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Heavy Metal Bioremediation Potential of Mangrove Rhizobacteria from Cochin Backwaters, Kerala

Wafha K.A[a], Gayathri S.[b], Baby Divya[c]

[a]Department of Zoology, St. Xavier's College for Women, Aluva, Ernakulam, Kerala

[b]Department of Microbiology, St. Xavier's College for Women, Aluva, Ernakulam, Kerala,

[c]Department of Zoology, St. Xavier's College for Women, Aluva, Ernakulam,

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Corresponding Author:
E-Mail: wafhaka1994@gmail.com

Abstract:

Heavy metal pollution as a result of urbanization and industrialization has posed a serious threat to Cochin backwaters. Microbes possess enormous potential to detoxify heavy metals by the property of biosorption. Microbial biosorption is an efficient, economical, eco-friendly, and convenient technique for remediating heavy metal pollution. In the present study, twenty-three bacterial strains isolated from rhizosphere sediments associated with mangroves of Puthuvypin and Panambukad, Cochin were examined for heavy metal tolerance against Cadmium, Cobalt, Nickel, Copper, and Zinc at different concentrations ranging from 500 ppm to 4000 ppm. About 43.4% and 21.7 % of the isolates showed tolerance to all concentrations of Ni and Cu respectively. However, 43% of the isolates could tolerate Zn and 26 % Co concentrations from 500-2000 ppm. Interestingly, bacterial strain 3PE06 showed multi-metal tolerance against Ni, Cu, and Zn at all concentrations of heavy metals. The isolate 3PE06 showed the highest biosorption for Nickel (90 %), followed by Cadmium (56.8 %), Copper (32.6%), Zinc (30.6%), and Cobalt (12.8%). The molecular identification of bacterial isolate 3PE06 revealed it as *Bacillus cereus*. This investigation has proved that various dominant bacterial isolates from mangrove rhizosphere have metal tolerance ability and the rhizobacterium *Bacillus cereus* has huge potential in the biosorption of heavy metals.

Keywords:*Bacillus, Heavy Metal tolerance, Biosorption, Bacteria, Mangrove, Cochin, Bioremediation*

1. Introduction

Heavy metals are the most serious threat to environment which causes many deleterious effects on mangroves as a result bioaccumulation by anthropogenic factors like mining, oil pollution etc.[1]. The city of

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Cochin being the industrial capital of Kerala, the rapid urbanization showed a drastic decline in the mangrove patches of Ernakulam district especially in Panambukad and Puthuvypin [2]. The effluents discharged from the industries in the banks of the backwaters which accounts to about 260 million on a daily basis has aggravated the decline of mangroves [3,4]. viz., Travancore Cochin Chemicals, Zinc-Alumina ore smelting, Cochin Refineries Ltd, Indian Rare Earths, Fertilizers and Chemicals Travancore Ltd, and Hindustan Insecticides. In Kerala, Cochin backwaters have the characteristic features of wide salinity gradient and varying habitat types such as low lying swamps, tidal creeks and mangrove patches that support diverse flora and fauna. The major mangrove patch of Cochin is distributed along Puthuvypin region towards the north of the development sites. The mangrove cover of the area could be categorized into three, they are moderately dense, interspersed with settlements and under degradation due to athropogenic activitiesactivities [5]. Puthuvypin comprises unique species of mangroves with *Acanthus ilicifolius* and *Excoecariaagallocha* as the dominant species [6].

According to a News Report of "The new indian express", the mangrove cover at Puthuvypin has depleted from 127 hectares to 74 hectares over the last seven years in the area between the LNG Terminal and Goshree Junction, two important landmarks of Cochin. Development of infrastructure and other projects made the mangroves at Puthuvypin among the fastest depleting wetlands [7]. "Deccan Chronicle" also reported that, Panambukad mangrove region is also an issue by anthropogenic activities [8].Chakraborty et al., 2014 in a study examined the bioaccumulation of zinc, copper and lead heavy metals mainly accumulated in various parts of *E. agallocha*when compare to other mangroves as result of these urbanisation and human activities [9].

