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## Isolation, Molecular identification of metal tolerant bacteria and its heavy metal removal capacity

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### Abstract

The cadmium removal by isolated cadmium tolerant bacteria *Pseudomonas* sp isolated from electronic waste (e-waste) recycling facility soil was studied in a laboratory simulated environment. The bacterium was identified by biochemical and 16S rRNA molecular studies. The DNA was isolated and amplified using universal bacterial primers. The phylogeny tree suggested the isolated cadmium tolerant bacterium as *Pseudomonas aeruginosa*. The partial 16S rRNA sequences were submitted to NCBI Genbank and the accession number JN102340 was assigned to the submitted sequences. Various parameters were optimized for cadmium removal such as pH, temperature, kinetics studies and metal tolerant assay. The metal removal studies suggested that the cadmium tolerant *Pseudomonas aeruginosa* JN102340 able to remove cadmium 92% at pH 6, 89% at 35°C, 90% after 24h incubation in the optimized pH and temperature. The bacterium *Pseudomonas aeruginosa* JN102340 tolerate and survive up to 600mg L<sup>-1</sup> cadmium. The abstract should not exceed 250 words, and should concisely summarize the basic content of the paper. Experimental details should not be presented in the abstract. Avoid specialized terms, abbreviations, diagrams, and references.

Keywords: Bioremediation; Cadmium removal; *Pseudomonas aeruginosa*; Bacteria; e-waste; 16S rRNA

### Introduction

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Copper, chromium, cadmium and nickel are known to be the most commonly heavy metals used and the more widespread contaminants of the environment<sup>1, 2, 3</sup>. Traces of these heavy metals are necessary as co-factors of enzymatic reactions, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions. The microorganisms respond to these heavy metals by several processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions<sup>4,5,6,7,8,9</sup>. The bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Metal accumulative bioprocess generally falls into one of two categories, biosorptive (passive) uptake by nonliving, nongrowing biomass or biomass products and bioaccumulation by living cells<sup>10</sup>. Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation<sup>11</sup>. Bahig et al., (2008)<sup>12</sup> reported that the previous studies have shown that long term and short term stresses such as high temperature, extremes of pH or chemical pollution often result in altered metabolism, species diversity and plasmid incidence of soil bacteria populations. Bacteria are among the most abundant organism that occurs everywhere on earth. Heavy metals are increasingly found in microbial habitats due to several natural and anthropogenic processes; therefore, microbes have evolved mechanisms to tolerate the presence of heavy metals by efflux, complexation, or reduction of metal ions or to use them as terminal electron

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acceptors in anaerobic respiration<sup>13</sup>. Most mechanism studied till date involves the efflux of metal ions outside the cell, and genes for tolerance mechanisms have been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of those metal ions<sup>13</sup>. To understand the contamination status, Ha et al., (2009)<sup>14</sup> measured trace elements (TEs) in soil, air dust, and human hair collected from e-waste recycling sites (a recycling facility and backyard recycling units) and the reference sites in Bangalore and Chennai in India. Concentrations of Cu, Zn, Ag, Cd, In, Sn, Sb, Hg, Pb, and Bi were higher in soil from e-waste recycling sites compared to reference sites. For Cu, Sb, Hg, and Pb in some soils from e-waste sites, the levels exceeded screening values proposed by US Environmental Protection Agency (EPA). Concentrations of Cr, Mn, Co, Cu, In, Sn, Sb, Tl, Pb and Bi in air from the e-waste recycling facility were relatively higher than the levels in Chennai city. High levels of Cu, Mo, Ag, Cd, In, Sb, Tl, and Pb were observed in hair of male workers from e-waste recycling sites. Ha et al., (2009)<sup>14</sup> results suggest that e-waste recycling and its disposal may cadmium to the environmental and human contamination by some TEs. This is the first study on TE contamination at e-waste recycling sites in Bangalore, India.

In this study, e-waste recycling facility surface soil was used to evaluate the sorption capacity of cadmium of the isolated cadmium metal tolerant bacteria in a laboratory simulated environment.

## Materials and methods

### Samples

Soil sample was collected from an e-waste recycling facility surface soil from Peenya Industrial Estate, Bangalore, Karnataka, India (Fig. 1) and stored at -20°C. Soil was dried to remove the moisture content.

