwa Ghat, Sample 2- Gau Ghat, Sample 3- Saraswati ghat, and Sample 4- Arail Ghat.

These Ghats are situated southern part of Allahabad city. The pH values of the effluent wastewater were within the optimal range (Table-1). The BOD of sample 1 and sample 3 was found to be greater than the control indicating that the pollution strength is higher in the water samples. As the BOD values increases, the microbial content in the sample seems to be higher. TDS is the total dissolved solids present in the samples. The more the TS value, the more contaminated the water. The TDS of the sample 4 was greater than all the samples and much higher than the control. The COD reading of all 4 water samples was in the range which shows that the chemical oxygen demand was found to be inside the limit. The COD of sample 4 was found to be greater than the other 3 samples including the control. The high content of the COD in sample 4 may be due to the chemical composition of the water sample. The heavy metals like zinc, lead and chromium was detected in all the water samples. The concentrations of the heavy metals in all the samples were found to be more than the concentration present in the control.

5.2 Isolation and characterization of municipal wastewater isolates

Total seven bacterial isolates were identified on the basis of morphological and biochemical characteristics. It includes Bacillus licheniformis A3, Pseudomonas aeruginosa B4, Pseudomonas sp.C6, Planococcus salinarum D7, Stenotrophomonas maltophilia R9, Paenibacillus sp. S11 and Paenibacillus borealis T12.

5.3 Construction of Bacterial Consortia

From seven different bacterial isolates, three consortia were prepared those are namely Consortia 1(A3 + B4), Consortia 2(C6 + D7) and Consortia 3 (R9 + S11 + T12).

5.4 Screening for Bio Surfactant Producing Capacity

Bacterial isolates and consortia were streaked on Muller Hinton Agar plates & the strains were named as A3, B4, C6, D7, R9, S11, T12, CO1, CO2 and CO3 respectively. The isolated colonies were sub-cultured on Muller Hinton Agar plates to obtain pure cultures.

5.4.1 Oil Spreading Technique

The supernatant of the ten strains (Seven isolates and three defined consortia) were added to the plates containing oil. The strain A3, B4, C6, D7, R9, S11, T12, CO1, CO2 and CO3 displaced the oil showing a zone of displacement with SDS as the control. The results were shown in the (Table- 2). The strain C6 showed the higher zone

of displacement compared to the control. For comparison purposes, commercially available synthetic surfactants were used indicating the efficiency in displacing oil layer due to the production of bio surfactant followed by the strain T12. Least zone of displacement was found by the strain D7.

Table-2: Oil Displacement Activity of Isolates

Bacterial Strain	Zone of Displacement (mm)	Inference
Control (SDS)	13	Positive
A3	8	Positive
B4	5	Positive
C6	14	Positive
D7	7	Positive
R9	6	Positive
S11	10	Positive
T12	5	Positive
CO1	4	Positive
CO2	4	Positive
CO3	3	Positive

5.4.2 Blood Hemolysis Test

Blood agar method is often used for a preliminary screening of microorganisms for the ability to produce bio surfactants on hydrophilic media [31]. In the current studies all the strains were streaked on blood agar plates. All the ten strains showed positive results for haemolytic activity. Formation of a clear zone could be seen around the colonies (Table -3). The positive result of blood hemolysis is due to the lysis of the RBC's present in the medium.

Table-3: Blood Hemolysis Results

Bacterial strains and Consortia	Observation	Results
A3	β hemolysis	Positive
B4	β hemolysis	Positive
C6	β hemolysis	Positive
D7	β hemolysis	Positive
R9	β hemolysis	Positive
S11	β hemolysis	Positive
T12	β hemolysis	Positive
CO1	β hemolysis	Positive
CO2	β hemolysis	Positive
CO3	β hemolysis	Positive

Blood agar is an enriched and selective medium which allow only hemolytic organisms to grow by utilizing blood and hence the production of bio surfactants cause the lysis of cells which is an indicator of production of bio surfactants by these organisms. The organisms showed complete zone of hemolysis.