The mangrove ecosystem act as a niche for various bacteria, fungi, microalgae and invertebrates and it provides habitat for marine organisms to breed, grow, and feed [10]. One of the characteristic features of mangroves is its ability to hold large microbial communities [11] which in turn can generate mutualistic relationships with the mangrove trees. Metals have a significant role in the growth and metabolism of microbes [12]. In spite when the metal concentration goes beyond, the microbes thriving in such polluted areas exhibit a variety of mechanisms to tolerate the heavy metals to reduce the effect of toxicity [12]. Adsorption or Accumulation are the mechanism adopted by the bacteria to remove the heavy metal from the environment [13,14]. Hence, rapid urbanisation and anthropogenic activities have led to an increased occurrence of heavy metals and metal-resistant bacteria [15].

Mangrove ecosystems are vulnerable to heavy metal bioaccumulation and act as metal sinks of coastal areas and thus the microbial population in the mangrove sediments develop multifarious metal tolerance mechanisms to combat metal toxicity. The interactions between bacteria and metal ions are of great interest in potential bioremedial technology. The present study deals with the isolation and screening of heavy metal tolerant rhizobacteria from mangroves of Cochin

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backwaters and its use in bioremediation of heavy metals.

2. Methodology

2.1 Collection of samples

Sediment samples were collected from mangroves of Puthuvypin and Panambukad (*Avicennia sp., Acanthus sp., Bruguiera sp., Rhizophora sp., and Excoecaria sp.*) located in Cochin backwaters. The samples were collected in a sterile container using sterile spatula and immediately brought to the laboratory for further study.

2.2 Isolation and Characterization Bacteria

The samples were weighed as much as 10 grams and then suspended in 90 ml of autoclaved water (ambient water) and homogenized using a shaker. About, 1 ml of the suspension was serially diluted to 10^{-5}. About 100 µl of the samples from dilutions 10^{-3} and 10^{-4} were plated onto nutrient agar plates and incubated at 28°C for 48 hours. After the incubation, the morphological characteristics of bacterial colonies recorded and subcultured in nutrient agar plates.

2.3 Morphological, biochemical and molecular characterization

The colonies grown on nutrient agar plates were examined to know the morphological characters such as size, shape, colour, elevation, margin, pigmentation and recorded. Morphologically different isolates were purified and preserved in 20 % glycerol stock. The isolates were subjected to biochemical identification such as, sugar fermentation tests using glucose, lactose, sucrose, and maltose, IMViC tests, TSI agar test, urease test, nitrate reduction test, mannitol motility test, catalase, oxidase tests, gram staining and motility.

For molecular identification DNA was extracted from each sample using the salting-out method and was amplified using universal 16SrRNA primers (Forward: 5'AGATTTGATCMTGGCTCAG3' Reverse: 5'CGGTTACCTTGTTACGACTT3') with PCR conditions, denaturation (95 °C - 3 min, 94 0C-1 min and 30 sec), annealing (54 °C- 40 sec), and polymerization (72 °C-1 min and 30 sec, 72 0C-10 min) for 30 cycles. Then the amplicons were electrophoresed in a 1% Agarose gel and visualized under UV (Biotech R and D laboratories, Salem, India). The PCR products were sequenced by Sanger's method at EnfysLifesciences Pvt Ltd, India. For molecular identification, a homology comparison of 16S rRNA sequences of four different bacterial strains was performed using NCBI BLAST (Basic Local Alignment Search Tool) http://www.ncbi.nlm.nih.gov/.

2.4 Metal Tolerance analysis by Agar diffusion method

For tolerance study, 24 hour old bacterial cultures swabbed on nutrient agar well plates and 0.1 ml of various concentrations (500 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm, 3500 ppm and 4000 ppm) of Cadmium, Cobalt, Nickel, Copper and Zinc heavy metal solutions to the respective wells. Followed by the incubation at 28°C for 48 hours, the absence of zone that indicates tolerant to that metal recorded.

2.5 Growth curve

For the growth curve, 50 ml of nutrient broth with 500 ppm of different heavy metals (Cadmium, Cobalt, Nickel, Copper and Zinc) on each conical flask were inoculated with 1 ml of culture (25 X 10^{-6} cfu/ ml) and incubated at room temperature for 10 days. Aliquot 4 ml of the culture

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suspension centrifuged at 8000rpm for 10 minutes at an interval of every 24 hours and the pellet dissolved in fresh nutrient broth and record the optical density (OD) at a wavelength of 600 nm using spectrophotometer. At the end of experiment, graph plotted by time on X axis versus optical density on Y axis.

