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## Biosorption Of Heavy Metals By Biomass Of Enterobacter Cloacae Isolated From Metal-Polluted Soils

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**Abstract:** Heavy metal resistant bacteria were isolated from SIPCOT industrial effluent, Cuddalore. Their maximum tolerable concentration (MTC) was calculated. 7 strains were identified based on MTC. Among the 7 strains, most potential strain *E.cloacae* AB6 was identified by 16s rRNA sequencing. Biosorption potential of *E.cloacae* was tested against Pb(II), Cu(II), Cr(VI), Hg(II) and Cd(II). The maximum biosorption capacities of biomass *E.cloacae* AB6 for Pb(II), Cd(II), Cr(II), Hg(II) and Cu(II) ions were determined as 65.68% at 200 mg l<sup>-1</sup>, 56.56% at 150 mg l<sup>-1</sup>, 54.28% at 100 mg l<sup>-1</sup>, 45.57% at 100mg l<sup>-1</sup> and 74.46 % at 300 mg l<sup>-1</sup> of initial metal ion concentrations under optimized conditions.

**Keywords:** Heavy metals, bacteria, biosorption, *Enterobacter cloacae*.

### 1. Introduction

Bacteria are generally the first organisms to be affected by discharges of heavy metals into the environment, resulting in an increase of metal resistant bacteria in these environments<sup>1</sup>. Resistant of toxic metals in bacteria probably reflects the degree of environmental contamination with these substances and may be directly related to exposure of bacteria to them<sup>2</sup>. Microorganisms have developed several mechanisms to tolerate high concentrations of heavy metals. Biomass of algae, fungi and bacteria has been known to readily adsorb or accumulate metal ions<sup>3,4</sup>. The ability of metal uptake by those microorganisms (known as biosorption or bioaccumulation) has caught great attention due to its potential to provide an effective and economic means for heavy-metal remediation<sup>3</sup>.

Biosorption is dependent on the affinity between the metallic species or its ionic forms and the binding sites on the molecular structure of the cellular membrane, cell wall and capsule<sup>5</sup>. Such processes are of industrial interest because the removal of potentially hazardous heavy metals and radionuclides from industrial effluents and wastewaters by microbial biomass can lead to detoxification and also to recovery of valuable elements such as gold and silver after appropriate treatment of the loaded biomass<sup>6</sup>.

The cell surfaces of all microorganisms are negatively charged owing to the presence of various anionic structures. This gives bacteria the ability to bind metal cations. A characteristic component of Gram-positive

cells are teichoic acids and acids associated to the cell wall, whose phosphate groups are key components for the uptake of metals. Carboxyl groups are the main agents in the uptake of heavy metals. The sources of these carboxyl groups are the teichoic acids, associated to the peptidoglycan layers of the cell wall<sup>7</sup>. The outer membrane of a Gram-negative bacterium is composed of lipopolysaccharides (LPS), phospholipids, and lipoproteins. It plays a very important role in the survival of the bacterium under environmental pressure. For example, it prevents the whole bacterium from heavy metals<sup>8</sup>.

Therefore, this study was navigated to isolate the most promising metal resistant bacteria from heavy metal contaminated soils and further, to assess their metal accumulating ability.

## 2. Materials And Methods

### 2.1. Isolation of heavy metal resistant bacteria

Heavy metal resistant bacteria were isolated from heavy metal polluted sediment sample (Uppanar estuary (11°43'N lat. and 79°49'E long), Cuddalore, Tamil Nadu). In order to minimize the complexation of heavy metals, the isolates were grown in Tris minimal medium (Tris-HCl-100(pH-7.2), Glucose-11, NH<sub>4</sub>Cl<sub>2</sub>-4, MgCl<sub>2</sub>-10, CaCl<sub>2</sub>-0.1, KH<sub>2</sub>PO<sub>4</sub>-0.1, in millimoles per litre of deionized water and Agar-15g/L) (Mergeay, 1995). For isolation of bacteria, samples were serially diluted in sterile distilled water and plated on Tris minimal medium supplemented with 0.5mM of heavy metals such as HgCl<sub>2</sub>, Pb(NO<sub>3</sub>)<sub>2</sub>, CdSO<sub>4</sub>, CuSO<sub>4</sub> and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> one metal at a time by standard pour plate method. Plates were incubated for 72 hrs at 37°C. All the determinations were carried out in triplicates. After the incubation, the plates were examined and counted the number of colonies per plate.

