BIOREMEDIATION OF HEAVY METALS USING BIOSURFACTANT PRODUCING MICROORGANISMS

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Abstract

The present study was carried out to evaluate degradation of heavy metals in effluent waste water samples using microorganisms. The physical and chemical properties of the effluent samples were analyzed using standard methods. The soil sample collected from the heavy metal contaminated sites was subjected to serial dilution and streak-plating methods and six different strains were isolated from the samples. The activity of the isolates for hemolysis was studied on the Blood-Agar plates. The isolated strains were studied for its biochemical and morphological characteristics. The dark-blue colonies were observed by CTAB method, which confirmed the anionic bio surfactant produced by the isolate. The isolates were subjected to other screening tests like emulsification activity and oil displacement technique. These strains were used in the degradation of heavy metals present in the effluent waste water samples. The organism KDM 4 showed better degradation with 93.18% ability in reducing zinc when incubated for 72 hours and 86.36% when incubated for 24 hours in sample 3. The lead reduction was found to be 84.13% by the organism KDM3 when incubated at 37°C for 72 hours incubation. The chromium was reduced by the organism KDM 6 with 87.9% 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. The least amount of degradation was found in the organism KDM5 with only 27.08%. The percentage of reduction of heavy metals varies from one sample to another sample.

Keywords: Bio surfactants, Metal analysis, Lead, Zinc, Biodegradation

INTRODUCTION

Pollution due to chemicals including heavy metals is a problem that may have negative consequences on the biosphere. The levels of metals in all environments, including air, water and soil are increasing in some cases to toxic levels, with contributions from wide variety of industrial and domestic sources. Metal contaminated environments pose serious threat to health and ecosystems. The most abundant pollutants in waste water and in sewage are heavy metals (Hong *et al.*, 1996).The environmental pollution by heavy metals 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 (Meghraj *et al.*,2013) Some of the negative impacts of heavy metals on plants include decrease of seed germination and lipid content by cadmium, decreased enzyme activity and plant growth by chromium, the inhibition of photosynthesis by copper and mercury, the reduction of seed germination by nickel and the reduction of chlorophyll production and plant growth by lead (Gardea-Torresdey *et al*.,2005). The impacts on animals include reduced growth and development, cancer, organ damage, nervous system damage and in extreme cases, death. Heavy metals contaminate the drinking water reservoirs, fresh water habitats and can alter microbial communities.

Chemical precipitation of heavy metals in water has been practiced as a prime method of treatment in industrial waters for many years. A combination of precipitation with other chemical treatment techniques, such as ion exchange has been reported to be effective in heavy metal removal in polluted waters (Akpor *et al*., 2010).Adsorption is a widely used method for the treatment of industrial wastewater containing color, heavy metals and other inorganic and organic impurities This method suffers from low adsorption capacity and in some cases complete removal is not possible and high cost of the adsorbent (Patel *et al*., 2010). Chemical oxidation is a process in which the waste materials from the industrial waste water 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 (Dias-Machado *et al*., 2006; Ksibi 2006). Phytoremediation is a remediation process that entails the use of plants to partially or substantially remediate selected substances in contaminated soil, sludge, sediment, groundwater, surface water and wastewater. It is also referred to as green remediation, botano- remediation, agro-remediation or vegetative remediation (Pivetz 2001).

All these methods have some limitations and the common problems that are associated with these methods are expensive and can themselves produce other waste disposal problems, which have limited their industrial applications(DebayanDas 2012).Among the available treatment processes, the application of the biological

processes is gradually getting momentum due to the following reasons: Chemical's requirement for the whole treatment process is reduced, Low operating cost, Eco-friendly and cost effective alternative of conventional techniques, efficient at lower levels of contamination.

Bioremediation is the use of microorganisms or their enzymes to break down and thereby detoxify dangerous chemicals in the environment (Obayori *et al*., 2009). It plays a major role in making the environment clean from pollutants and contamination (Arun *et al.,* 2014). Microbial activity is thought to play a key role in the detoxification of metals in water. In heavy metal remediation biosurfactant have a role for removal process of the metals, including wetting, contact of biosurfactant to the surface of the sediments and detachment of the metals from the sediment.