### Preparation of metal adsorbates

Different metal concentrations were prepared by dissolving Cadmium, in double distilled water to get metal concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg L<sup>-1</sup>. A stock solution of 1000 mg L<sup>-1</sup> was prepared; all other concentrations are obtained from it. Prior to experiment all the glasswares were treated with 0.1 M HCl before and after the biosorption experiments to avoid binding of metals to it. Cadmium was estimated using PAR Indicator method and absorbance was measured at 520 nm.

### Enumeration and screening of metal tolerant bacteria

Cadmium metal tolerant bacterial (MTB) strains were isolated from the soil samples using bacterial medium. Approximately 10 g of soil sample was serially diluted with peptone water and 100 µl of suspension from 10<sup>-4</sup> dilutions from the broth was spread on to the nutrient agar plates (standard spread plate technique) amended with different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg L<sup>-1</sup>) cadmium to isolate MTB strains. The plates were incubated at room temperature (30–

35°C) for 2-3 days. After incubation, the numbers of colonies were counted. Heavy metal resistant bacteria were screened by selecting the metal tolerant identical colonies from metal amended nutrient agar plate. The isolated colonies were grown in LB broth and these colonies were further characterized and employed for heavy metal tolerance studies. The pure cultures were isolated from the plate by inoculating the individual colonies into the sterile Luria Bertani Agar plates. The plates were incubated at 37°C for 24 h.

### Identification of the isolated MTB strains

Morphological, physiological, and biochemical characteristics of the isolated MTB strains were performed by following tests according to Bergey's manual<sup>15</sup> of systematic bacteriology.

### Molecular identification of MTB by 16S rRNA

The ready to amplify DNA was isolated from the MTB by using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corporation, USA) by following the manufacturer's instructions. Isolated MTB DNA was run on 0.8% Agarose Gel Electrophoresis to check the presence of DNA. MTB 16S rRNA genes were amplified by using the Universal primers (8 F' 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492 R' 5'-TACGGCTACCTTGTGTTACGACTT-3'). PCR was performed using the following reaction mixtures: 1 µl of DNA, 17 µl of 1x Prime Taq™ DNA polymerase (Genet Bio, Republic of Korea), 1 µl of each primer (Genei, Bangalore, India), to give a final volume of 20 µl. Thermal cycling was carried out under the following conditions: Denaturation at 94°C for 3 min, Annealing at 92°C for 1 min, 52°C for 1 min, 72°C for 1.30 min followed by 30 cycles of 72°C for 5 min. The amplified MTB DNA was run on 1% Agarose Gel electrophoresis. The product was eluted and purified using HiYield™ Gel/PCR Large DNA Extraction kit (Real Biotech Corporation, Taiwan) by following the instructions manual. The purified MTB 16S rRNA was sequenced at Ocimum Biosolutions, Hyderabad, India. The MTB 16S rRNA gene sequences were initially analyzed using the BLASTn search facility. Phylogenetic tree was constructed using the software MEGA version 5.0. The sequences were submitted to the NCBI (National Centre for Biotechnology and Information) GenBank for obtaining accession numbers.

### Biosorbent quantification

The MTB biosorbents was quantified by withdrawing 2.5 ml of broth culture from the bacterial medium and the absorbance was measured using spectrophotometer at 595 nm<sup>16</sup>. Uninoculated growth medium was used as blank.

### Optimization of pH for heavy metal removal

The MTB biosorbents was inoculated into a series of 250 ml conical flasks containing heavy metal (100 mg L<sup>-1</sup>) at different pH (2, 4, 6, 8 and 10) and kept in orbital shaker (120 rpm) for 24 hrs incubation. After 24 h incubation, the biosorbent were separated by centrifugation at 3,000 rpm for 15 min and the heavy metal concentration was determined

spectrophotometrically. The initial and the final concentration of heavy metal used in batch mode studies were calculated by estimating the concentration of heavy metal spectrophotometrically. From the difference in concentration the removal efficiencies of the biosorbent was calculated. Based upon the heavy metal removal and biosorbent data, the optimum pH was determined. To avoid precipitation of the heavy metal ions at high pH, all experiments were carried out only upto pH 10. The use of buffers was avoided to eliminate unknown effects of their components in the presence of metallic ions<sup>17</sup>. The pH was adjusted with 1 N NaOH and 1 N HCl as required.

