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Reduction of Heavy Metals in Steel Industrial Effluent by *Bacillus subtilis* Bacteria

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ABSTRACT

Bio-degradation is a new biological method and various low cost bio-adsorbents are used for maximum removal of heavy metals from waste water.Main importance of the present study was to identify the heavy metals in industrial waste water and to screen the heavy metal degraders. Based on analytical analysis with AAS the concentration of the heavy metals in the effluent is Cu-5.8mg/l, Cd-1.09mg/l, Cr-5.97mg/l, Pb-1.9mg/l. The copper concentrations in the effluent were decreased after the inoculations of the isolated bacterial strains were 2.6mg/l, 1.6mg/l, 2.0mg/l, 1.7mg/l. The Cadmium concentration in the effluent were significantly decreased as 0.76mg/l, 0.6mg/l, 0.17mg/l, 1.1mg/l. The Chromium concentrations in after the inoculation of the bacterial strains were 5.2mg/l, 2.9mg/l, 3.7mg/l, 2.5mg/l. The lead concentration in the effluent after inoculation with bio-degraders were 0.38mg/l, 0.6mg/l, 0.41mg/l, 0.8mg/l. The four bacterial isolate were screened from the waste effluent and isolate no.2 shows efficient degradation that were identified by 16SrRNA sequencing and documented as Bacillus subtilis. Conclude that bio based methods are eco-friendly best solutions for removing toxic to nontoxic from waste water rather than physical and chemical methods.

KEYWORDS: Bio-degraders, heavy metals, chromium, copper, effluents

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INTRODUCTION

Heavy metals from industrial waste are of special concern because, they produce water of chronic poisoning in aquatic animals[9].While some heavy metals are purely toxic with no cellular role[24], other metals are essential for life at low concentration but become toxic high concentration of all heavy inhibits activity of sensitive enzymes [13].

Heavy metal can damage the cell membrane, alter enzyme specificity, disrupt cellular function and damage the structure of the DNA[1], The toxic effect of Arsenic, Mercury and Lead were known to be ancient, but methodical studies of the toxicity of some heavy metal appear to date from only 1868 [26] in human, heavy metal poisoning is generally treated by the administration of chelating agents [27,24]. Some elements otherwise regarded as toxic metals are essential in small quantities [8].

The toxic heavy metals are relatively dense metal or metalloid that is noted for its potential toxicity, especially in environmental context[28].The term has particular application to Cadium, Mercury, Lead and Arsenic all of which appearing the world health organization examples include Manganese, Zinc, Selenium, Silver, Antimony and Thallium[8].Uncontrolled discharges of large quantity of heavymetals containing waste create huge economic and health care burden [25,20].

Thetoxic metal pollutant like Lead, Nickel and Cadium enter to the water bodies through industrial waste water [10].Among the heavy metal, Lead is a nonessential heavy metal and general toxicant[17].The toxicity of these heavy metal occur through the displacement of essential metal from their native binding site or through ligand interaction[22,14]. The toxicity can occur as a alteration in the conformational structure of the nucleicacid and protein and interference with oxidative phosphorylation and osmoticbalance[16,11].In the biosorption mechanisms, the complex structure of microorganisms implies that the manyways for the metal to be taken up by the microbial cell[20.]

The bio adsorption mechanisms are various, they may be classified according to the dependence on the cellmetabolismand bio adsorption mechanism can be divided into: Metabolism dependent and non –metabolism dependent. According to the location where the metal removed from the solution is found bio adsorption can be classified as (1)Extra cellular accumulation/precipitation (2) Cell surface sorption /precipitation: and (3) intracellular accumulation. Heavy metals are not biodegradable and tend to be accumulated in organism and because of numerous disease and disorder[24].To survive under metal -stressed condition, bacteria have evolved several types of mechanism to tolerate the uptake of heavy metal ions[18,19]. These metal mechanisms include the effluxofions outside the cell, accumulation and complexation of metal ions

inside the cell [20]. The objective of this work is to isolate the metal resistant bacteria from the industrial effluent by the determination of physio-chemical properties of the effluent and the biochemical characterization of the metal resistantbacteria.

Heavy metal pollution caused by mining, steel production, and electroplating has induced an adverse impact on the environment, which threatens the health of human beings and the stability of ecosystem[1]. Heavy metal ions can be accumulated through food chain, which is believed to be a risk for human beings even at trace level[29]. Additionally, heavy metal ions can affect cellular organelles, components, and induce oxidative stress. For instance, arsenic can induce DNA hypomethylation gene expression[21]; chromium can induce DNA damage and oxidative stress in Sprague–Dawley rats and in human liver carcinoma cells, copper has been reported to take part in the oncogenic BRAF signaling which is related to various cancers..