### 2.2. Determination of Maximum Tolerable Concentration (MTC)

Maximum resistance of the selected isolates against increasing concentrations of Hg(II), Pb(II), Cd(II), Cu(II) and Cr(VI) on Tris minimal medium plates were evaluated until the strains unable to give colonies on the agar plates<sup>9</sup>. The results obtained in the solid media were validated by the tube dilution method<sup>10</sup>. Positive controls consisted of a metal-free medium inoculated with the microorganism, while negative controls consisted of a metal-supplemented medium without the microorganism. Culture turbidity was measured at 600 nm in a spectrophotometer.

### 2.3. Subcellular fractionation assay

Subcellular fractionation was obtained by the method of Kumar and Upreti<sup>11</sup> (2000).

### 2.4. Biosorption studies

Biomass was harvested from the medium by centrifugation at 10000rpm for 10 min. The supernatant was discarded and the cells were resuspended in purified water for washing and again centrifuged as above to make sure that no media remain on the cell surface and then the biomass were autoclaved at 121°C for 15mins. This biomass was used for the adsorption experiments. Biosorption studies were done using biomass as a function of various parameters such as a) pH b) Biomass concentration c) Contact time d) Initial metal concentration.

#### a) Effect of pH

The metal absorption monitored for pH range 1.0 to 7.0. NaOH and HCl were used as pH regulators. 1 mg/ml biomass was dispersed in 100 ml of the solution containing 100 mg/L of each metal concentration and incubated for 12hrs. Solutions were centrifuged as above and the supernatant was analysed for the residual concentrations of the metal ions using ICP-OES (Perkin Elmer, Optima 1200dv).

#### b) Effect of biomass concentration

Biomass was centrifuged at 10000 rpm and different weights of the biomass ranging from 0.5 to 3mg/ml were dispersed in solutions containing 100 mg/L of each metal concentration. The solutions were adjusted to the optimum pH in which maximum biosorption of the metal ion occurred. Flasks were left for equilibration. The solutions were later centrifuged at 10000 rpm and the metal ion concentrations were determined by using the procedures described earlier.

### c) Effect of contact time

The cell pellet dispersed in metal solution of 100 mg/L concentration with a working volume of 100 ml. The experiment was carried out at the optimum pH system. Flasks were allowed to attain equilibrium on rotary shaker at 240 rpm and samples were collected at regular time intervals (0, 15, 30, 45, 60, 75, 90, 105, 120 min). Centrifugation at 10000 rpm was done and the supernatant was analysed for the residual metal content.

### 2.5. Biosorption of metal ions by *Enterobacter cloacae*

Test solutions containing heavy metal ions were prepared from analytical grade chemicals. The concentrations of metal ions prepared from stock solution ranged from 25 to 350 mg l<sup>-1</sup>. Before mixing the microorganisms, the pH of each test solution was adjusted to the required value by using 1M NaOH and HCl. The optimized biomass of isolate was added to the above metal solution and incubates for optimized time interval. And then the samples were centrifuged at 10000g for 20 min and supernatants were diluted in deionized water with 5% nitric acid for estimation of the metal ions from the medium. The pellets were treated overnight using 1M HCl and then were lysis in sonicator, twice for 45 sec followed by centrifugation at 10000 rpm for 5 min. The supernatant was collected and digested with 10% HNO<sub>3</sub> for estimation of heavy metals accumulated by the cells and then metal concentration was measured with Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). The cell pellets were dried for 48 hrs at 70°C and weighed for noting bacterial biomass.

The amount of metal uptake was calculated using the following mass balance equation:

$$\text{Biosorption (\%)} = \frac{C_0 - C_e}{C_0} \times 100$$

Where C<sub>0</sub> (mgL<sup>-1</sup>) is the initial metal ion concentration, C<sub>e</sub> (mgL<sup>-1</sup>) is the metal ion concentration after adsorption.