5.4.3 Emulsification Index (E24)

The isolated strains showed positive results were tested for their abilities emulsify crude oil and in this study olive oil was take for the study of emulsification index. The results were noted down in (Table- 4). It showed that the strain C3 showed the maximum emulsification activity with olive oil after 24 hours of incubation followed by the strain R9 and the least activity was shown by the organism D7.

Table-4: Emulsification Activities of Bacterial Strains And Consortia

Bacterial strains and Consortia	E_{24} (%)
A3	73.4
B4	62.3
C6	92.4
D7	87.5
R9	66.8
S11	83.3
T12	88.1
CO1	90.2
CO2	91.3
CO3	94.5

From this assay the ability to degrade the hydrocarbon by producing bio surfactants which is the property of the bio surfactant producing organism can be determined.

5.5 Production of Bio Surfactant

Mineral salt medium supplemented with 1% glycerol produced high amount of bio surfactant which was collected using acid precipitation and purified by chloroform methanol solvents. White coloured precipitate was seen at the junction of

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the two immiscible liquids chloroform: methanol. This white precipitate, which was the bio surfactant, was observed in all the ten strains (Seven isolates and three defined consortia).

5.6 Biochemical Properties of Bio Surfactant

Biochemical composition of the bio surfactant and the presence of carbohydrates, proteins and lipids were detected in various strains using standard method and are tabulated (Table -5).

Table-5: Biochemical Properties of Bio Surfactant Produced By Isolated Strains And Consortia

Bacterial Strains and Consortia	Biochemical Properties		
	Carbohydrate	Lipid	Protein
A3	++	+	+
B4	-	+	+
C6	+	++	+
D7	+	+	-
R9	-	+	-
S11	-	+	-
T12	+	++	+
CO1	+	++	+
CO2	+	+	++
CO3	+	+	++
Symbols: ++ highly positive, + slightly positive, - negative			

A3 contains all the 3 components but the carbohydrates in higher amounts than the other two. The strain C6 contains lipid in greater amounts along with carbohydrates and lipids in small amounts.

5.7 Reduction of Heavy Metals from Water samples

The sample when treated with municipal wastewater isolated organisms, after treatment the sample showed considerable reduction in the heavy metals were shown in (Fig.1, Fig.2, Fig.3, & Fig.4). Water sample was treated with the isolated organisms almost all the organisms showed reduction in heavy metals.