2.6 Screening of isolate for heavy metal biosorption

For biosorption studies, 45 ml Nutrient Broth with 500 ppm of different heavy metals (Cadmium, Cobalt, Nickel, Copper and Zinc) on each conical flask were inoculated with 2ml of bacterial culture (25 X 10^{-6} cfu/ml) and incubated at 28^{0}C for 7 days. After the incubation, the culture centrifuged at 10000 rpm for 10 minutes and the supernatant subjected to Atomic Absorption Spectrophotometer analysis to detect the heavy metal concentration.

The percentage of biosorption of each heavy metal in supernatant was determined by using the following formula.

$$\text{Percentage of biosorption} = \frac{\text{Initial concentration} \times \text{Final concentration}}{\text{Initial concentration}} \times 100$$

3. Results and Discussion

With the help of basic microbiological techniques for the isolation and characterisation of bacteria, a total of 18 dominant bacterial isolates were obtained from the mangrove rhizosphere sediment of Puthuvypin and Panambukadu mangroves. This was followed by screening to study the tolerance towards the heavy metals (Cadmium, Cobalt, Nickel, Copper and Zinc) using agar well diffusion method, ranging from 500 ppm to 4000 ppm. On an average, 43.4%and 21.7 % of the isolates showed tolerance to all concentrations of Ni and Cu respectively. About, 43% of the isolates could tolerate Zn and 26 % Co concentrations from 500-2000 ppm. The tolerance levels of each bacteria on five heavy metals at various concentration is showed in Figure 1.

Interestingly among 18 isolates, 12 bacterial isolates were tolerant at 4000ppm of heavy metals (Figure 2). Then the potent 12 bacterial isolates subjected to biochemical and molecular characterization and were identified as, 3PE06 (*Bacillus cereus*), 1PR10 (*Bacillus albus*), 1MA27 (*Bacillus paramycoides*), 1MB07 (*Bacillus cereus*), 3PB09 (*Rossellomorea aquimaris*), 3MM05 (Bacillus cereus), 3MR02 (*Bacillus velezensis*), 1BM22 (*Aeromonashydrophila*), 1MA03 (*Klebsiella pneumoniae*), 1PB07 (*Bacillus thuringiensis*), 1MR06 (*Staphylococcus saprophyticus*), and 1PE12 (*lysinibacillus macrolides*). They belonged to the mangrove rhizosphere of *Exceocaria sp., Acanthus sp., Brugeria sp., Rhizophora sp., and Avicennia sp.*. Nucleotide Gene Bank accession number for strain 3PE06, 1PR10, 1MB07, 3PB09, 3MR02, 1MA03, 1PB07, 1MR06, and 1PE12 are OQ439620, OQ439616, OQ439611, OQ439619, OQ439618, OQ439610, OQ439615, and OQ443075 respectively.

The isolate 3PE06 (*Bacillus cereus*) was identified with highest tolerance levels among 18 isolates with score value of 27 out of 40(Figure 2). It also showed multiple metal tolerance to three heavy metals Nickel, Zinc and Copper at 4000 ppm.Similarly, Sahoo and Goli (2020) reported *Bacillus pumilus* as multi-metal tolerant halophilic bacteria against Cd2+, Cu2+, Fe3+, and Ba2+ from mangroves of

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Karnataka [16].Nomani et al., (2019), reported a bacterium MK02 as multimetal tolerant against metals Zinc, Cadmium, Iron and Lead up to 1000 mg/L from Mirza Khan Pond of Darbhanga, India [17].

Growth pattern of the isolate 3PE06 in the media amended with five heavy metals (500 ppm) under study , showed that there wasgrowth inhibition in the presence of heavy metals in the order of Cd >Zi> Cu > Ni > Co. The control (nutrient broth without heavy metals) showeddrastic increase in their growth when compared to the experiment with heavy metals (500 ppm) and entered to stationary phase from 2nd day onwards. The growth of bacterial isolate 3PE06 in Cadmium amended broth showed sudden increase compared to other heavy metals and became consistent from 5th day of incubation onwards. While the growth of bacterial isolate 3PE06 in broth amended with other heavy metals (Nickel, Copper, Zinc and Cobalt) showedgradual increase and then became consistent.

