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Optimization of Cu, Hg and Cd removal by *Enterobacter cloacae* by ferric ammonium citrate precipitation

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Abstract. Iron precipitating organisms play a significant role in the formation of ferric hydroxide precipitate, which acts as strong adsorbent for toxic metal. In this respect four different iron precipitating cultures were isolated from Hutti gold mine surface winze water sample on citrate agar medium. The best isolate was screened out for metal removal study on the basis of fast visual iron precipitation. The selected isolate was identified as *Enterobacter* sp. based on routine biochemical tests and Biolog GN microplate results and as *Enterobacter cloacae* subsp. *dissolvens* by 16S rRNA gene sequence analysis (GenBank accession number EU429448). Influence of medium composition, medium initial pH, the influence of inoculum size, effect of various media and ferric ammonium citrate concentration were studied on metal removal in shake flask experiments. Under the optimized conditions studied, *E. cloacae* showed 94 ± 2, 95 ± 2 and 70 ± 2% of cadmium, copper and mercury removal from a simulated waste in shake flask studies. In lab scale column reactor more than 85% of copper and mercury removal was achieved.

Keywords: biosorption; iron precipitation; Enterobacter cloacae; copper; mercury; cadmium

1. Introduction

Today indiscriminate and uncontrolled discharge of metal contaminated industrial effluents into the environment has become an issue of major concern (Hakeem and Bhatnagar 2010). Rapid industrialization and urbanization have resulted in elevated levels of toxic heavy metals entering the biosphere (Safavi *et al*. 2011). Heavy metals like copper, cadmium, mercury, chromium, iron, cobalt, nickel, and lead are detrimental to human health and ecosystem stability (Rajasimman and Murugaiyan 2010). The permissible limit for copper, cadmium and mercury in drinking water as per World Health Organization guidelines is 1.0, 0.005 and 0.001 mg/L respectively (Kumar and Puri 2012). Copper, finds its way to the water stream from electroplating, mining, electrical, electronics, printing, photography and metal finishing industries (Shetty and Rajkumar 2009). Ingesting of heavy metals can cause headaches, dizziness, nausea, vomiting, diarrhoea, hypertension, emphysema, damage to vital organs and can even cause death (Tilaki and Ali 2003, Mortazavi *et al*. 2005, Rajeshkumar and Kartic 2011, Sinha and Khare 2012).

Methods, such as chemical precipitation, oxidation or reduction, electrochemical treatment,

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evaporative recovery, filtration, ion exchange and membrane technologies are widely used to remove heavy metal ions from industrial wastewater (Hakeem and Bhatnagar 2010, Olguin and Sanchez-Galvan 2012). However, application of these treatment processes has been found to be sometimes restricted, because of investment, operational costs and the potential generation of secondary pollution. These processes may be ineffective or expensive, especially when the heavy metal ion concentrations in solutions are 1-100 mg/L (Samarth *et al*. 2012). Biosorption has been defined as the property of certain biomolecules or types of biomass to bind and concentrates selected ions or other molecules from aqueous solution (Volesky 2007). Biosorption involves physical and chemical mechanisms such as: physical adsorption, ion exchange, complexation, precipitation, co-precipitation and transport across a cell membrane (Jencarova and Luptakova 2012). Biosorption is an ideal process for the treatment of high volume, low concentration complex waste and was successfully applied in the removal of several heavy metals (Volesky 1986, Dave 2008). Dead biomass are usually utilised as adsorbents for different metal ions, while living biomass is seldom employed because of metal toxicity (Solisio *et al*. 2000).

