Indian Journal of Science and Technology, Vol 9(47), DOI: 10.17485/ijst/2016/v9i47/106911, December 2016
ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

Comparative Study of Heavy Metal Bioremediation in Soil by Bacillus Subtilis and Saccharomyces Cerevisiae

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Abstract

Background/Objectives: The objective of this present investigation is focused to determine metal tolerating capacity and bio-sorption capacity of two microbial isolates i.e. Saccharomyces cerevisiae and Bacillus subtilis. **Method/Statistical Analysis**: Bioremediation is a process which involves all methods and action for reduction of environmental pollutants with the help of biological entities. Microbial cultures were maintained and scaled up using sterile techniques. Metal tolerance test was done by using MIC test. **Findings:** Presence of heavy metals in the mentioned species was confirmed by Atomic Absorption Spectroscopy. The results of biosorption studies explains that B. subtilis was most efficient in the removal of Hg2+ and Cd 2+ from the contaminated soil. After 5 days Bacillus subtilis immersed 75.76% while S. cerevisiae immersed 69.56% Cd 2+ from contaminated soil. And even after 5 days Saccharomyces cerevisiae was able to accumulate 19.5 % Hg2+ while 29.9 % Hg2+ was absorbed by Bacillus subtilis from contaminated soil. Saccharomyces cerevisiae was able to accumulate 92.68% of Cd 2+ and 90.48% of Hg2+ from contaminated soil after 21 days of incubation. **Applications/ Improvements:** Metal remediation through common physio-chemical techniques is expensive and unsuitable in treating large contaminated area effectively. Bioremediation offers a promising means to reclaim such contaminated soil in an economical and eco-friendly way. Furthermore, we are working on remediation of heavy metals in soil and water through Nanomaterials which is sought to be more promising technique for remediation in years to come.

Keywords: Bacillus Subtilis, Bioremediation, Biosorption, Heavy metals, Saccharomyces Cerevisiae, Soil Pollution

1. Introduction

Bioremediation is a process which involves all methods and action for reduction of environmental pollutants with the help of biological transformation or naturally occurring process which involves uses of fungi, microorganisms, green plants or their enzymes to return natural environment altered by contaminants to its original condition. There are so many approaches that have been implemented for the bioremediation of heavy metals, organic toxicity, and other toxicity. Heavy metals are a big threat for the environment. Heavy metal contamination means a condition having abnormally high levels of toxic metals in the environment. Heavy metals are penetrating and silent killers. Both the rapidly expanding industrial and domestic activities is a result of addition of heavy metals to environment[1,2]. Process of bioremediation is of two types, In-situ and other one is Ex-situ, In In-situ type remediation is done at the site of contamination. Further In-situ bioremediation can be classified into three types that are bio-stimulation, bio-attenuation, bio-augmentation. Bio-stimulation is process in which chemical stimulus is given in environment to increase the rate of bioremediation, chemicals may be some nutrients like Nitrogen, Phosphorous etc. in controlled concentration[3]. Bio-stimulation is ejecting methane in Trichloroethylene-contaminated groundwater which was demonstrated as small field experiment and which

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was funded by environmental agency and by ministry of international industry and trade. Bioattenuation is another type of In-situ bioremediation in which monitoring on the natural process of degradation of contaminant is done to insure the decrease in the concentration of pollutant, this method is widely used in USA for cleanup of petroleum contamination from ground water and soil[4]. Bio-augmentation is third type of In-situ bioremediation in which micro-organism is used to remove contamination, this method is most effective and practiced in nowadays frequently. Historically, first bioremediation was reported in 1972, for the removal of sun oil pipeline spill in Almer Pennsylvania. Bioremediation became a popular method for the removal of diesel, gasoline, and other easily degraded petroleum product. (National Research Council 47). Micro-organisms have a great ability to decrease the amount of the heavy metal from environment[5–8]. Micro-organism remediate heavy metal by different mechanism like reduction, alkylation, and precipitation[9–15]. The *Saccharomyces spies* is considered as one of the best studied and commercially explored of all micro-organisms. Ability of *Saccharomyces cerevisiae* to reduce the heavy metal contamination from environment is very good and can be applied as as tool for bioremediation[16–21]. *Saccharomyces cerevisiae* is used in large scale fermentation. Annually production of *Saccharomyces cerevisiae* is millions of tons, thus it is proved as cheap biomass[22]. Bacteria also considered as very good in heavy metal remediation[23,24]. There are number of species of bacteria reported for the bio remediation of heavy metals[25–27]. *Bacillus* is a genus of Gram-positive, rod-shaped bacteria and they are member of the phylum Formicate *Saccharomyces Bacillus* species are facultative anaerobes, and provide a positive result against enzyme catalase test[28]. It was found that *Bacillus subtilis* can be grown in presence of a wide range of metals namely zinc, copper, and iron, so it can be considered for the remediation of heavy metals[29]. Present work is done to study the bioremediation of Cd and Hg using *Bacillus* subtilis and *Saccharomyces* cerevisiae, also to compare the efficiency of both the micro-organism along with tolerance. Result was in favour of bacterial species and suggested that *Bacillus* subtilis is holds more potential in remediation of Hg and Cd.