As a consequence, numerous diseases such as Menkes disease, Alzheimer's disease and cancers can be induced by the excessive intake of heavy metal ions[4]. Therefore, heavy metal pollution has already become one of the most serious environmental problems and the remediationofactiveheavymetalloidsofgreatimportance.Traditional heavy metal remediation methods have many limitations including production of toxic chemical sludge and not eco-friendly. Therefore, simple and low-cost remediation method is urgently needed.

Nowadays, bioremediation technologies based on microorganisms have attracted scientists' great interest because of their outstanding advantages including high efficiency, low-cost and eco-friendly, especially at low metal concentrations.Therefore, we have summarized the heavy metal remediation and their detoxification pathways of microorganisms to uncover the interrelationship, which will contribute to finding more efficient bioremediation strategies and developing the next-generation bioremediation technology.

MATERIALS AND METHODS

Sample Collection

Industrial waste water sample were collected from a Stainless steel industry in and around Coimbatore in sterile glass bottles and were brought to the lab aseptically in the ice cold box. The samples were stored at 4° C for further use. Heavy metals (Chromium, Copper, Lead and Cadmium) in the sediment were determined. 1g of sediment sample with concentrated HNO3 made 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[15].

Physic-chemical Analysis

These collected water samples were subjected to various analytical analysis of Physicochemical parameters such as temperature, pH, turbidity, colour, odour, electrical conductivity, TDS, TSS, BOD, COD and DO of the water sample were determined APHA [2].

Isolation of metal resistant bacteria

In the present study heavy metal resistant bacterial species were isolated from the industrial effluent by serial dilution and pour plating method using Nutrient agar supplemented with different heavy metal salts. Strains were maintained in agar slants containing nutrient agar. They were characterized morphologically and on the basis of biochemical reactions. They were transferred weekly to new medium in order to keep metabolic activity and checked for purity by microscopic examination.

Screening of metal resistant bacteria

Heavy metals tolerant bacteria were isolated on nutrient agar supplemented with 5µg/ml to 100µg/ml of Cr, Cd, Pb and Cu. The nutrient agar was sterilized at 121°C for 15min and allowed to cool 40- 45°C. Then the metals were added to the nutrient agar and transfer into petri plates. The waste water sample was serially diluted in which 9ml of sterile saline water in 6 tubes and 1ml of samples were added to the first tube to have 10^{-1}repeated up to 10^{-6}. The 0.1ml of the dilution were spread on the surface of the agar plates and incubated at 37 ℃ for 2days colonies differing in morphological appearance were selected for further studies and sub-cultured on same media[12].

Heavy metal analysis in industrial effluent

The 2 ml of treated dye effluent was taken in a boiling tube and was digested using 10 ml of triple acid solution (HNO3, H2SO4 and HCIO4 in 9:2:1 proportion respectively) till the effluentbecomes colorless.The digested sample was filtered using whatman number 1filter paper for two times made up to 50 ml and subjected for heavy metal assay using Atomic Absorption Spectroscopy (Mac: SL 176 Double beam Spectrophotometer) as per the standard method recommended by APHA[2]. The three replications were maintained for each treatment. The

percentage of degradation was calculated from the following equation,

% Degradation=initial amount final amount/Initial Amount×100

Statistical Analysis Heavy metal tolerance

Four heavymetals which were at higher concentrations in the collected effluent, they were viz chromium, cadmium, lead and copper whereas zinc and manganese were considerable low in their presence.So, the heavy metal tolerance of the bacterial consortium was studied with these four heavy metals which were used at the range between 5µg/ml to 100µg/ml with an interval of 25mg/l.Using each of four heavy metals, the tolerance was examined. The used heavy metal substitutes were chromium metal powder, nickel sulfate, lead (II) phosphate and copper sulfate. Heavy metal substitutes were prepared at the concentration using the formula(X);

X = Molecular weight of compound/ Molecular weight of heavy metal × amount of sample required µg/ml.

Heavy metal bioremediation using microbial consortium

The heavy metal contaminated industrial effluent was treated with the bacterial consortium developed in this study with the standardized growth conditions except the heavy metal tolerance parameter. The bacterial consortium was identified for its peak time of heavy metal bioremediation with reference to its cell growth. The enhanced bioremediation was monitored using a portion of cultured broth followed by the separation of cell free supernatant and cell pellet using centrifugation at 3000rpm for 15 min.The fermentation process was monitored for 102hrs with an interval of 6hrs starting from the lag phase to decline phase under batch culture conditions.Individually estimation was monitored for the bacterial growth using the dry weight ofcellbiomass(g/L)as described earlier and heavy metals in the cell free supernatant and cell pellet was determined using AAS as described below.