Iron precipitation and accumulation are not limited to iron bacteria. There are certain rod bacteria, such as *Pseudomonas* that can also precipitate iron (Staley *et al*. 1989, Eaton *et al*. 1995). Using synthetic media containing ferric ammonium citrate, they found that the isolates of *Aerobacter aerogens*, *Serratia indica* and *Bacillus pumulis* could also precipitate iron mainly through the utilization of citrate as an energy source (Cullimore and McCann 1978). The adsorption behaviour of amorphous hydroxide type adsorbents, i.e., ferric hydroxide, ferric cupric hydroxide and ferric lead hydroxide, were reported for removing a wide range of metals and metalloid like arsenic, selenium, cobalt, nickel, cadmium and zinc from aqueous solution (Chakravorty and van Grieken 1986, Fujita *et al*. 2006). Freshly precipitated ferric hydroxide is amorphous and it has the ability to sorb heavy metals and many organics. The use of iron oxidizing bacteria to remove pollutant ions from water may present an alternative removal method. Biogenic iron oxides are produced by auto-lithotrophic microbes that oxidize soluble Fe^{2+} to Fe^{3+} creating a hydrated iron hydroxide precipitate in an organic matrix (Leake 2009) as well as copper, cadmium, lead and zinc were co-precipitated with ferric hydroxide and the binding of the metal in the ferric hydroxide depended on the type of metal present (Lee and Lee 2005).

In this context, the present study was conducted to isolate iron precipitating bacteria and investigate its bioprecipitation for its role in bioremoval of copper, mercury and cadmium from aqueous solution. Moreover, apart from bioremoval optimization studies at a shake flask scale also to transfer the process to a lab-scale indigenously designed column bioreactor.

2. Materials and methods

2.1 Isolation, identification and inoculum preparation of iron precipitating organisms

A water sample was collected from Surface winze water from Hutti Gold mine, Raichur, Karnataka, India. The sample was transported in possible minimum time (48 h) and was preserved at 4 ± 2°C until further processing. The water sample was serially diluted up to 10^{-5} in sterile distilled water and 0.1 mL of each dilution was used for isolation of heterotrophic iron precipitating organisms using spread plate techniques on citrate agar (HiMedia, India) plate. The isolate, which showed the highest iron precipitation in citrate broth (g/L): ferric ammonium citrate (FAC) 10.0; ammonium sulphate 0.5; sodium nitrate 0.5; magnesium sulphate 0.5; dipotassium

phosphate 0.5; calcium chloride 0.2 and pH 6.6 ± 0.2, HiMedia, India) was selected for further study. The selected isolate was identified based on routine biochemical test, Biolog® identification system (Biolog 2001) and also by 16S rRNA gene sequence analysis. For optimization study in the modified citrate broth medium was modified by reducing the amount of ferric ammonium citrate (FAC) from 10.0 g/L to 1.0 g/L and pH adjusted to 5.0 with 0.1N HCl.

2.2 Batch study for optimization of bioremoval of different metals

To prepare a stock solution of metals 100 ppm (i.e., 100 mg/L) of copper, mercury and cadmium 39.2 mg, 13.5 mg and 22.8 mg of analytical grade $CuSO_4.5H_2O$, $HgCl_2$ and $CdSO_4.8H_2O$ were dissolved respectively in 100 mL of pH (5.5) distilled water. The working standards were prepared freshly in distilled water (pH 5.5) from the stock solution.

If otherwise mentioned, all the experiments were conducted in 250 mL Erlenmeyer flask with working volume of 50 mL of optimized citrate broth containing either copper (10 ppm), mercury (5 ppm) or cadmium (10 ppm). All the flasks were inoculated with actively growing 10% (v/v) culture, having ~4.1×10^8 cells/mL. All the flasks were incubated in environmental orbital shaker (Newtronics, India) rotating at 150 rpm at 32 ± 2°C. Samples were withdrawn at regular time interval and centrifuged at 9000 g for 10 min (Remi C24, India). The biomass was separated and desired amount of supernatant was taken for remaining copper and mercury analysis.

To check the pH optima initial pH of the medium was adjusted to 3, 5 and 7 using 0.1 N NaOH or HCl. In this experiment, the metal bioremoval was studied with 10 mg/L copper, 10 mg/L of cadmium and 5 mg/L mercury. The influence of the FAC concentration on copper, mercury and cadmium bioremoval was studied using 1.0, 0.1 and 0.01 g/L FAC in citrate broth. To access the effect of different media on bioremoval of mercury and cadmium, nutrient broth, nutrient broth containing FAC (0.1g/L) and citrate broth were studied. To optimize the inoculum size, 2×10^9, 4×10^9 and 6×10^9 cells were used as inoculum for mercury and cadmium bioremoval study.