All the biosorption experiments were repeated three times to confirm the results. Also, blank experiments were conducted to ensure that no adsorption had taken place on the walls of the apparatus used.

### 2.6. Scanning Electron Microscopy (SEM) analysis

Investigation of absorption of the copper after fixation and dehydration was conducted with SEM equipped with Energy Dispersive Spectroscopy (EDS). The cells grown with and without Cu(II) were washed with ultrapure water and smeared onto glass slides and dried. Then it was fixed in 2.5% glutaraldehyde for 12 h at 4 °C followed by rinsing in distilled water three times to remove traces of glutaraldehyde. Later it was dehydrated in a series of ethanol concentrations (30%, 50%, 75%, 85%, 95% and 100%), dried and kept in desiccators until use. The samples were subsequently mounted on aluminium stubs and sputter coated with gold. Specimens were examined using Scanning Electron Microscope equipped with an Energy Dispersive Spectroscopy.

### 2.7. FT-IR analysis of *Enterobacter cloacae* cells

Fourier Transform Infrared (FT-IR) spectra of untreated and copper treated cells (3mM) were recorded on a Perkin Elmer FT-IR spectrometer in the region of 400–4000 cm<sup>-1</sup>. The cells grown overnight in the absence and presence of copper were harvested by centrifugation. Then they were dried in the hot air oven at 60°C to complete dryness. The dried biomass is ground to a fine powder using a mortar and pestle. The powdered sample was pressed into spectroscopic quality KBr pellet with a sample/KBr ratio of 1/100.

### 2.8. Statistical analysis

The data obtained on the adsorption of different metals at different time interval, by the bacterium *E. cloacae* was subjected to post hoc Tukey test to analyze the differences in the rate of adsorption between the metals. Statistical analysis was done using SPSS ver10 software.

### 3. Results And Discussion

#### 3.1. Primary screening for potential strain

A total of 251 heavy metal resistant bacteria belonging to 6 genera were isolated (Table.1). Among the 251 strains, 13 strains were selected based on different morphology. They were purified and checked for their Maximum Tolerable Concentration (MTC).

**Table 1: Number and type of bacterial isolates**

Type of bacteria	No.of strains isolated
<i>Bacillus</i> sp.	154
<i>Enterobacter</i> sp.	36
<i>E.coli</i>	2
<i>Achromobacter</i> sp.	12
<i>Staphylococcus</i> sp.	5
<i>Pseudomonas</i> sp.	32

#### 3.2. Maximum Tolerable concentration (MTC)

The tolerance test indicated that among five experimented heavy metals, maximum tolerance to copper showing the growth of microorganisms up to 14 mM and minimum tolerance to mercury showing no growth above 2 mM. The microbial tolerance at each concentration of heavy metal was depicted by the microbial load on Tris minimal media plate. The microbial load decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms. About 7 strains were selected based on the MTC (Table.2).

**Table 2: Maximum Tolerable Concentration of bacterial isolates against metal ions**

Strain	MTC of metal ions (mM)				
	Cu(II)	Cd(II)	Cr(VI)	Pb(II)	Hg(II)
AB02	3	2	1	2	0.5
AB04	3	1	2	4	0.5
AB06	14	3	5	11	2
AB09	2	6	3	3	0.5
AB11	7	4	4	5	1
AB13	11	3	2	9	2
AB16	5	2	1	4	0.5
AB19	8	5	3	1	0.5
AB20	4	3	4	7	2
AB22	10	1	2	10	1
AB23	3	4	5	2	0.5
AB25	2	1	3	8	1
AB27	4	3	6	3	0.5

#### 3.3. Identification of potential strains using biochemical tests and 16s rRNA sequencing

All the 7 isolates were identified using biochemical tests as per Bergey's manual of systematic bacteriology. The most potential strain AB6 was identified as *Enterobacter cloacae*. The name of the strain was designated as *E.cloacae* AB6. 16s rRNA sequencing was done for this strain alone and comparative analysis of the sequence with already available database and phylogeny based on ClustalX (Fig.1) clearly indicates that AB6 was close to the member of *Enterobacter cloacae*. This sequence had been submitted to Gen Bank (Accession Number: JQ640581). The copper resistant bacteria *Enterobacter cloacae* was finally selected as the most active strain for the treatment of heavy metal polluted water based on the relative ability of the Maximum tolerable concentration of the tested strains and exhibiting co-resistance against Hg(II), Pb(II), Cd(II) and Cr(VI).