Estimation of heavy metals using AAS in the treated broth

Heavy metals in the cell free supernatant weredeterminedwitha100mladdedwith3mlHNO3 followed by complete dryness on a hot plate whereas the cell pellet was lyophilized or freeze dried. Further, the same applied conditions in the AAS for the estimation of heavy metals in the effluents were applied here for the determination of four heavy metals viz. Chromium (357.9nm), Lead(283.3nm), Cadmium (228.8nm) and Copper (324.7nm).

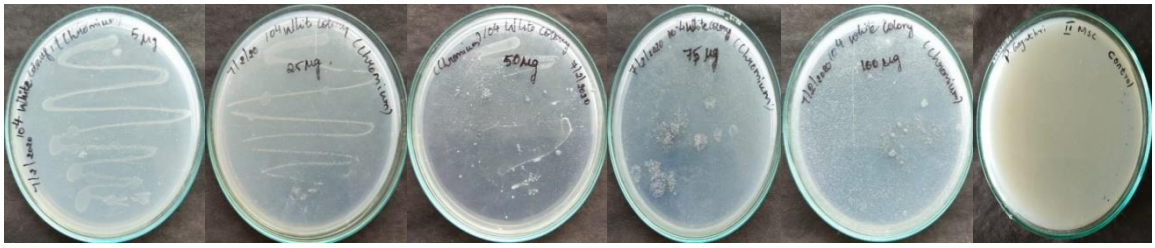
RESULTS AND DISCUSSION

Morphology of the Isolates

The present study deals with isolation, identification and characterization of heavy metal resistant bacteria were isolated from Stainless steel industrial effluent collected in and around Coimbatore. The 24hrs cultures were gram stained and observed under microscope for gram reaction. Violet coloured rod shaped cells were indicated as gram positive rods. Heavy metals are natural constituents of the environment, but indiscriminate use for human purposes has altered their geochemical cycles and biochemical balance. This result in excess release of heavy metals such as cadmium, copper, lead, chromium, zinc etc., into natural resources like the soil and aquatic environments. Prolonged exposure and higher accumulation of such heavy metals can have deleterious health effects on human life and aquatic biota.

Screening and selection of metal tolerant bacteria

Purified isolates were screened for metal tolerance by growing on LB medium amended with varying concentration of different metals (5µg/ml to 100µg/ml in the interval of 25µg/ml) at 28+ 1°C.These plates were incubated for 24hrs. Hence our study is to isolate chromium resistant microbial consortia and to treat the effluent.Growth observed (5µg/ml to 100µg/ml) concentration of heavy metals Cr(III) with different strains:



5µg/ml 25µg/ml 50µg/ml 75µg/ml 100µg/ml Control

Fig No: 1 Screening and selection of Cr (III) tolerant bacteria:

Measurement of physico-chemical parameters

The physico-chemical parameters of the industrial effluent were given in a Table 1. All the samples collected from the area indicated high level of pollution. The physico-chemical of sample collected from industrial effluent show that the milky white in color having pH range in 7.18 may be due to the presence of sodium, potassium and chromium etc. The temperature of the sample was 20-30°C.The sample were found turbid and the turbidity are 86 NTU.

Four heavy metals (Cu, Cd, Cr and Pb) in the industrial effluent were determined using Atomic Adsorption Spectrophotometry (Perkin Elmer) after digestion with microwaves digester following the manual instructions with the addition of nitric acid, hydrofluoric acid and hydrogen peroxide for sample digestion[5,6]. The concentration of the heavy metals in the effluent is Cu-5.8mg/l, Cd-1.09mg/l, Cr-5.97mg/l, Pb-1.9mg/l. The copper concentrations in the effluent were decreased after the inoculations of the isolated bacterial strains are 2.6mg/l, 1.6mg/l, 2.0mg/l, 1.7mg/l. The Cadmium concentration in the effluent there was a significant decrease of cadmium content after the inoculation of bacterial strains are 0.76mg/l, 0.6mg/l, 0.17mg/l, 1.1mg/l. The Chromium concentrations in the effluent were decreased after the inoculation of the bacterial strains from 5.2mg/l, 2.9mg/l, 3.7mg/l, 2.5mg/l. The lead concentration in the effluent after inoculation of heavy metal strains from 0.38mg/l, 0.6mg/l, 0.41mg/l, 0.8mg/l.