2.3 Development of lab scale column reactor for copper and mercury removal

In glass column of 38 cm length, 2.74 cm of inner diameter and of 3.6 cm of outer diameter glass wool was inserted at the lower end of the column up to the 10 cm height, above these polystyrene beads having the average weight, length and width of 0.0246 g, 4.12 mm and 2.99 mm respectively were added. The approximate surface area and volume of bead was 134 mm^2 and 116 mm^3 respectively. The total volume of column reactor was 130 mL with working volume of 50 mL. The experiments were performed with aeration and without aeration. Aeration was provided to the column by the aerator. For the development of the biofilm actively growing culture of *Enterobacter cloacae* in citrate broth medium was passed through the column for nine days. The bacterial biofilm was formed on the support material simultaneously with the precipitation of iron. Bacteria deposited ferric hydroxide precipitate, giving a brown or rust red colour around the beads, leaving the water in the form of clear supernatant. After biofilm formation used up medium was drained gradually and the same volume of copper solution of 20 to 200 mg/L was added in the column both under the influence of aeration and without aeration and the remaining copper in the effluent after the treatment was estimated from each cycle. In case of mercury, the biofilm development was carried out in the same manner as mentioned for copper. Synthetic wastewater containing 5 mg/L of mercury was added in the column for ten cycles. Samples were collected from the column at regular interval of time and were analysed for mercury removal.

2.4 Analytical procedure

Copper and mercury were analysed from the supernatant by measuring optical density spectrophotometrically at 560 and 610 nm by using diethyldithiocarbamate complex and malachite green complex method respectively (Vogel 1961). Cadmium analysis was carried out by atomic absorption spectrophotometer (Elico India, model SL 191) by preparing appropriate dilution by using acidified distilled water of pH 4.5.

3. Results and discussion

3.1 Isolation and screening and identification of iron precipitating organisms

The bacteria which precipitate ferric hydroxide from the medium around cells showed brown or rusty red colour colonies. Based on the iron precipitation four different cultures were isolated from Hutti gold mine surface winze water sample on citrate agar medium. When studied in liquid medium they showed 30, 35, 37 and 93% iron precipitation from citrate broth in 24 h of incubation (data not shown). The isolate which showed 93% iron precipitation was selected and identified as *Enterobacter* sp. based on routine biochemical tests and Biolog GN microplate results. Further identity of the selected isolate was confirmed as *Enterobacter cloacae* subsp. *dissolvens* by 16S rRNA gene sequence analysis (GenBank accession number EU429448).

3.2 Optimization study for bioremoval of different metals

The pH condition is of prime importance in determining the mobility of metal (Tansupo *et al*. 2008). Thus, influence of pH on copper, mercury and cadmium removal by *E. cloacae* was studied and the results are listed in Table 1. At 24 h of incubation, 84.2% copper was removed from a medium containing 20 mg/L copper at pH 7.0. Moreover, 78.3% and 64.5% copper removal was observed at pH 5.0 and pH 3.0 respectively. The trend continues similar even for prolonged incubation of 96 h. However, the difference in the copper removal between pH 3.0 and pH 7.0 was narrowed down at 96 h as compared to the result obtained at 24 h of incubation. This is obviously due to less amount of copper remained as the incubation time increases. In case of mercury and cadmium also, as the pH was raised towards the alkaline side their bioremoval increased.