The biosorption capacity of the bacterial isolate 3PE06 against heavy metals (Nickel, Copper, Zinc, Cobalt and Copper at 500 ppm) showed that there was a decrease in the metal concentration. Concentration of Nickel decreased from 500 ppm to 50 ppm, Cadmium to 216 ppm, Copper to 337 ppm, Zinc to 345 ppm and Cobalt to 436 ppm. The bacterial isolate 3PE06 had high ability to remove Nickel compared to other heavy metals tested. The heavy metal biosorption efficiency of isolate 3PE06 was in the order Ni > Cd > Cu > Zn > Co. The percentage of biosorption was also calculated based on the initial and final concentrations of heavy metals. The percentage of biosorption of the bacterial isolate is 90 % for Nickel, while it is 56.8 % for Cadmium, 32.6 % for Copper, 31 % for Zinc and 12.8 % for Cobalt. In a study by Rexet al., 2019, 66 bacterial isolates showed Nickel tolerance at 6000 ppm and 16 isolates at 10,000 ppm of nickel from Bayto River in Santa Cruz. [18].Pandit et al., 2013 reported six bacterial strains tolerant to Nickel at 200 ppm from industrial effluents, AmalakhadiAnkleshwar, Gujarat [19]. On the other hand, Nickel is an important trace element act as cofactor by several well-characterized microbial enzymes (Nickel superoxide dismutase, acireductonedioxygenase, Iron hydrogenase, methyl coenzyme M reductase etc.) [20]. Multi metal tolerant bacteria *Bacillus cereus* was reported to be isolated from bauxite mines, Kolli hills, Tamil Nadu and their efficiency was found to be 91.98% (Cu), and 77.44% (Zn) respectively [21].

The biosorption ability was compared with growth curve of the isolate. The growth of the isolate in Cadmium amended medium showed the highest growth with 56% bisorption ability (Figure 3). This denoted that along with the increase in the cell number of bacteria absorbance ability increases. However in the other heavy metals (Zinc, Copper and Cobalt)biosorbtion ability decreases corresponding to their cell number. Interestingly, the highest biosorption capability of 90% was seen in Nickel though cell number was less. For the reason that, the concentration microbial cell proportional to metal uptake which restricts the access to binding site for metal ions [22-23].This showed that 3PE06 isolate have high efficiency in nickel biosorption even at small concentration of bacterial cells.

The bacterial strain 3PE06 (*Bacillus cereus*) was isolated from sediment samples of

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Puthuvypin mangroves. If an areas of study were heavily polluted with heavy metals, the bacterial strains isolated from those areas will tolerant to that heavy metals [24]. Many species of *Bacillus* utilized globally for many applications[25]. Among which extensively investigated role is the mitigation of heavy metals from contaminated environments via biosorption and bioaccumulation

Here *Bacillus cereus* showed high tolerance to Nickel and also high biosorption percentage against nickel (90%). Heavy metal pollution is one of the major concern in these days of increasing urbanization and industrialization. Cheap and efficient methods such as bioremediation using microorganisms can effectively bioremediate these heavy metals. So *Bacillus cereus* isolated from the mangrove sediment in this study can be used as an effective agent for bioremediation of Nickel.

4. Conclusion

Coastal areas are highly vulnerable to the pollution by heavy metals as a consequence of anthropogenic activities. Heavy metals are exceptionally stable and represent recalcitrant pollutants that greatly affect the aquatic environments. Mangroves are bioremediators for contaminated environments. They act as a natural filter between land and water thus retaining the pollutants such as heavy metals. Hence the microbial communities held by the mangrove sediments have developed novel metal resistance mechanisms.In order to overcome the metal pollution, bioremediation is one of effective methods that can be used. From the present study *Bacillus cereus* possess multimetal tolerance against Nickel, Zinc and Copper. It is also efficient for biosorption of Nickel (90%) than other heavy metals (Cadmium,Zinc,Copper and Cobalt) tested. The bacteria *Bacillus cereus* isolated from mangrove sediment have an efficient Nickel biosorption capacity, which make possible its pottential use in heavy metal bioremediation. Further experimental studies are required to exploit *Bacillus* cereus for heavy metal bioremediation.