### Optimization of temperature for heavy metal removal

The MTB biosorbents was inoculated into a series of 250 ml conical flasks containing different heavy metals (100 mg L<sup>-1</sup>). The flasks were incubated at different temperatures (25, 30, 35, 40 and 45°C) and kept in orbital shaker (120 rpm) for 24 hrs incubation. After 24 hrs incubation, the biosorbent were separated by centrifugation at 3,000 rpm for 15 min and the heavy metal concentration and bacterial biosorbent were determined by spectrophotometrically. From the heavy metal removal and biosorbent data, the optimum temperature was determined.

### Measurement of the kinetics of broth cellular growth and heavy metal removal

The MTB biosorbent was inoculated into a 250 ml conical flask containing heavy metal (100 mg L<sup>-1</sup>). The flask was kept in orbital shaker (120 rpm) at optimum pH and temperature for 24 hrs. During the incubation period, heavy metal concentration and biosorbent were monitored for every two hours interval (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hrs) until heavy metal removal attains a saturation level.

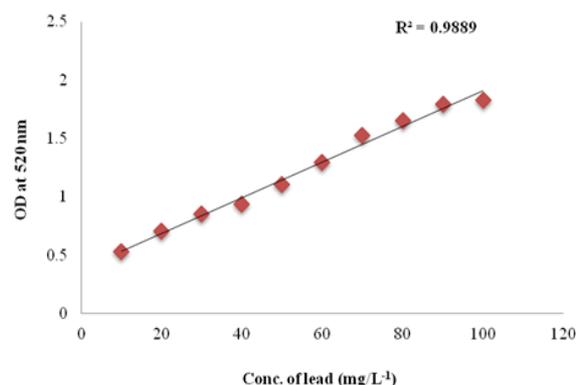
### Heavy metal tolerance assay

Bacterial isolates were added into a 250 ml flask containing nutrient broth amended with heavy metal (ranging from 100 to 1000 mg L<sup>-1</sup>). The biosorbents were shaken in a rotary shaker (120 rpm) in a temperature controlled water bath at pH 7 and 35°C for 24 hrs. After 24 hrs incubation, the biosorbent was measured. The extent of tolerance was compared and the “normalized” biosorbent was calculated, i.e., biomass at each heavy metal concentration per biosorbent using a control. Bacterial biosorbent were quantified by spectrophotometer at 595 nm. All the experiments were carried out in triplicates.

## Results and discussion

### Heavy metal quantification

A Standard graph was plotted against the concentration of standard cadmium and absorbance. From the standard graph, the concentration of the sample can be calculated (Fig 2).



**Figure 2.** Calibration of Cadmium (PAR Indicator Method)

### Enumeration and screening of MTB

The population of MTB was reduced due to the increasing concentration of metals (Table 1) that exerted more stress in the medium. Brown et al., (1994)<sup>18</sup> concluded that the microbes continued to be metabolically active in the presence of higher concentration of heavy metals, but the number might be reduced. In general the maximum number of population was observed under control and as the concentration of heavy metals increased from 0 to 100 ppm, the population decreased sharply in all the isolates.

### Identification of the isolated MTB strains

To determine the genus to which the strains belong, a series of biochemical tests were performed. Morphological, physiological, biochemical profile was subsequently examined according to Bergeys' manual, the isolated potential organisms identified which is shown in Table 2.

### Identification of MTB strain by 16S rRNA

The DNA of cadmium tolerant bacterial strain *Pseudomonas* sp was amplified with primers 1492R and 27F. The PCR amplified products were detected by 0.8% agarose gel electrophoresis with ultraviolet (UV). The length of object fragment is about ~1, 500 bp. Sequence analysis of the 16S rRNA gene has been considered a fast and accurate method to identify the phylogenetic position of bacteria. Partial 16S rRNA of *Pseudomonas* sp were sequenced and used to construct phylogenetic development trees (Fig 3).

Comparative analysis of the sequences with already available database showed that the strains were closed to the members of genus and it was classified in the branch *Pseudomonas aeruginosa*. The accession number JN102340 was assigned by NCBI Genbank.