Table 1 Effect of pH on copper, mercury and cadmium bioremoval by *E. cloacae*

Incubation period (h)	% Metal removal								
	pH								
	3	5	7	3	5	7	3	5	7
	Cu			Hg			Cd		
24	64.5	79.2	84.2	10.3	30.1	42.1	18.3	21.5	19.9
48	77.1	83.7	89.4	33.9	38.46	57.23	38.7	68.3	67.1
72	81.7	89.1	92.1	41.5	53.8	61.84	60.2	96.1	95.3
96	88.5	94.5	96.8	44.5	56.4	64.8	62.3	97.2	96.2

This could be due to increased FAC precipitation at neutral pH as well as better growth of *E. cloacae* at pH 7.0 as compared to these activities at pH 3.0 and pH 5.0. Metal removal enhanced with increase in medium pH as medium pH affects the solubility of metal ions and ionization state of the functional groups (Reddy *et al*. 1995). At low pH values, cell surface being positively charged would not be favourable for the attachment of positively charged metal ion due to repulsion. It can also be explained as low amount of metal ion retained by the biosorbent at pH values below 4, because most functional group expected to dissociate only at neutral pH values. The increase in the biosorption level observed with increasing pH can be explained by a strong relation of biosorption to the number of surface negative charges, which depends on the dissociation of functional groups. The high adsorption believed to be associated with the formation of positively charged metal hydroxyl species, having a strong affinity for surface functional group (Okoronkwo *et al*. 2007). Cadmium bioremoval was all most similar both at pH 5.0 and 7.0 and reached to more than 95%. At low pH, metal ions had to compete with H^+ ions for adsorption sites on the adsorbent surface. As the pH increased this competition weakens and more metal ions were able to replace H^+ ions from the adsorbent surface (Ibrahim *et al*. 2006). The obtained result suggests the applicability of the organisms in a wide range of pH for cadmium, copper and mercury removal.

Effect FAC in the range of 0.01 to 1 g/L in citrate broth on copper, mercury and cadmium bioremoval by *E. cloacae* was studied and the results are shown in Fig. 1. Bioremoval of all the metals studied were in direct proportional to the FAC concentration in the medium.

However, 1.0 g/L FAC resulted in heavy precipitation of iron in the medium and gave darker colour to the spent medium. Thus, 0.1 g/L ferric ammonium citrate was considered to be concentration of choice in terms of the metals removed, amount of precipitate formed and extant of decolourization of the medium. The copper removal was found to be 85.0, 82.0 and 72.2% at 1.0, 0.1 and 0.01 g/L FAC respective whereas, cadmium removal was 95.0, 93.3 and 63.2%.

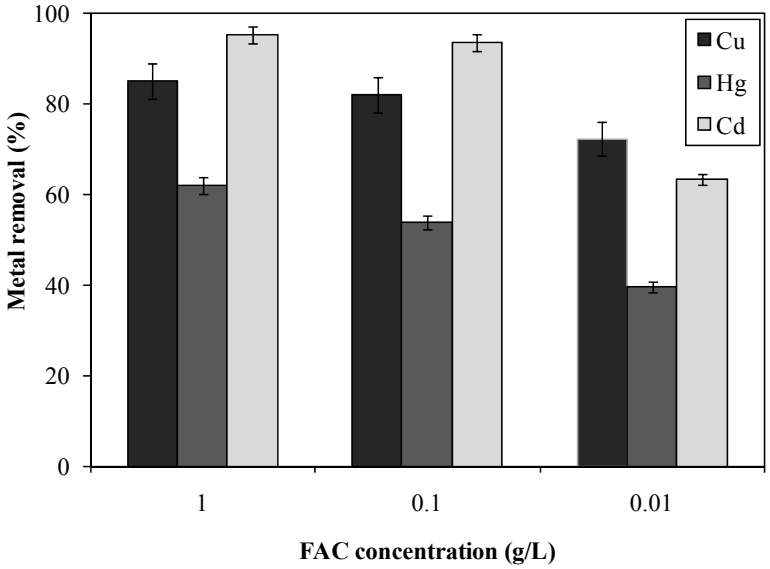

Fig. 1 Effect of FAC concentration on metal bioremoval by *E. cloacae*. (Removal of Cu, Hg and Cd was between 2.2 to 4.5% irrespective of FAC concentration in the absence of inoculum)