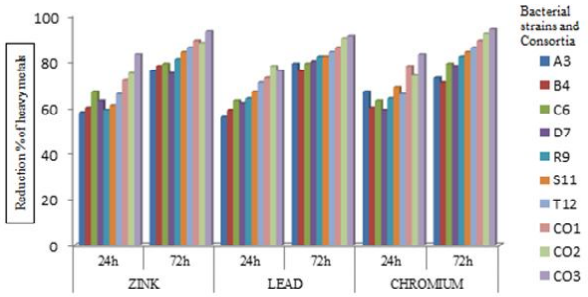

Fig.1:: Heavy Metal % Reduction in Sample 1

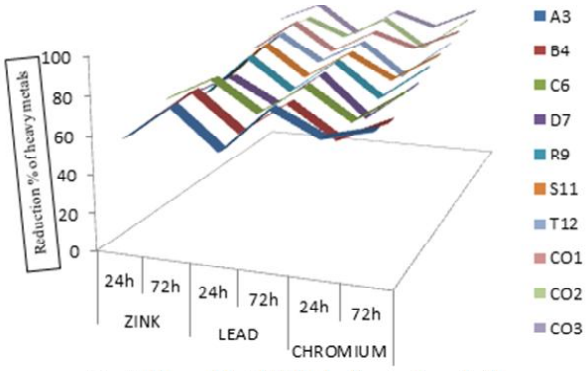

Fig.2: Heavy Metal % Reduction in Sample 2

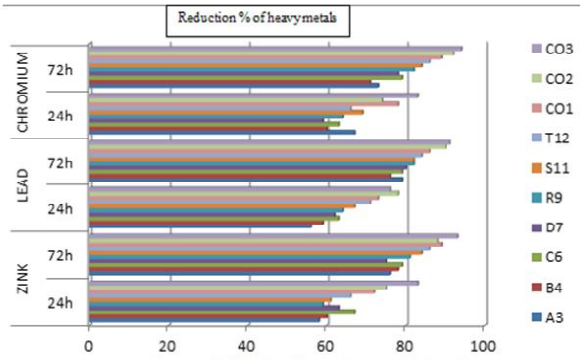

Fig.3: Heavy Metal % Reduction in Sample 3

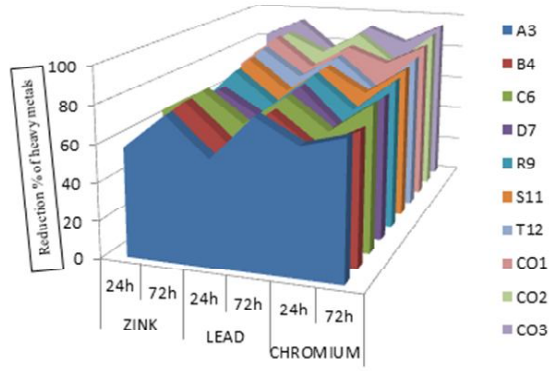

Fig.4: Heavy Metal % Reduction in Sample 4

The organism A3 and R9 showed highest percentage of degradation of zinc when incubated for 72 hours with 80.26% ability, whereas the C6 shows the highest percentage of degradation of lead which when incubated for 72 hours with 84.13% ability and the degradation of chromium was at the same percentage by the organism A3, B4 and S11 at 72 hours. All the parameters like temperature, pH, time duration and amount of the inoculum plays an important role in determining the efficiency.

5.8 *Degradation of Zinc, Lead and Chromium*

In the present study the maximum amount of degradation was found to be 86.36% at 24 hours incubation and 93.18% when incubated for 72 hours at 37°C by D7 in sample 3 which indicates that as the time duration increases, the increase in the amount of degradation of Zinc was observed. Lead removal ability from all the water samples at 37°C for 5 days which was higher than the ability of the C6 whose maximum reduction found to be 84.13% when incubated for 3 days. Whereas results showed that, bio-surfactants showed lesser activity in reduction of Pb from the metal contaminated waste water. T12 organism showed maximum amount of degradation of Cr in sample4 with 87.9% efficiency after 72hrs at 37°C incubation followed by A3 and B4 with the efficiency of 79.0% in sample 2. The highest amount of degradation found by S11 may be due to the high content of Cr in the car paint sample which could be easily degraded and also the duration of time and temperature. The least amount of degradation was found by 27.08% by R9. Consortia 3 (R9 + S11 + T12) showed better degradation with 93.78% ability in reducing zinc when incubated for 72 hours and 86.16% when incubated for 24 hours. The lead reduction was found to be 84.33% by Consortia 1(A3 + B4) when incubated at 37°C for 72 hours incubation. The chromium was reduced by Consortia 2(C6 + D7) with 87.61% ability when incubated for 72 hours.

6. CONCLUSION

Heavy metal contaminated sites were screened for Microbial strains and isolates activity for haemolysis was studied on the Blood -Agar plates. The isolated strains were studied for its biochemical and moprphological characteristics and also checked their bio surfactant producing capacity. The isolates were determined to other screening tests like emulsification activity and oil displacement technique. Seven isolates and three defined consortia were tested for degradation of heavy metals (zinc, lead and chromium) present in the river water samples. The percentage of reduction of heavy metals varies from one sample to another sample. Consortia 3 (R9 + S11 + T12) showed better degradation with 93.78% ability in reducing zinc when incubated for 72 hours and 86.16% when incubated for 24 hours. The lead reduction was found to be 84.33% by Consortia 1(A3 + B4) when incubated at 37°C for 72 hours incubation. The chromium was reduced by Consortia 2(C6 + D7) with 87.61% ability when incubated for 72 hours. The organisms had capacity to reduce the heavy metals depending on the factors like time and concentration of inoculum. As the time of incubation increases, more reduction was observed. This study gives us insight in to the inherent potential of the Consortia to bio remediate toxic heavy metals.

Further identification of organism at genus and species level will help in heavy metal reduction in highly polluted effluent treatments.

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