**Table 1. Enumeration of cadmium resistant bacterial species at different concentrations**

| Concentration of cadmium (mg L <sup>-1</sup> ) | No. of Colonies | Colony forming unit (CFU/ml) |
|--|-----------------|------------------------------|
| 0  | TNTC            | -                            |
| 10   | TNTC            | -                            |
| 20   | TNTC            | -                            |
| 30   | TNTC            | -                            |
| 40   | TNTC            | -                            |
| 50   | 32              | 32 x 10 <sup>-4</sup>        |
| 60   | 29              | 29 x 10 <sup>-4</sup>        |
| 70   | 16 (TFTC)       | 16 x 10 <sup>-4</sup>        |
| 80   | 11 (TFTC)       | 11 x 10 <sup>-4</sup>        |
| 90   | 9 (TFTC)        | 9 x 10 <sup>-4</sup>         |
| 100  | 4 (TFTC)        | 4 x 10 <sup>-4</sup>         |

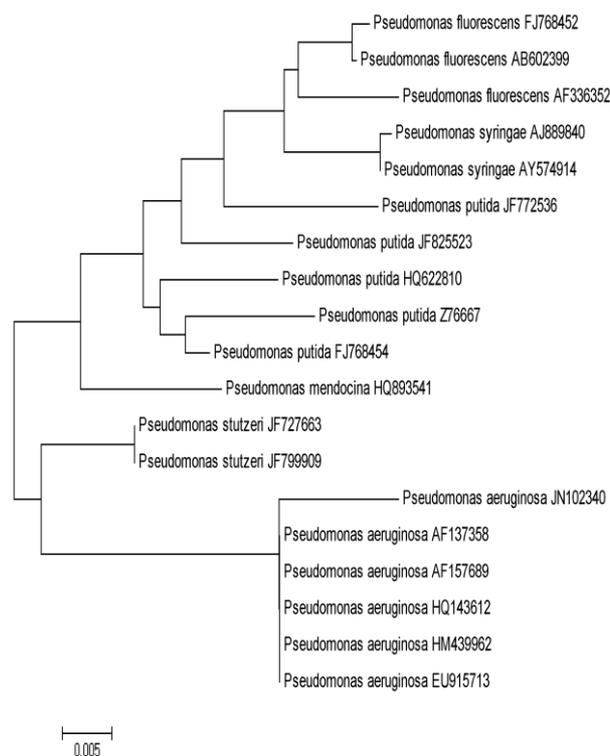
**Table 2. Morphological, physiological and biochemical characteristics of the isolated bacterial species**

| Morphological/Physiological/Biochemical characteristics | Isolated Cadmium MTB strain |
|---|-----------------------------|
| Gram's staining   | -                           |
| Cell shape  | Rod                         |
| Indole production test                                  | -                           |
| Methyl red test   | -                           |
| Voges- Proskauer test                                   | -                           |
| Citrate test  | +                           |
| Triple sugar iron test                                  | K/A                         |
| Gas production  | -                           |
| Urease test   | -                           |
| Motility test   | -                           |
| Catalase test   | +                           |
| Oxidase test  | +                           |
| ONPG test   | -                           |
| Nitrate test  | +                           |
| Gelatin hydrolysis test                                 | +                           |
| Starch hydrolysis test                                  | -                           |
| Strain name   | <i>Pseudomonas</i> sp       |

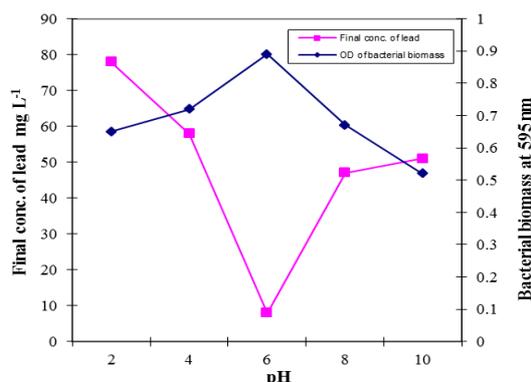
**Optimization of pH for heavy metal removal by MTB strains**

Hydrogen ion concentration in the adsorption is considered as one of the most important parameters that influence the adsorption behavior of metal ions in aqueous solutions<sup>19</sup>. It affects the solubility of the metal ions in the solution, replaces some of the positive ions found in the active sites and affects the degree of ionization of the adsorbate during the reaction<sup>20</sup>. Tests were conducted with initial heavy metal levels of 100 mg L<sup>-1</sup>, at a temperature of 35°C and equilibration time of 24 hrs. The effect of pH on the biosorption of heavy metal onto MTB strain was evaluated within the pH range of 2-10. *Pseudomonas aeruginosa* JN102340 reported a maximum removal of 92% at pH 6 at a concentration of 100 mg L<sup>-1</sup>. The influence of pH on the percentage sorption of heavy metals by *Pseudomonas*

*aeruginosa* JN102340 was depicted in the Fig 4. This pH dependency of biosorption efficiency could be explained by the functional groups involved in metal uptake and metal chemistry. Above pH 5, the percent removal of cadmium increased rapidly by the isolated *Pseudomonas aeruginosa* JN102340 strain.