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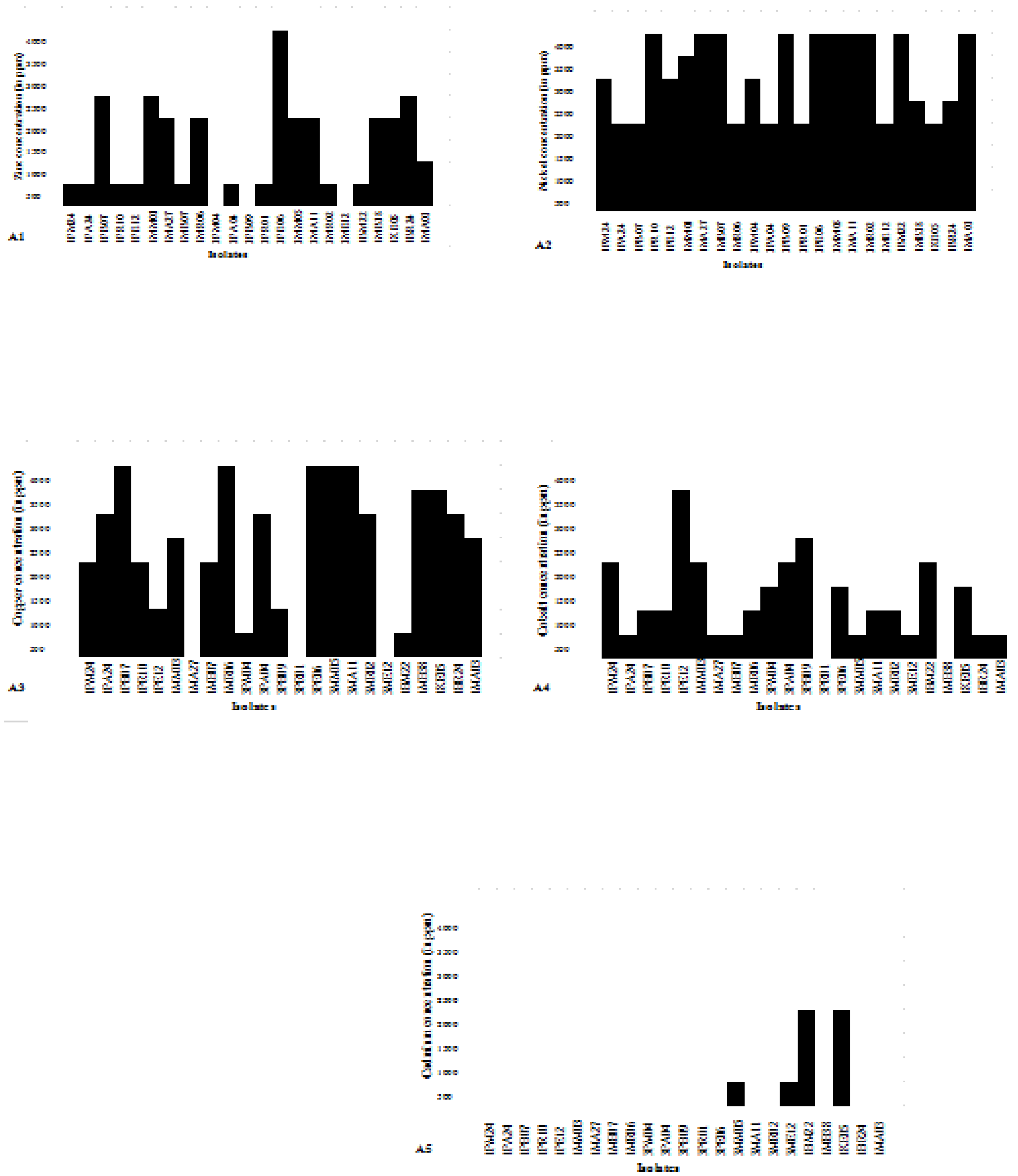

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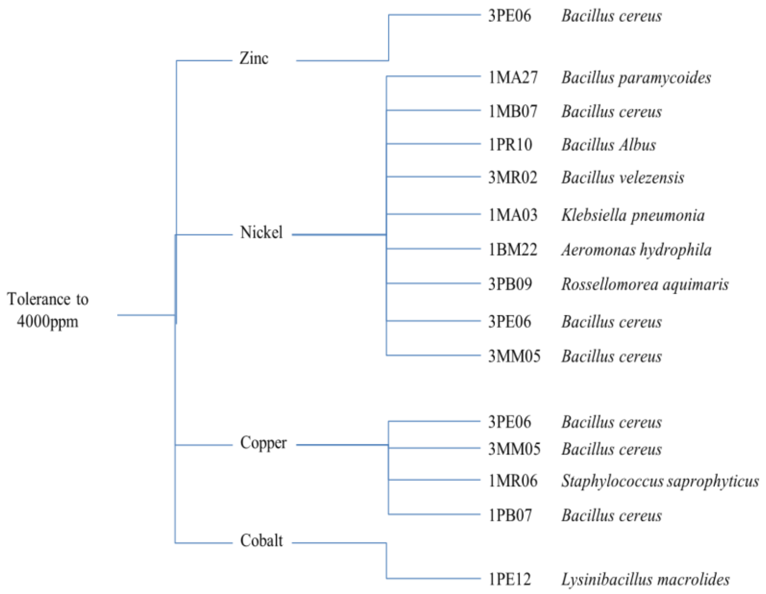