2. Material and Method

To carry out present study Agar Powder (Moly Chem., India), Dextrose (Moly Chem., India), Cadmium Chloride (CDH Pvt. Ltd., India), Mercuric Chloride (CDH Pvt. Ltd., India), Nickel Chloride (CDH Pvt. Ltd., India), Nutrient Agar (Titan Biotech Ltd., India), Peptone Thomas (Baker and Chemicals, India), Potassium Chromate (CDH Pvt. Ltd. CDH Pvt. Ltd., India), Yeast extract (CDH Pvt. Ltd., India) chemicals were purchased. Spectrophotometer-Model LT-31(Labtronics), Laminar Air Flow-Yorko Scientific Industries, Incubator- Model C1-168(REMI), Atomic Absorption Spectrophotometer- Model No. AA240FS (VARIAN) were also used. *Saccharomyces cerevisiae (*MTCC 464), *Bacillus subtilis (*MTCC 10619) were taken from MTCC Chandigarh.

Microbial cultures were maintained and scaled up using sterile techniques. Metal tolerance test was done by using MIC test. Soil sample was taken from university garden and then sterilized. Soil was contaminated manually by using heavy metals salts separately. Contaminated soil was packed in soil reactor and incubated at room temperature. Soil sample was taken out from the soil reactor at different interval of time and analysed by AAS MIC test of *saccharomyces cerevisiae* and *Bacillus subtilis*: Petri plates, test tubes, laminar hood, incubator, Yeast extract-peptone-dextrose growth medium (YEPD) for *Saccharomyces cerevisiae* and Nutrient agar for bacteria, Agar-Agar powder, 70% ethanol, distilled water, Heavy metal salts (Hg, Cd), Inoculum of microorganism.10ml of sterile YEPD media (or Nutrient agar for bacteria) was taken into the sterile test tubes, then 6000 ppm stock solutions of 2 heavy metals (Hg, Cd) were prepared in different flasks by using the salts of $HgCl_2$ and $CdCl_2$ respectively. Different volume of stock solution was added to the media so that the final concentration of heavy metal would be 100ppm, 200ppm, 300ppm, 400ppm and 500ppm.Using sterile technique 1ml inoculum was added to each test tube. Test tubes were kept in shaker incubator (37^0C for *Bacillus subtilis,* and 28^oC for Yeast) for 24hrs. Next day YEPD agar media (or Nutrient agar for bacteria) was prepared.1 ml inoculum was taken from the test tube and streaking was done on the YEPD media or nutrient agar petri plate. Saccharomyces Petri plates were incubated (37^0C for *BACILLUS subtilis,* and 28^oC for Yeast) for 24hrs and observed[30].

2.1 Bio-sorption Experiment

Soil was collected from average depths of 4 cm, 10 cm, and 20 cm from Lovely Professional University garden. The collection method consisted of using an open core and sectioning off the appropriate depth Saccharomyces

After collection the soil was stored in polythene bags at room temperature.5kg of both the top soil and the deep soil was dried at 100^{0}C for 24 hours and autoclaved for 30 minutes at 124^{o}C and 15 psi in Electric Pressure Steam Sterilizer to kill the indigenous bacteria. These soil samples were then subsequently used for the checking the amount of bioremediation to be done by microorganism.