### 3.4 Subcellular fractionation assays

The results of distribution and uptake of metal ions by *E. cloacae* AB6 were presented in Table.3. Subcellular fractionation studies revealed that more than 85% of the metal ions were taken up by the cell wall of *E.cloacae* AB6 compared to cytoplasmic and membrane fractions. The biosorption removal process is rapid; it takes only a few minutes and takes place under normal pressure and normal temperature conditions. Biosorption is in fact a passive immobilization of metals by biomass.

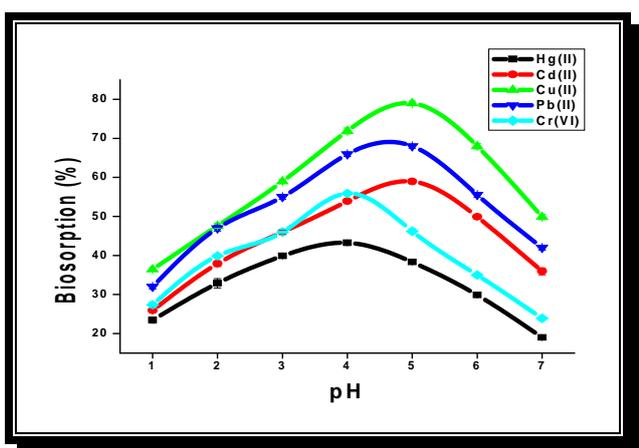
**Table 3: Distribution and uptake of metal ions by *E.cloacae* AB6**

Metal ion	Distribution and uptake of metal ions by <i>E.cloacae</i> AB6 (%)		
	Cell wall	Cytoplasm	Membrane fraction
Cu(II)	89.3	3.2	7.5
Pb(II)	94.6	2.7	2.7
Cd(II)	88.8	7.5	3.7
Cr(VI)	85.8	8.1	6.1
Hg(II)	96.2	1.3	2.5

### 3.5 Biosorption studies

#### a) pH

The most important single parameter influencing the sorption capacity is the pH of the adsorption medium<sup>12</sup>. The charge of the adsorbate and the adsorbent often depends on the pH of the solution. It is known that in acidic pH, metals exist as free ions, but at alkaline pH the ions precipitate as insoluble hydroxides or oxides. Therefore, the availability of metal ions depends upon the pH of the medium or solution<sup>13</sup>. In the present study, it was noticed that increase in the pH from 2.0 to 6.0, decreased the availability of metal ions. At pH 7.0 and above, there was precipitate formation. The influences of pH on the percentage sorption of metals were depicted in the Fig.2. pH 4.0 is optimum for the biosorption of Hg(II), Cr(VI) whereas maximum biosorption of Cu(II), Pb(II) and Cd(II) ions were obtained at pH 5.0. The maximum adsorption capacities (%) for the different metals by biomass of *E.cloacae* AB6 were 78.98% for Cu(II)(at pH5.0) >67.98% for Pb(II) (at pH 5.0) >58.93 % for Cd(II) (at pH 5.0) >55.87 % for Cr(VI) (at pH 4.0) > 43.23% Hg(II) (at pH 4.0). The different pH binding profiles for different metal ions are due to the nature of the chemical interactions of metal with the bacterial cells<sup>14</sup>.



**Fig.2. Optimization of pH for heavy metal biosorption**

#### b) Biomass concentration

The influence of biomass concentration on the percentage sorption of metal ions is depicted in Fig.3. To achieve the maximum biosorption capacity of the biosorbent for metal ions, the biomass concentration was varied from 0.5 to 3.0mg/ml and it was found that a concentration of 1.5mg/ml was sufficient for maximum biosorption of around 74.87% under the reported experimental conditions for Cu(II) ions. It is also seen from this figure the sorption percentage increases from 0.5 to 1.5mg/ml and further increase in biomass does not

affect the sorption percentage greatly for all the metal ions. Increase in biomass concentration generally increases the level of biosorption of metal ions because of an overall increase in the surface area of the biomass, which in turn increases the number of binding sites<sup>15</sup>. The interference onto binding sites due to increased biomass dosages cannot be over ruled and thus results in the low specific uptake rate<sup>16</sup>.