Cadmium removal was highest where as mercury removal was the lowest irrespective of the FAC concentration compared. Irrespective of metals studied when FAC concentration was increased 10 fold higher than 0.1 g/L only 0.93 ± 0.06 fold increase in removal was noticed while when 10 fold lower FAC concentration was taken 1.31 ± 0.18 fold lower removed was achieved. So, the 0.1 g/L concentration of FAC in the growth medium was taken as optimum. Under the experimental conditions, as compared to the test flask in control flask when no inoculum was added the metal

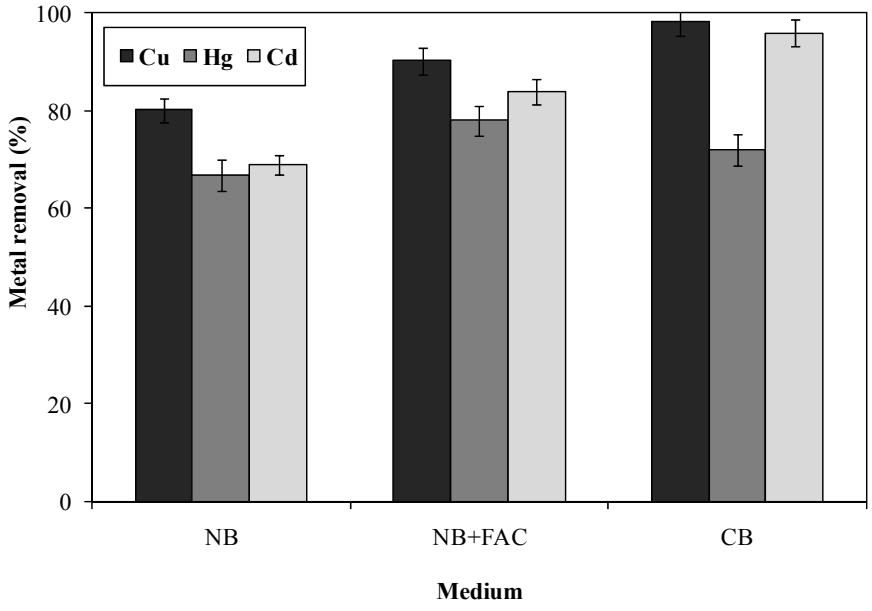

Fig. 2 Effect of nutrient broth (NB), citrate broth (CB) on metal removal by *E. cloacae*

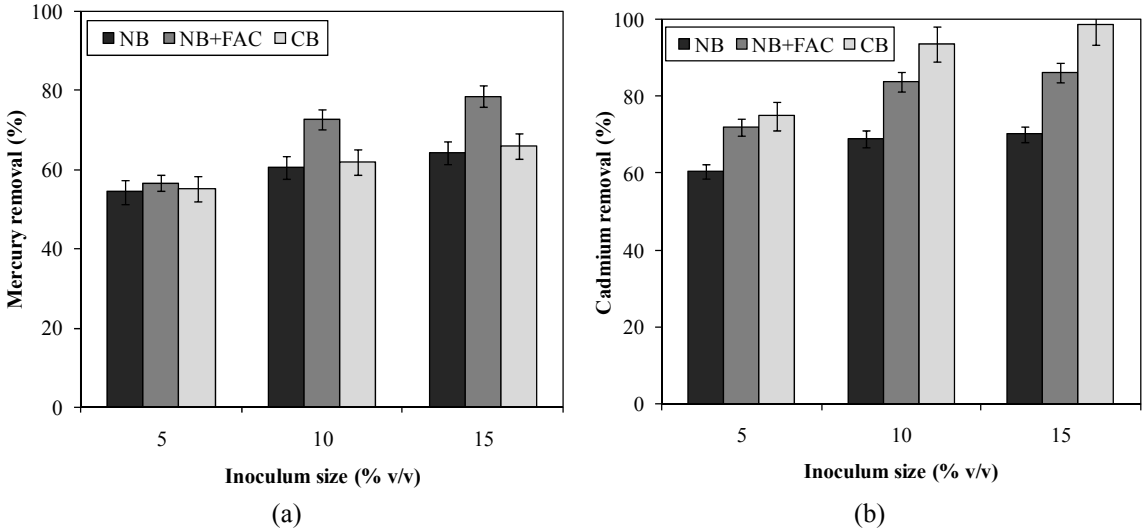

Fig. 3 Influence of inoculum size on bioremoval of (a) mercury and (b) cadmium by *E. cloacae*