MATERIALS & METHODS

ANALYSIS OF CONSTITUENTS IN THE EFFLUENT WASTE WATER

Sample Collection: The sample was collected from places in and around Bangalore. Welding shop located in Hennur, bronze mending shop in Gedalahalli, galvanizing shop in Horamavu, car paint workshop in Kacharkanhalli and the last sample from the rail and wheel axle industry Yelahanka. The samples were labeled as sample 1, sample 2, sample 3, sample 4 and sample 5 respectively. Two liters of the effluent waste water were collected in polyethylene bottles previously washed with 8M HNO_3 and distilled water. The total volume of the bottle was filled completely and a cap was locked enough, so that no air space can be remained inside the bottles.

Effluent Analysis: These effluent waste water samples were subjected to various analyses like COD, BOD, TDS, metal analysis. The physical-chemical parameters including Microbial count and calcium content were estimated using standard methods (AmeerBasha & Rajaganesh, 2014).

Metal analysis: Complexometric titrations are commonly known as EDTA titrations since EDTA acts as a most versatile complexation agent of all the commercially available complexing substances. A wide range of metal ions including Zn,Pb,Cr removed as complexes with least difficulty using modified method (PranveraLazo, 2009).

ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA

Soil samples were collected from different sites in the locality of Bangalore which is contaminated with metals. 1 g of each soil samples were inoculated in 50ml of Mineral Salt Medium (MSM).All the flasks were incubated for 72 hours at 37°C in the shaker incubator. 1ml of the culture is serially diluted up to 10^{-6} dilution. 0.1 ml of which was transferred to sterile petriplates containing nutrient agar and spread plate was performed. The plates were inverted and incubated at 37°C, for 48 hours. After incubation morphologically distinct colonies were selected for further studies (Sneha 2012).

Isolation of pure bacterial cultures: Isolated cultures were grown on the NA plates and were sub-cultured on fresh MHA plates from the master plates to get the pure cultures.

SCREENING FOR BIOSURFACTANT PRODUCING BACTERIA

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following methods.

Oil spreading assay: In oil spreading assay10 µl 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 µl of culture or culture supernatant were gently placed on the Centre of the oil layer. The presence of biosurfactant 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 (Morikawa *et al*., 1993).

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 biosurfactant producing organisms. Results were recorded based on the type of clear zone observed (Sarvanan 2012; Anandaraj & Thivakaran 2010).

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 (Saravanan & Vijayakumar 2012; Satpute *et al*., 2008).

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 (Bodour *et al*., 2004). It is expressed as percentage.

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 (Suganya 2013). 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.

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 (Chandran & Das 2011; Heyd *et al*., 2008; Thavasi *et al*., 2010). 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}) (Sarvanan 2012).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 (Thavasi *et al*., 2010).

Characterization of Bio surfactant Producing Organism: The screened bio surfactant producing organism was then characterized by using different morphological and biochemical tests, includes Gram staining, Motility Test, Indole Test, Methyl Red Test, Voges-Proskauer Test, Citrate Test, Spore Staining, Starch Hydrolysis, Casein Hydrolysis, Gelatin Hydrolysis, Lipid Hydrolysis, Gelatin liquefaction Test, Oxidase Test, Catalase Test.

EFFECT OF MICROORGANISMS ON THE EFFLUENT WASTE WATER SAMPLE

Removal of Heavy Metals in Effluent samples : Degradation of heavy metals experiments were carried out in 250 ml of separate flasks containing 100 ml of effluent waste water collected from study sites S1, S2, S3, S4 and S5. The pH was adjusted to 7 +/- 2 using NaOH and H_2SO_4. Then, the flasks were autoclaved at 121^{o}C 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 37^{o}C for 24 hours and 72 hrs. The effluent waste water without bio surfactant producing organism served as control (Sankar Narayan Sinha *et al*., 2012).

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 (Arun *et al*., 2014).