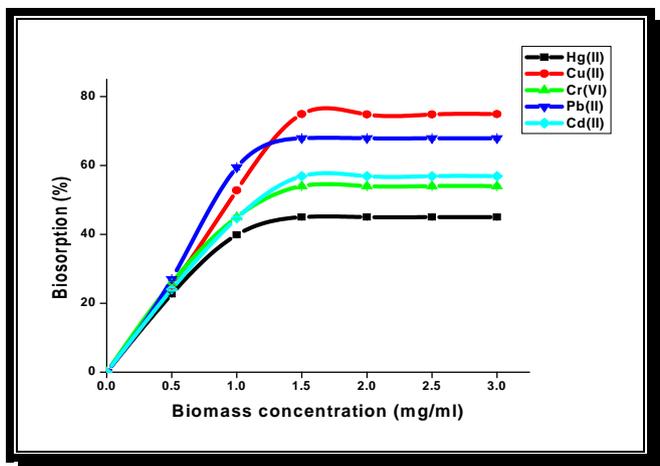


Fig.3.Optimization of biomass concentration for heavy metal biosorption

#### c) Contact time

The optimum biomass concentration from Fig.3 taken for *E.cloacae* AB6 and it is time to reach maximum sorption was monitored. The adsorption experiments of metal ions were carried out for different contact times with a fixed adsorbent dose of 1.5mg/ml concentration at pH 5.0 for Cu(II), Pb(II) and Cd(II) ions and pH4.0 for Hg(II) and Cr(VI) at 30°C. The results are plotted in Fig.4, which indicate that maximum sorption attained at 60 min for all metal ions. These results indicated that the adsorption sites were bind up in the initial 60 min by the metal ions passively. After this, the increase in contact time might not help for more adsorption of metal ions with this biosorbent<sup>17</sup>. As a result, 60 min was chosen as optimum contact time for further studies. Pardo et al.<sup>18</sup> reported that the percentage removal of Cd(II), Cu(II), Pb(II) and Zn(II) from aqueous solution by *Pseudomonas putida* was 80%, in this study the percentage adsorption by *E.cloacae* was 46%, 54%, 57%, 66% and 74% for Hg(II), Cr(VI), Cd(II), Pb(II) and Cu(II) respectively.

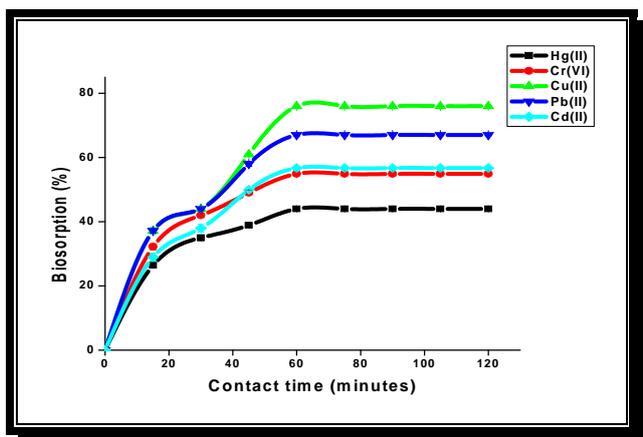


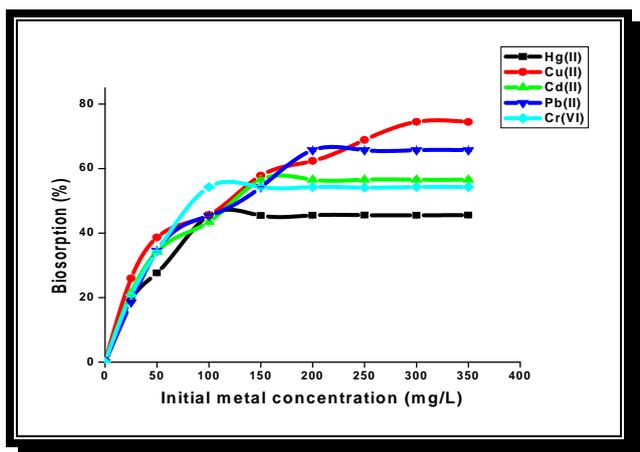
Fig.4. Optimization of contact time for heavy metal biosorption