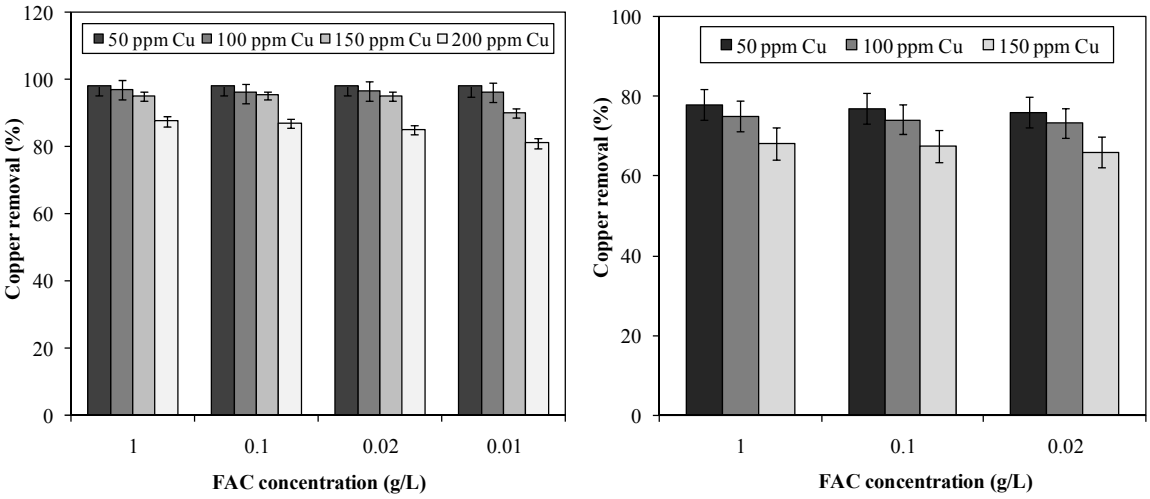

Fig. 4 Lab scale column bioreactor study for copper removal by *E. cloacae*: (a) with aeration; and (b) without aeration

removal ranged between 2.2 to 4.5% only irrespective of FAC concentration or the type of the metal studied.

The influence of various media was studied for cadmium, copper and mercury removal. As can be seen from Fig. 2 and Figs. 3(a) and (b), citrate broth was showed the highest copper and cadmium removal where as for mercury, NB+FAC was the best. As depicted in Figs. 3(a) and (b) metals bioremoval was in direct proportion to the amount of inoculum added. It is obvious that due to higher biomass, the mercury and cadmium bioremoval were high. *E. cloacae* showed the highest mercury bioremoval which comes out to be 79% in NB + FAC, whereas it showed as high as 98.6% cadmium bioremoval in citrate broth as compared to NB + FAC. This could be due to higher cell mass and protective role played by synergistic effect of FAC and peptone.

3.3 Lab scale column reactor for copper and mercury bioremoval

Lab scale column study was performed in two different modes. In case of column reactor, the influence of FAC concentration was studied with and without aeration for removal copper at different copper concentrations and results are shown in Figs. 4(a) and (b).

Under the experimental condition, the concentration range of FAC studied showed less than 15% difference in copper removal, when FAC concentration was decreased from 1 g/L to as low as 0.01 g/L in the system irrespective of the amount of copper present in the solution i.e. 50-200 ppm, but no significant difference was noticed when FAC concentration was studied in the range of 1.0 to 0.02 g/L. This finding showed that more than 81% copper was removed at 24 h of incubation period even, when as low as 0.01 g/L of the FAC was present in the medium. The copper removal rate was directly proportional to the amount of copper concentration in the system (Table 2).