Table No:1 Measurement of Physico-chemical parameters

S.NO	PHYSICO-CHEMICAL PARAMETERS	UNIT	RESULTS
1	Colour	Hazen	250
2	Odour	Mg/l	Disagreeable
3	Turbidity	NTU	86
4	pH@25°C	-	7.18
5	Electrical conductivity@25°C	µS/cm	1540
6	Total Dissolved Solids@180°C	Mg/l	1020
7	Biochemical Oxygen Demand(3 Days for 27°C)	Mg/l	3650
8	Chemical Oxygen demand	Mg/l	80,000
9	Total Suspended Solids	Mg/l	596
10	Temperature	°C	30
11	Copper as (Cu)	Mg/l	5.8
12	Cadmium (Cd)	Mg/l	1.09
13	Chromium (Cr)	Mg/l	5.97
14	Lead (Pb)	Mg/l	1.2

Estimation of heavy metals using AAS (atomic absorption spectroscopy) in the treated broth

Atomic Absorption Spectroscopy used for the estimation of the heavy metals in the effluents where inoculated with metal resistant strains and incubated at 37°C for 7 days were applied here for the determination of four heavy metals Cr, Cd, Cu, and Pb.

Fig no: 2 Samples for AAS Analysis

Table No: 2Estimation of heavy metals using AAS

S.No	Heavy Metals	Unit	Before treatment	After treatment with isolates			
				Isolate**1**	Isolate**2**	Isolate**3**	Isolate**4**
1	Copper	Mg/l	5.8	2.6	1.6	5.0	1.7
2	Cadmium	Mg/l	1.09	1.1	0.17	0.4	0.76
3	Chromium	Mg/l	5.97	5.2	2.9	3.7	2.5
4	Lead	Mg/l	1.2	0.8	0.41	0.6	0.38

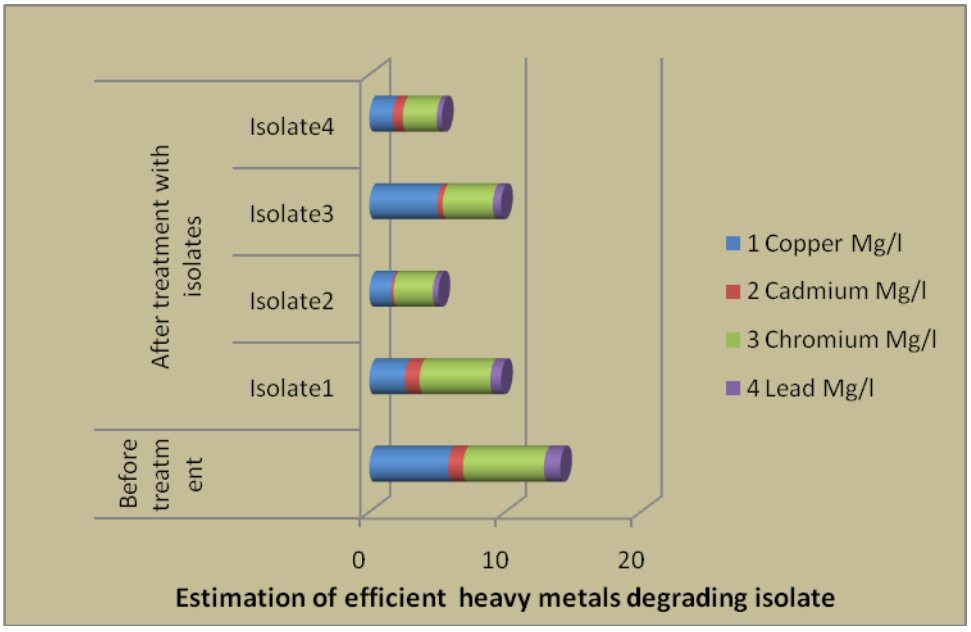


Fig.No:3 Statistical Analysis shows the Efficient Heavy metal degrading Isolate

Based on the statistical analysis of AAS Isolate 2 shows the efficient degradation of heavy metals compare to other 3 isolate. The efficient strains were identified by 16rRNA sequencing.