#### d) Initial metal ion concentration

The effect of initial metal ion concentration on the biosorption capacity of *E.cloacae* AB6 was studied at optimum pH values and contact time. These experiments were carried out using single metal ion solution (25–350 mg l<sup>-1</sup>). The amount of metal ions adsorbed per unit mass of bacterial biosorbent increased first with

increasing of the initial metal ion concentration and reached to a saturation value. Then the value did not change with the initial metal ion concentration (Fig.5).

As seen from Fig.5, the maximum biosorption capacities of biomass *E.cloacae* AB6 for Pb(II), Cd(II), Cr(II), Hg(II) and Cu(II) ions were determined as 65.68% at 200 mgL<sup>-1</sup>, 56.56% at 150 mgL<sup>-1</sup>, 54.28% at 100 mgL<sup>-1</sup>, 45.57% at 100mg L<sup>-1</sup> and 74.46 % at 300 mgL<sup>-1</sup> of initial metal ion concentrations. The enhancement in metal sorption could be due to an increase in electrostatic interactions, involving sites of progressively lower affinity for metal ions<sup>19</sup>. Macaskie and Strandberg et al. provided detailed description of mechanisms involved in metal microbe interactions. Tobin et al. demonstrated that ions having a smaller ionic radius could be more quickly adsorbed onto a fixed area of adsorbent.



**Fig.5. Optimization of biosorbent concentration for heavy metal biosorption**

### 3.6 FTIR

The FTIR spectra of non-metal and metal-loaded biosorbents, in the range of 400–4000 cm<sup>-1</sup>, were taken to confirm the presence of functional groups that are usually responsible for the biosorption process Fig.6a and b. The metal ion displays a number of absorption peaks, reflecting the complex nature of the biomass. Peaks in the region of 3500–3200 cm<sup>-1</sup> are due to the stretching of the N–H bond of the amino group and indicates the presence of bonded hydroxyl groups. The peak found at 2962 and 1458 cm<sup>-1</sup> represents the presence of C–H group of the control sample. The characteristic peaks at 1645 and 1573 cm<sup>-1</sup> correspond to amide and amide II respectively. The peak at 1406 and 1112 cm<sup>-1</sup> represents C–O stretching of the control sample. Fig.6b spectra of stress induced sample shows characteristic peaks at 3431, 2960, 2355, 1641, 1448, 1400 and 1126 cm<sup>-1</sup>. The peaks at 1400 and 1126 cm<sup>-1</sup> are due to the presence of C–O stretching of the sample. The broad band at 3431 cm<sup>-1</sup> corresponds to hydroxyl group while the band at 2960 and 2355 cm<sup>-1</sup> represents the presence of aliphatic groups (CH<sub>2</sub> and CH<sub>3</sub>). The bands at 1641 and 1448 cm<sup>-1</sup> are due to amide and C–H stretching, respectively. It has been noted that the Fig.6b exhibits specific peak at 2355 cm<sup>-1</sup> and hidden the amide II peak compared to the control (Fig.6a). It reveals that the functional groups and chemical alteration has been occurred by the copper stress induction in the microbial sample. The spectral data thus confirms the presence of amine, hydroxyl and carboxyl in the biomass.

The FTIR spectra show little differences in the nature of functional groups and the overall charging behaviour between control and Cu(II) treated cells of *E.cloacae* AB6. As expected, the main functional groups are associated with proteins, phospholipids and polysaccharides<sup>20</sup>. Similar result was observed by Pandiyan and Mahendradas<sup>23</sup> for nickel removal using *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*.