Under the condition studied, the copper removal rate varied between 2 to 6.5 mg/L/h for 50 and 150 ppm of copper concentration respectively in aerobic condition. In case of absence of aeration

Table 2 Overall copper removal rate achieved by *E. cloacae* with aeration and without aeration in lab scale column bioreactor (0-22 h incubation)

Copper concentration (ppm)	Copper removal rate (mg/L/h)					
	FAC concentration (g/L)					
	1.0		0.1		0.02	
	WA	WOA	WA	WOA	WA	WOA
50	2.23	1.77	2.23	1.75	2.23	1.73
100	4.41	3.41	4.36	3.37	4.38	3.33
150	6.49	4.65	6.49	4.60	6.48	4.50

WA: with aeration; WOA: without aeration

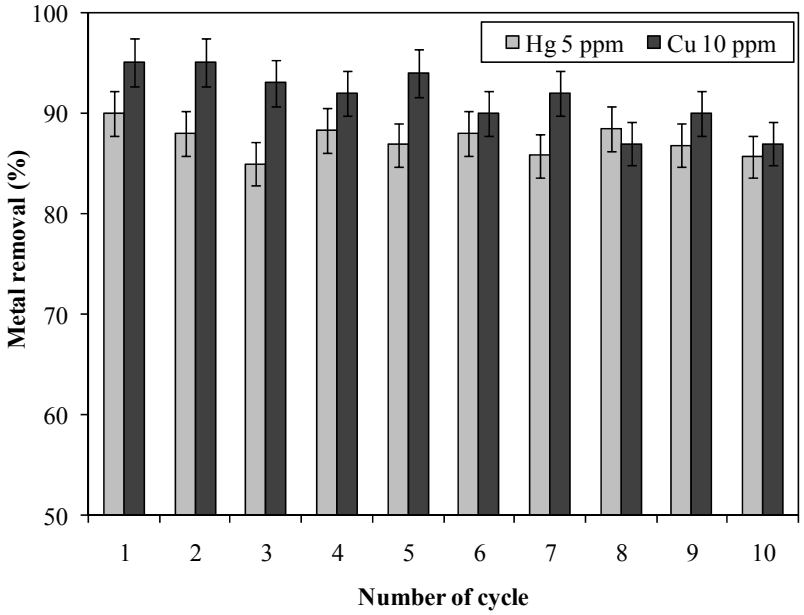

Fig. 5 Lab scale column bioreactor study for mercury and copper bioremoval by *E. cloacae*

as shown in Fig. 4(b), the highest copper removal was 77.8% for 50 ppm of copper and 68.16% for 150 ppm of copper with 1g/L FAC in 24 h of contact time, which was 20 and 27% less as compared to copper removal in the presence of aeration respectively. Similarly, a decreased copper removal rate of 1.73 and 4.5 mg/L/h was also noticed in the absence of aeration compared to aerobic condition at 50 and 150 ppm concentration respectively (Table 2). This could be due to the influence of aeration, which enhanced growth as well as iron precipitation that could be responsible for higher copper removal and enhanced rate of removal. So, aeration condition proved to be better as compared to the absence of aeration.

Copper and mercury removal were studied for 10 cycles in the lab scale column reactor and results are shown in Fig. 5. As can be seen from the result, in all the 10 cycles, mercury bioremoval was 87.5 ± 2.5% in 18 h of contact time, whereas, copper removal was 91.0 ± 4.0% in a similar experiment. More than 85% metals were removed even in the 10^{th} cycle indicates the

suitability of continuous process due to formation of the biofilm on the supporting material used in the column for metal remediation.

4. Conclusions

From the results of the study conducted it is concluded that

- *E. cloacae* play significant role in iron precipitation.
- Use of microbially formed ferric hydroxide precipitate proved to be an alternative with a good potential for removal of copper, mercury and cadmium present even at a low concentration in the aqueous system.
- *E. cloacae* was successfully exploited for the remediation of heavy metals from aqueous solution at shake flask and lab scale air lift column reactor.
- The developed process indicates the feasibility of this organism and column reactor, as a potential technique for bioremediation of heavy metals.

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