2.2 Bio-sorption in Miniature Soil Bioreactor (Figure 1)

Conical flasks, Sterile and dry soil, Heavy metal (Hg, Cd), Inoculum of microorganism, YEPD media, AAS.

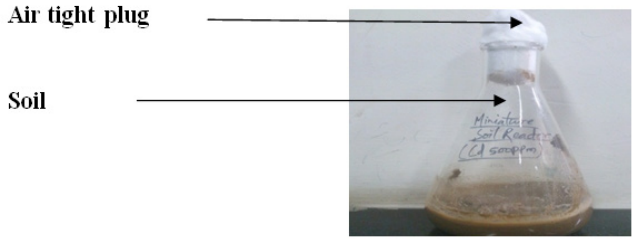

Figure 1. Miniature soil bioreactor.

Soil sample was collected from the University garden below 10 cm of the surface.250gm of soil sample was taken in 2 different 500ml conical flasks and contaminated with heavy metals (mercury and cadmium) separately having initial concentration of 500ppm.Contaminated sample was inoculated with 10 ml of *Saccharomyces cerevisiae* culture and 50 ml YEPD media was added. Contaminated sample was inoculated with 10 ml of *Saccharomyces cerevisiae* culture and 50 ml YEPD media was added. The setup was incubated at room temperature for 5 days and the pH of soil was 7.2. After 5 days, the sample was taken out and analysed in AAS.

2.3 Bio-sorption using Soil Reactor (Figure 2)

The reactors were inclusive of 4 inch PVC pipe. The top chamber was of 25 cm height and 10 cm diameter with 2 sample collection ports located at 10 and 15 cm from the top. The bottom chamber was 5cm in height and 10cm in diameter which was used as leachate collector. Filter media consisting of gravel, glass beads, and sand were packed to a height of 4.5 cm from the bottom of the top compartment over which 3 kg of the contaminated soil was loosely packed.

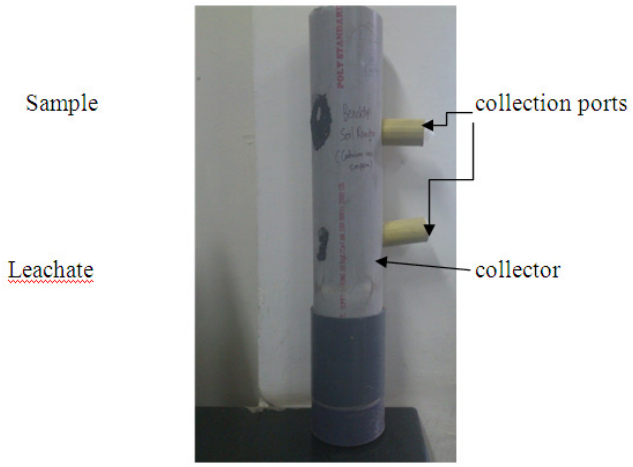

Figure 2. Soil reactor.

Sterile and dry soil, Heavy metal (Hg, Cd), Inoculum of microorganism, YEPD media (or nutrient broth), AAS Soil sample was collected from the University garden below 10 cm of the surface. The samples were dried in atmospheric condition for 48hrs to remove the solvent (water) and then autoclaved.3 kg of soil was taken in 2 different tray Saccharomyces One sample was contaminated by using Mercuric chloride and another by using Cadmium chloride both having initial concentration of 500ppm. For soil experiments cultures were grown at 30^{0}C at pH 5.5 in a liquid medium. A 24 hrs old culture of microorganism was taken and inoculated in the soil samples of various metal concentrations. The inoculation was done by spraying 30 ml of the pre-cultured liquid media in the soil samples. The contaminated soil was packed in soil reactor and incubated at room temperature. The soil sample was taken out in inconsistent interval of days and pH was mesured and concentration of heavy metals were estimated by AAS [31].

3. Results and Discussions

3.1 MIC (Minimal Inhibitory Concentration)

3.1.1 MIC test of Saccharomyces cerevisiae

After the incubation time of 24 hours' growth was observed in the presence of mercury up to 400ppm, but at 500ppm growth was inhibited (Figure 3). Similarly, for

Cd growth was repressed completely at concentration of 400ppm of cadmium ions (Figure 4).