**Figure 3. Neighbor-Joining tree deduced from partial sequences of 16S rRNA gene of cadmium MTB *Pseudomonas* sp EWR44 isolated from the e-waste recycling facility surface soil**



**Figure 4. Cellular growth and cadmium removal by *Pseudomonas aeruginosa* in response to various pH**

The low bioaccumulation capacity at pH values below six is attributed to the competition of hydrogen ion with metal ion on the sorption site. Thus, at lower pH, due to the protonation of binding site resulting from high concentration of proton, negative charge intensity on the site is reduced which results in the reduction or inhibition for the binding of metal ion. Most of the microbial surfaces are negatively charged due to the ionization of functional group, thereby contributing to metal

binding. At low pH, some of the functional groups will be positive charged and may not interact with metal ions<sup>21</sup>. The increase in percent removal of metal with increase in pH from two to five is due to the strong relations of bioaccumulation to the number of surface negative charge, which depends on the dissociation of functional group<sup>22</sup>. As the pH is increased above the zeta potential of the biosorbent, there is a reduction in the electrostatic attraction between the heavy metals and the biosorbent surface, with a consequent decrease in percentage bioaccumulation. The rate of metal uptake and the extent were enhanced as the pH increases up to certain pH range. At low pH negligible removal of metals ions noted may be due to the competition between hydrogen and metal ions. With further increase in pH, there is an increase in metal removal, which may be due to the ionization of functional groups and an increase in the negative charge density on the cell surface. At higher alkaline pH values (8 and above), a reduction in the solubility of metals contributes to lower uptake rates.

### Optimization of temperature for metal removal by MTB species

The range of optimal temperature values (30–35°C) were comparable to the range of room temperature that was used when isolating the microorganisms, suggesting that the selection of these isolates might have been influenced not only with the heavy metals but also with the temperature used in the isolation procedure. The temperature of the adsorption medium could be important for energy dependent mechanisms in metal removal by microorganisms. Temperature is known to affect the stability of the cell wall, its configuration and can also cause ionization of chemical moieties. These factors may simultaneously affect the binding sites on isolated fungal and bacterial species causing reduction in heavy metal removal. Energy-independent mechanisms are less likely to be affected by temperature since the processes responsible for removal are largely physicochemical in nature<sup>23</sup>. Bioaccumulation of metal ions MTB species appears to be temperature dependent. Maximum removal of cadmium by *Pseudomonas aeruginosa* JN102340 was observed at 35°C. The temperature of the adsorption medium could be important for energy dependent mechanisms in metal biosorption. Energy-independent mechanisms are less likely to be affected by temperature, since the processes responsible for biosorption seems to be largely physicochemical (electrostatic forces) in nature<sup>24</sup>. A lot of experimental studies had been done on effects of temperature on heavy metal removal by microbes, and the researches were reported on effects of different microbial species and conditions<sup>25, 26</sup>, temperature affects biosorption of heavy metals and bacterial activity as well. To study the effect of temperature on heavy metal removal, we conducted tests with different equilibration temperatures. Initial heavy metal levels are 100 mg L<sup>-1</sup> for all four heavy metals. Experiments was carried out at pH 6.0 for 24 h and the result was shown as Fig. As indicated by Fig 5, when the equilibration temperature ascended from 25°C to 35°C, bacterial biomass and removal of cadmium also increased with temperature and afterwards declined when temperature became

even higher. Therefore, the best temperature for maximum removal of cadmium by *Pseudomonas aeruginosa* JN102340 is 35°C and the removal was 89%.

### Kinetics of cadmium removal and cellular growth of the MTB species

Experiment was conducted to investigate heavy metal removal courses and the result was shown as Fig 6. It was found from the Fig that within 24 hrs *Pseudomonas aeruginosa* JN102340 could remove 90% of cadmium. After 12 hrs, bacterial biomass of *Pseudomonas aeruginosa* JN102340 and removal of cadmium also increased very slightly with time and removal equilibrium. The time-course data for heavy metal removal and cellular growth were observed for each isolate under optimal pH and temperature conditions. When these isolates are applied in removing heavy metal from industrial wastewater, information regarding the effect of growth phase will be important in designing solid (sludge) retention time (SRT) for continuous flow completely stirred (CFCS) bioreactor, which is a general reactor type for wastewater treatment plants<sup>16</sup>. In the MTB isolates, specific metal bioaccumulation (accumulative biomass) increased when cells were in stationary phases. Therefore, expanded SRTs (stationary phase) may be recommended using the *Pseudomonas aeruginosa* JN102340 isolates in removing heavy metals from industrial wastewater however, a non-expanded SRT has to be designed for CFCS bioreactor so that a mid-log phase of cellular growth could be kept in the treatment system. The growth rate during the lag phase was very low because the isolated *Pseudomonas aeruginosa* JN102340 was adapting with the environment. After this stage, the isolates grew in logarithmic form using the nutrients