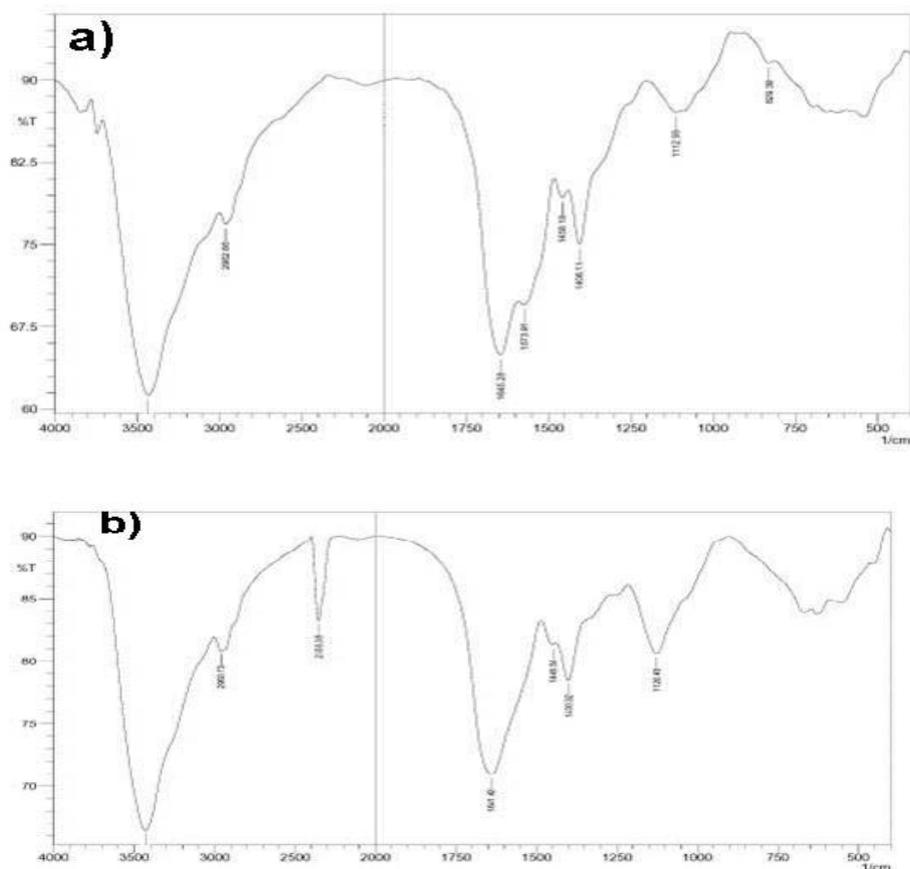
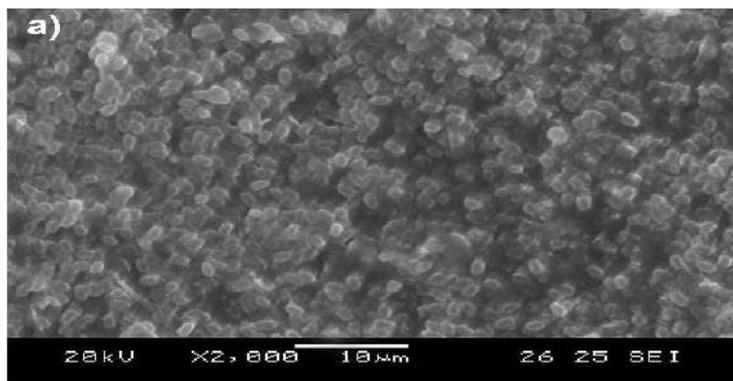
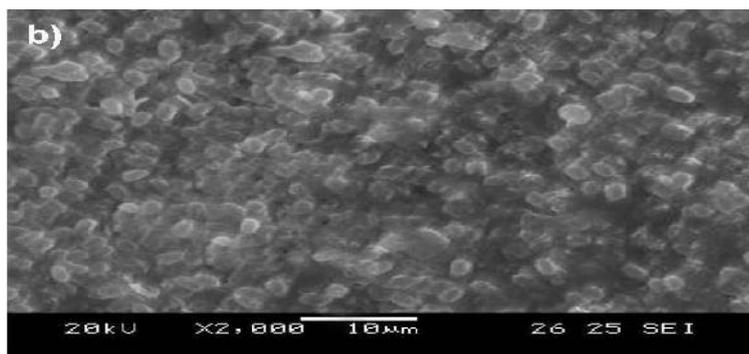