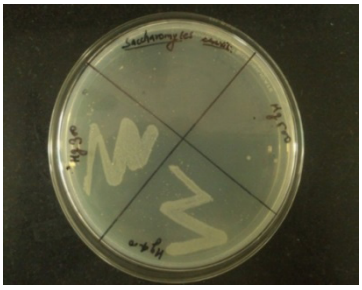

Figure 3. MIC test of *Saccharomyces Cerevisiae* (Hg)

Figure 4. MIC test of Saccharomyces *cerevisiae* (Cd)

From the above results, it was seen that the growth of *Saccharomyces cerevisiae* was greatly decreased in the higher concentration. *Saccharomyces cerevisiae* can tolerate the presence of certain heavy metals up to 500 ppm. This indicates that *S. cerevisiae* has great possibility to utilize heavy metals and reduce their toxicity level.

3.1.2 MIC test of Bacillus subtilis

After the 24 hours' incubation, the petri plates were observed, *B. subtilis* growth was decreases as the concentration of heavy metal was increased. The growth was completely prohibited in the presence of mercury at 500ppm (Figure 5). Similarly, growth was prohibited by 500ppm of Cd metal (Figure 6).

Figure 5. MIC test of *Bacillus subtilis* (Mercury).

Figure 6. MIC test of *Bacillus subtilis* (Cadmium)

The inhibition of the growth of *Bacillus subtilis* was observed in higher concentration of heavy metals. This indicates *B. subtilis* has higher capacity than *S. cerevisiae* to absorb metal ions (Table 1) (Figure 1).

Table 1. Minimal Inhibitory Concentrations (MICs) in ppm of the divalent metal ions during growth *Saccharomyces cerevisiae* and *Bacillus* subtilis cultured in broth and agar medium

Metal Ions	*Saccharomyces cerevisiae*		*Bacillus subtilis*	
	Broth	**Agar**	**Broth**	**Agar**
Hg^{2+}	200	300	400	500
Cd^{2+}	400	300	300	500

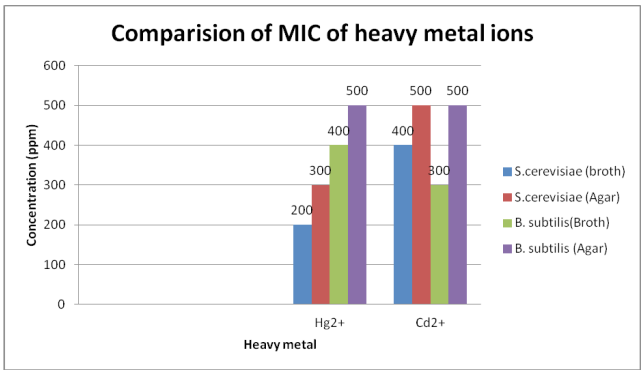

Figure 7. Comparison of Minimal inhibitory concentrations (MICs) in ppm of the divalent metal ions during growth *Saccharomyces cerevisiae* and *Bacillus subtilis* cultured in broth and agar medium.

3.2 AAS Analysis

3.2.1 Bio-sorption in Miniature Soil Bioreactor

Bio sorption of Cd and Hg was studied and it was found that there was a significant decrease in concentration of

Table 2. Results of AAS of contaminated soil inoculated with *Saccharomyces cerevisiae*

Metal	Mass of sample (mg)	Initial concentration (ppm)	Concentration after 5 days (ppm)	Mean value (ppm)
Cadmium	.5	500	134.4	132.2
	.5	500	130.00	
Mercury	.5	500	405.5	402.5
	.5	500	398.5	

Table 3. Results of AAS of contaminated soil inoculated with *Bacillus subtilis*

Metal	Mass of sample (mg)	Initial concentration (ppm)	Concentration after 5 days(ppm)	Mean value (ppm)
Cadmium	.5	500	120.4	121.2
	.5	500	122.0	
Mercury	.5	500	348	350.5
	.5	500	351	

heavy metal. Result was characterised by AAS (atomic absorbance spectroscopy) (Figure 2).