Figure 2. Bacterial Isolates that tolerant to 4000ppm of Heavy metals: Zinc, Nickel, Copper and cobalt

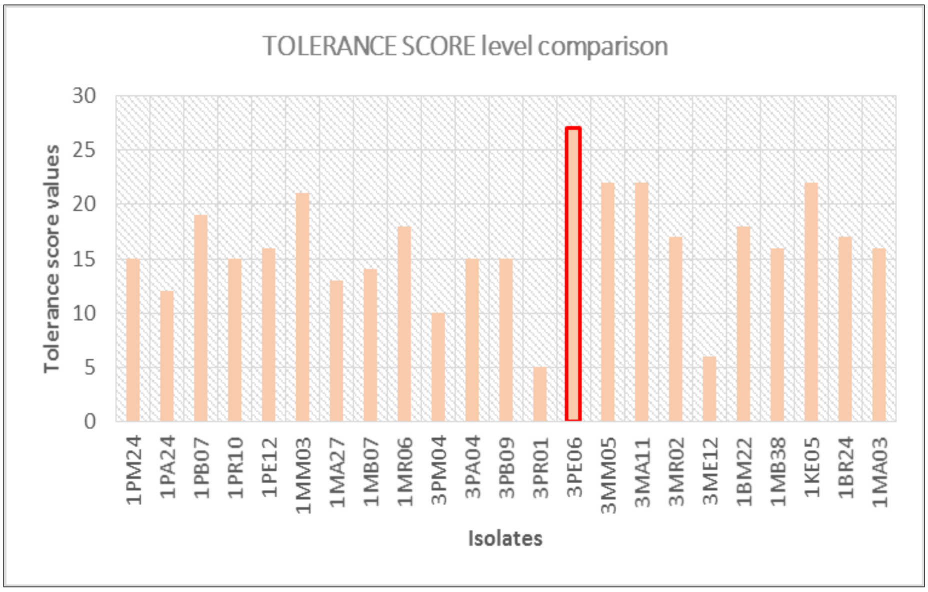

Figure 3 Heavy metal tolerance score level of bacterial isolates

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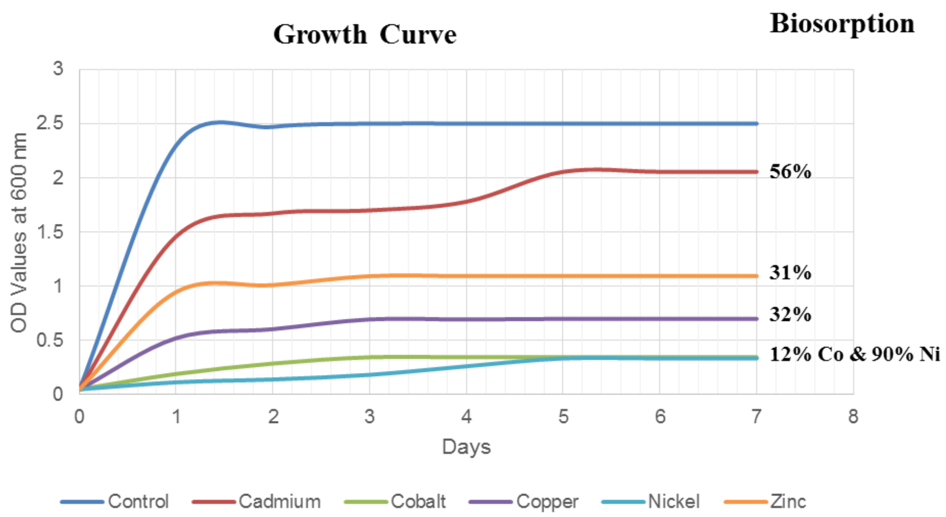

Figure 4. Growth curve of 3PE06 (*Bacillus cereus*) on each heavy metals (Cadmium, Cobalt, Nickel, Copper and Zinc) at 500ppm of concentration for 7 days and their biosorption ability on 7th day (in percentage)

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