Fig.6a and b showing the FTIR spectra of control and copper loaded *E.cloacae*

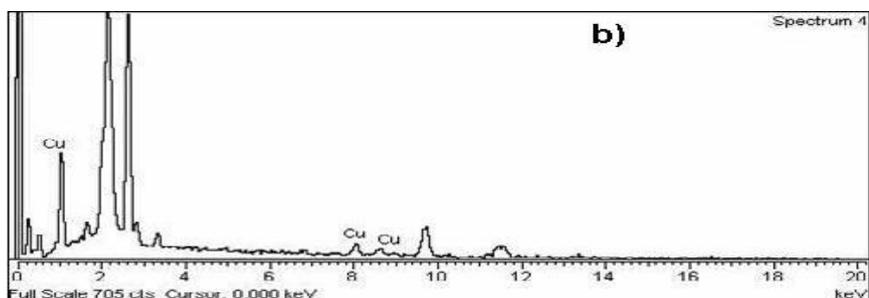
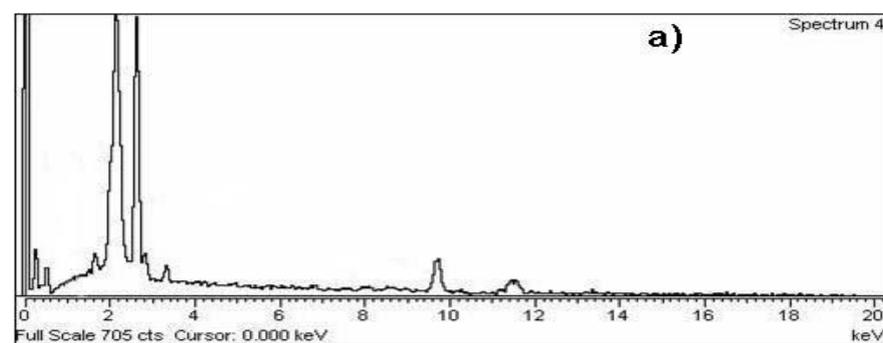
### 3.7 Scanning Electron Microscopy (SEM)

In order to understand the morphology of the biosorbent SEM analysis of bacterial samples before and after adsorption were carried out and are shown in Fig.7a and b respectively. The comparison of SEM pictures between the metal free and metal loaded biosorbent shows that the particle has undergone remarkable physical disintegration after adsorption in the biosorbents. It is observed that the cell-surface morphology considerably changed after metal adsorption. Moreover, the EDS analysis (Fig.8a and b) confirmed the presence of metal adsorbates on the cell mass, giving a direct detection of metals on cells. Electron microscopic observation carried out by Mullen et al<sup>21</sup> revealed the presence of Ag<sup>2+</sup> as discrete particles at or near the cell wall of both gram-positive and gram-negative bacteria and the presence of silver was confirmed by energy dispersive X-ray analysis (EDX). Large particles containing gold were localized in *Sargassumnatans* cells by EDX carried out in conjunction with scanning electron microscopy<sup>22</sup>.





**Fig.7. SEM micrograph of *E.cloacae* AB6: (a) control (before biosorption); (b) after Cu(II) biosorption**



**Fig.8. EDS analysis of *E.cloacae* AB6: (a) control (before biosorption); (b) after Cu(II) biosorption.**

#### 4. Conclusion

Bioremediation is an eco-friendly green technology used for the cleaning of environmental pollutants to safe levels. The present study was carried out to isolate and screen metal resistant bacteria from heavy metal polluted area and to study its bioremediation potential for heavy metal accumulation. This study confirmed that the biosorbent prepared from *E.cloacae*, a low cost, easily available and eco-friendly, could effectively remove pollutants from industrial effluents. The results would be useful for the commercial treatment of industrial effluent.

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