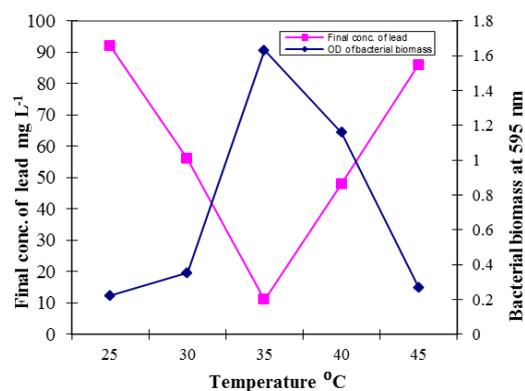
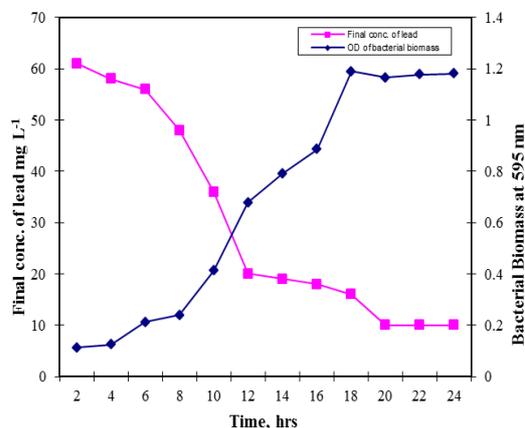


Figure 5. Cellular growth and cadmium removal by *Pseudomonas aeruginosa* in response to various temperatures

### Influence of initial metal concentration

The initial concentration of the cadmium in the solution remarkably influenced the equilibrium uptake. It was noticed that initial concentration increased the sorption of heavy metal as is generally expected due to equilibrium process. This

increase in uptake capacity of the *Pseudomonas aeruginosa* JN102340 with the increase in initial cadmium metal concentration is due to higher availability of metal ions for the sorption.



**Figure 6.** Kinetics of cellular growth and cadmium removal by *Pseudomonas aeruginosa*

Moreover, higher initial concentration provides increased driving force to overcome all mass transfer resistance of metal ions between the aqueous and solid phase resulting in higher probability of collision between metal ions and sorbents. This also results in higher metal uptake 27.

Tolerance implies a large change in sensitivity between sets of organisms to a particular toxicant. Tolerance can be adaptive, constitutive, or induced. Adaptive tolerance is where the organism colonizing a contaminated site is less insensitive than the same species colonizing uncontaminated sites and where this change in sensitivity is caused by the selection of genes that confer enhanced insensitivity. Induced tolerance, for which there is less evidence, is where particular enzymes that cause decreased sensitivity are induced on exposure to metal ions 28.

*Pseudomonas aeruginosa* JN102340 exhibited growth even at higher levels of metal ions and the biomass production decreased with increase in the metal concentration. A significant reduction of mean growth was achieved in all the *Pseudomonas aeruginosa* JN102340 with the increasing concentration of heavy metals in the medium. Konopka et al., (1999) 29 confirmed that the microbial biomass generation was decreased as the concentration of heavy metal increased. This is in agreement with the findings of Hussein et al., (2004) 30 who reported that the total amount of biomass production decreased while increasing the concentration of heavy metals. Bridge et al., (1999) 31 also confirmed that the microorganisms can release a diverse range of specific and nonspecific metal binding compounds in response to high levels of toxic metals which can ameliorate the effect of toxic metals and mediate the uptake process.

The present study evaluated and suggested that the cadmium metal tolerant bacterium *Pseudomonas aeruginosa* JN102340 can effectively remove cadmium in an optimized conditions.

Cadmium metal ion uptake by *Pseudomonas aeruginosa* JN102340 concede to look into process using this biomass for removing metal ions from industrial waste waters.

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