RESULTS & DISCUSSION

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

TABLE 1: Physical & chemical components of effluent waste water

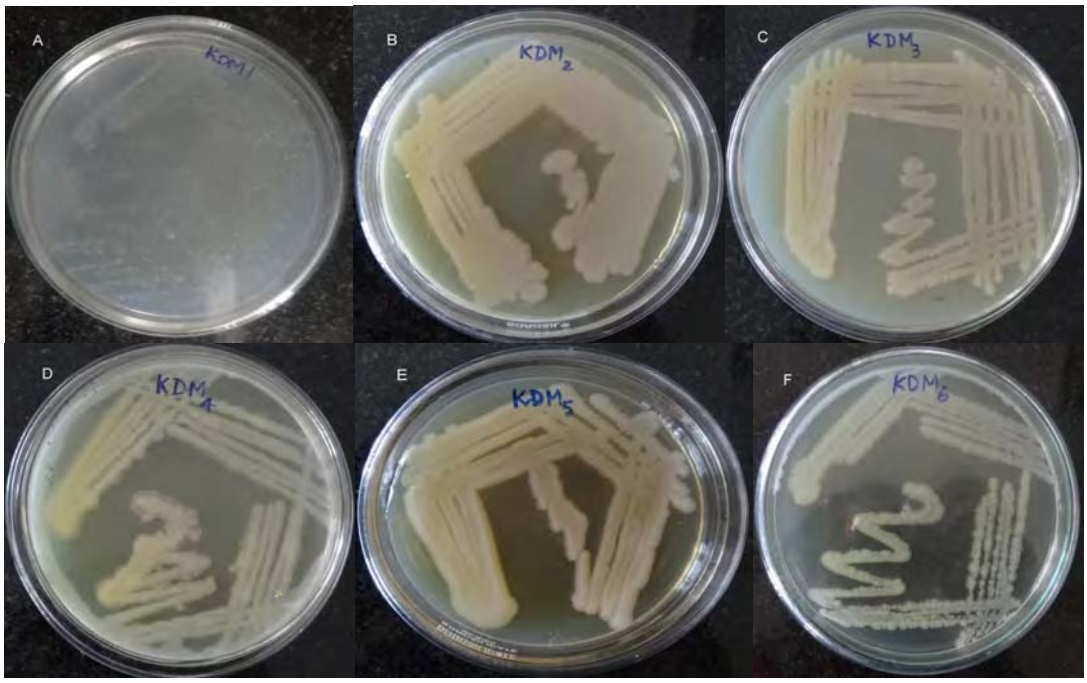
PARAMETERS	CONTROL	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE4	SAMPLE 5
Color	Colorless	Colorless	Colorless	Colorless	Slightly turbid	Colorless
pH	7	7.3	8.5	6.5	8.2	6.8
BOD(mg/l)	0.64	2.72	1.6	2.24	0.96	0.85
COD(mg/l)	12	17.6	40	32	54	20
TDS(mg/l)	238	2168	3658	3216	4312	1962
Ca(mg/l)	10	30	20	40	20	10
Zn(mg/l)	0.6	7.6	9.8	8.8	9.5	6.3
Pb(mg/l)	-	4.75	5.17	3.72	6.003	4.14
Cr(mg/l)	-	7.2	10.5	4.85	17.5	4.3

Control – Tap water, Sample 1- Welding shop, Sample 2- Bronze mending shop, Sample 3- Galvanizing work shop, Sample 4- Car paint workshop, Sample 5- Effluent from rail and wheel axle plant.

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 waste 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 5 effluent waste 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 4 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 the effluent waste water samples. The concentrations of the heavy metals in all the samples were found to be more than the concentration present in the control.

ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA: Biosurfactants producing bacterial isolates were streaked on Muller Hinton Agar plates&the strains were named as KDM1, KDM2, KDM3, KDM4, KDM5 and KDM6 respectively.The isolated colonies were sub-cultured on Muller Hinton Agar plates to obtain pure cultures (Plate -I).



Screening: Oil spreading technique: The supernatant of the six strains were added to the plates containing oil. The strain KDM1, KDM2, KDM3, KDM4, KDM5 and KDM6 displaced the oil showing a zone of displacement with SDS as the control. The results were shown in the (Table- 2).

TABLE 2: Oil displacement activity of isolated Biosurfactants

Bacterial strain	Zone of displacement (mm)	Inference
Control (SDS)	13	Positive
KDM 1	08	Positive
KDM2	05	Positive
KDM3	14	Positive
KDM4	07	Positive
KDM5	06	Positive
KDM6	10	Positive

The strain KDM 3 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 biosurfactant followed by the strain KDM6.Least zone of displacement was found by the strain KDM2.

Blood hemolysis test: Blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media (Schulz *et al*., 1991). In the current studies all the strains were streaked on blood agar plates. All the six strains showed positive results for haemolytic activity. Formation of a clear zone around the colonies (Table -3).

TABLE 3: The strains showing results for blood hemolysis test

ORGANISM	OBSERVATION	RESULT
KDM1	β hemolysis	Positive
KDM2	β hemolysis	Positive
KDM3	β hemolysis	Positive
KDM4	β hemolysis	Positive
KDM5	β hemolysis	Positive
KDM6	β hemolysis	Positive

The positive result of blood hemolysis is due to the lysis of the RBC's present in the medium. 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.