Identification efficient heavy metal degraders by sequencing

The efficient strain is identified as *Bacillus subtilis* (PGA-10_contig_1) by 16srRNA sequencing at Macrogen. Primer used for PCR 27F 5' AGA GTT TGA TCM TGG CTC AG 3'& 1492R 5' TAC GGY TAC CTT GTT ACG ACT T 3' and for sequencing universal primer 785F5'GGA TTA GAT ACC CTG GTA 3' & 907R 5' CCG TCA ATT CMT TTR AGT TT 3' .The 1344bp were used for sequencing and summation for NCBI. Identification of metal resistant bacteria that are adapted to the new toxic metal environment provides efficient potential candidates for heavy metal bio removal from contaminated media[30,7]. Most efficient heavy metal degraders of Isolate No.2 were selected for 16S rRNA gene sequencing in bacterial identification. Segments from these genes can be amplified by PCR using universal primers and sequenced. Sequence comparison of 16S rRNA has been used as a powerful tool for establishing phylogenetic and evolutionary relationships among organisms. The 16SrRNA gene sequencing and phylogeny analysis revealed that, the isolate were authentically identified as *Bacillus subtilis.*

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP020102.1	Bacillus subtilis	4215607	96453	97942	0	2732	0.0	1487/1490	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus subtilis

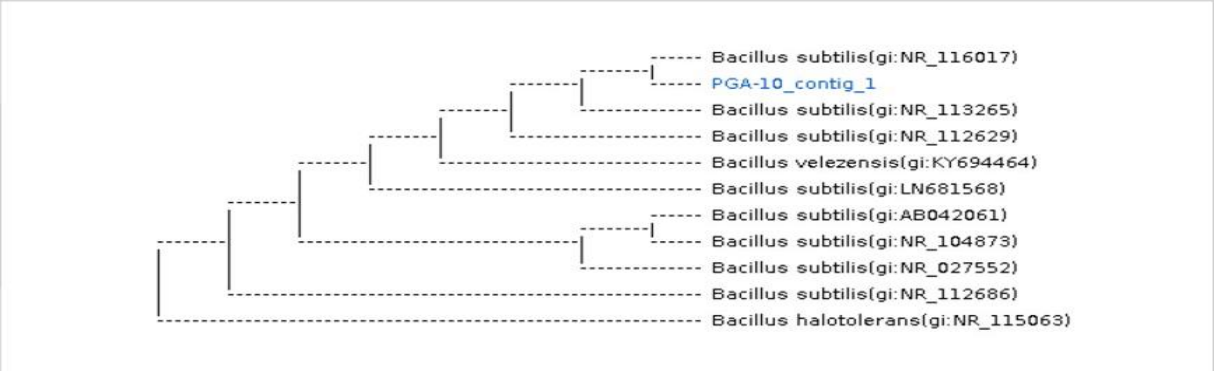


Fig.No:4. Phylogenetic and Evolutionary relationships

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CACGTCGTCTCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGG

GCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCA

GGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTT

ACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGC

ATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTC

CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGA

CTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGC

TTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTT

TCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCT

TCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGC

GTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCT

CCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTC

TCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTA

GCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTA

TGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCC

CGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGT

CCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCAT

GTATTAGGCACGCCGCCAGCGTTCGTCTGACAGAGAGAAAAACCAAAACA

AAAAAAGGAAAAAACTGGGGGGGGGGGGGGGGGGGGGAGGGGGGGGGG

GGGGGGGGGGGGGCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG

GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG

GGGGGGTGTGGTGCGGGGGGGGGGGGGGGGGGGGGGGGGGGGCGG

GGGGGGGGCGCCGCCCCCCGGGGGCCCGGGGCCCCGCGGGGGGGGGCGCC

GGGGCGGCGGGGGGGGGCCCCCCGGCCGGGGGGGGGGGGGGGGGGCCC

CCGCGCGCCCCCCCCCCCCCCCCGGGGGGGGGCGCGGCCCCCCCGGGGGG

CGGGGGGGGGGGGGGGGGGCGCGGGGGGCCGCGGGGGCGCGGCCGCCCC

CCCCCGCGGGCGGCGGCCGCGCGGCCCCGCCCGGCGGGGGGGGC

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None.

CONFLICT OF INTEREST

Author declares no conflicts of interest in this paper.

CONCLUSION

Results of the present study, the capability of bacteria resistance against different heavy metals may offer a beneficial tool for simultaneous monitoring of many contaminants and pollutants in the environment.Bacteria play a very important role in the removal of heavy metals from the industrial effluent. Therefore, the study is very useful to suggest that the possible impact of metal contaminated locations in human life may be greater the direct consequence of the pollution. The characterizations of metal resistant bacteria were done and the four bacterial strains isolated. Based on the result obtained *Bacillus subtilis* were able to tolerate up to 5µg/ml to 100µg/ml of chromium in the interval of 25µg/ml. Growth standardization of consortium in the medium using the highest heavy tolerance were done and reduced after treatment with bacterial consortium it was proved by quantitative analysis of heavy metals cell free supernatant of treated effluent.

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