Emulsification index: 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).

TABLE 4: Comparison of emulsification activities of all the strains

ORGANISM	E_{24} (%)
KDM1	73.4
KDM2	62.3
KDM3	92.4
KDM4	87.5
KDM5	66.8
KDM6	83.3

E24 = Emulsification index

It showed that the strain KDM3 showed the maximum emulsification activity with olive oil after 24 hours of incubation followed by the strain KDM4 and the least activity was shown by the organism KDM2. 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.

Methylene blue agar plate: Dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of anionic bio surfactant. KDM3 showed darker zone of color and hence the organism is an anionic bio surfactant and also produces rhamnolipid. All the six isolates showed positive result for blood hemolysis, oil displacement and emulsification index whereas KDM3 alone showed positive result for Bap test which produces anionic bio surfactant indicated by the halo zone around the colony (Table 5).

TABLE 5: Screening results of Bap test

Organisms	Methylene blue agar (Bap)
KDM1	-
KDM2	-
KDM3	+
KDM4	-
KDM5	-
KDM6	-

Symbols: + positive, - negative

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 the two immiscible liquids chloroform: methanol. This white precipitate, which was the bio surfactant, was observed in all the six strains

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 -6).

TABLE 6: Biochemical properties of bio surfactants produced by isolated strains

Biochemical properties	**KDM1**	**KDM2**	**KDM3**	**KDM4**	**KDM5**	**KDM6**
Carbohydrate	++	-	+	+	-	-
Lipid	+	+	++	+	+	++
Protein	+	+	+	-	++	+

Symbols: ++ highly positive, + slightly positive, - negative

KDM1 contains all the 3 components but the carbohydrates in higher amounts than the other two. The strain KDM 3 contains lipid in greater amounts along with carbohydrates and lipids in small amounts.

Characterization of isolated bacteria: The results obtained for the biochemical characterization of bacterial strains isolated from heavy-metal contaminated site is given in the (Table -7).

TABLE 7: Biochemical characterization of the isolated bio surfactants

PARAMETERS	**KDM 1**	**KDM2**	**KDM 3**	**KDM 4**	**KDM5**	**KDM 6**
Gram character	+ve	+ve	-ve	-ve	+ve	+ve
Shape	Bacilli	Small rods	Cocco bacilli	Bacilli	cocci	Cocci
Capsule	+	+	+	+	+	+
Motility	++	+	-	+	-	+
Indole	-	-	-	-	-	-
MR	-	+	+	+	-	-
VP	-	+	-	-	+	+
Citrate	-	+	+	+	+	+
Starch	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-
TSI	+	-	++	-	+	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+

Symbols: ++ highly positive, + positive, - negative

Reduction of heavy metals in effluent sample: The sample when treated with isolated organisms, after treatment the sample showed considerable reduction in the heavy metals were shown in (Figure No.1.a,2b,3c,4d,5e)

Heavy metal reduction in effluent sample

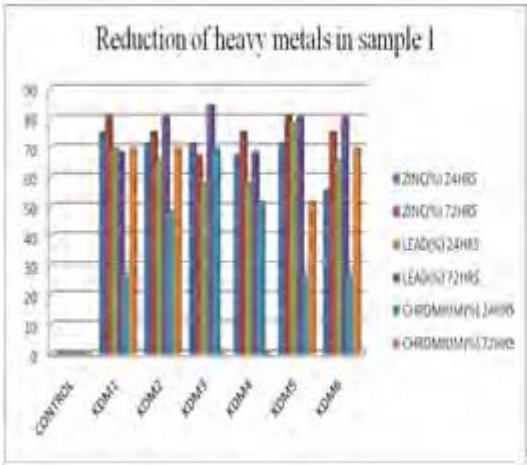


Figure 1 a

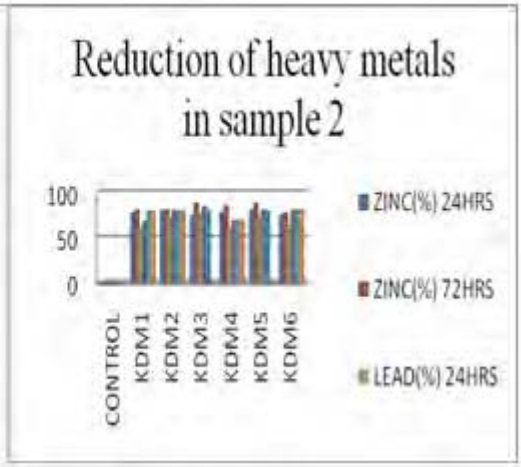


Figure 2 b

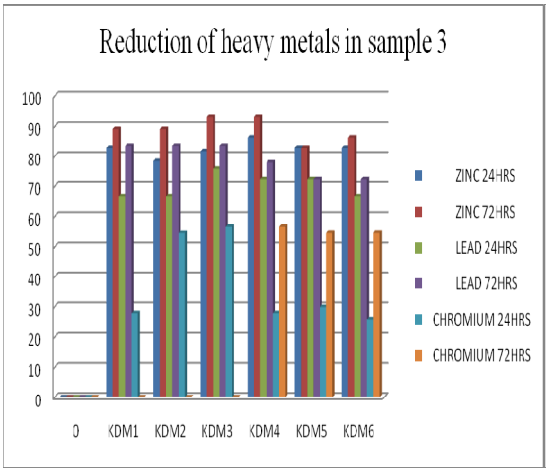


Figure 3 c

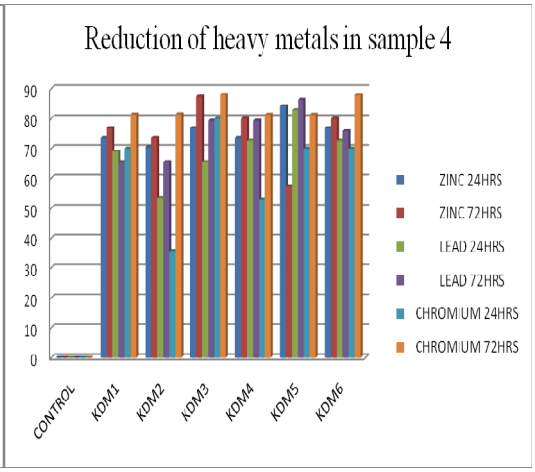


Figure 4 d

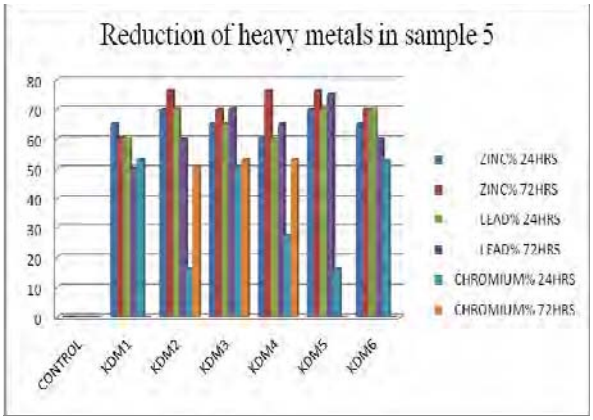


Figure 5 e

Effluent sample was treated with the isolated organisms almost all the organisms showed reduction in heavy metals. The organism KDM1 and KDM5 showed highest percentage of degradation of zinc when incubated for 72 hours with 80.26% ability, whereas the KDM3 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 KDM1, KDM2 and KDM6 at 72 hours. All the parameters like temperature, pH, time duration and amount of the inoculum plays an important role in determining the efficiency.

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^{o}C by KDM 4 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 textile dye effluent samples at 37^{o}C for 5 days which was higher than the ability of the KDM3 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. KDM6 organism showed maximum amount of degradation of Cr in sample4 with 87.9% efficiency after 72hrs at 37^{o}C incubation followed by KDM 1 and KDM 2 with the efficiency of 79.0% in sample 2. The highest amount of degradation found by KDM 6 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 KDM 5.

CONCLUSION

Heavy metal contaminated sites were screened for Microbial strains and the activity of the isolates for haemolysis was studied on the Blood-Agar plates. The isolated strains were studied for its biochemical and morphological characteristics and also confirmed the anionic biosurfactant produced by the isolate. The isolates were subjected to other screening tests like emulsification activity and oil displacement technique. These strains were used in the degradation of heavy metals present in the waste water samples. The percentage of reduction of heavy metals varies from one sample to another sample. The organism KDM 4 showed better degradation with 93.18% ability in reducing zinc when incubated for 72 hours and 86.36% when incubated for 24 hours in sample 3. The lead reduction was found to be 84.13% by the organism KDM3 when incubated at 37°C for 72 hours incubation. The chromium was reduced by the organism KDM 6 with 87.9% 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. Further identification of organism at genus and species level will help in heavy metal reduction in highly polluted effluent treatments.

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