Above results states that the accumulation of cadmium metal was higher than that of mercury by *Saccharomyces cerevisiae*. 69.56 % cadmium was aggregation by *Saccharomyces cerevisiae* in 5 days of incubation period. This indicates that the *Saccharomyces cerevisiae* cells can easily utilise cadmium for their metabolic process and degrade them. Only 19.5 % mercury was aggregation by *Saccharomyces cerevisiae* in 5 days (Table 2). This indicates that *Saccharomyces cerevisiae* poorly accumulates the mercury from soil. The reason for this poor performance was because of the death of cells by Hg toxicity or less utilization of Hg metals by the cells for their metabolism.

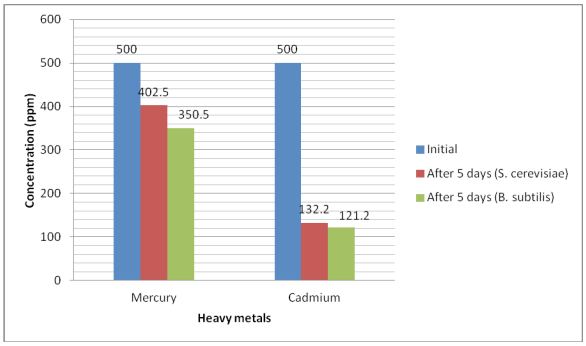

Figure 8. Analysis of biosorption.

The results indicate that the accumulation of cadmium metal was 75.76% after 5 days and that of mercury was 29.9%. (Table 3). Cadmium metal was highly absorbed and mercury was poorly absorbed by *Bacillus subtilis*. The toxicity of mercury seemed to be more than that of cadmium.

Table 4. Concentration of heavy metals during biosorption experiment

Heavy metals	Initial Concentration (ppm)	Final concentration (ppm)		
		7days	14 days	21 days
Cadmium	500	130.5	52.2	36.6
Mercury	500	395.5	152.37	47.57

The bioremediation capacity of *Saccharomyces cerevisiae* is nearly equal for mercury and cadmium. After 21 day 36.6ppm of cadmium metal and 47.57ppm of mercury metal was present in soil sample. 92.68% of cadmium was aggregated by *Saccharomyces cerevisiae* after 21 day of incubation. Alike 90.48 % of mercury was aggregated by the *Saccharomyces cerevisiae* after same period of incubation. The accumulation of cadmium was rapid in first 7 days i.e. around 73.9% while only 20.9 % mercury was aggregated. In next 7 day (14 day of incubation) (Table 4) the accumulation of mercury was rapid while the accumulation of cadmium was slow (Figure 3). The accumulation increased by nearly 2 times for mercury but the accumulation rate decreased for cadmium. Metal ion uptakes in yeast is known to involve and initial rapid biosorption of metal ion to negatively charge sites on the cell wall followed by a slower, energy-dependent entry into the cell.

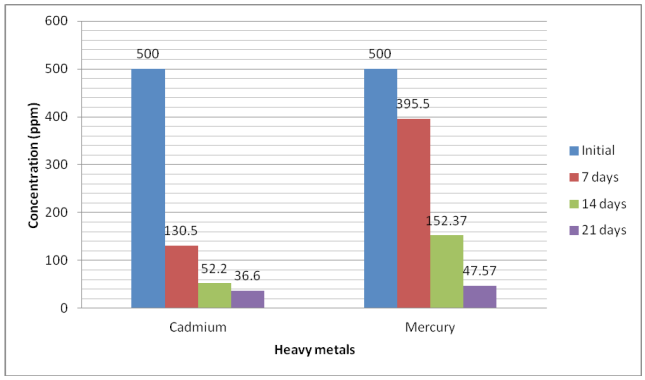

Figure 9. Analysis of contaminated soil inoculated with *Saccharomyces cerevisiae.*

Saccharomyces cerevisiae absorbed 69.56% cadmium while *Bacillus subtilis* absorbed 75.76%. Both microorganisms showed high potential of cadmium absorption. The result was quite low in the case of mercury ion Saccharomyces. *Saccharomyces cerevisiae* was able to accumulate only 19.5 % of mercury while *Bacillus subtilis* was able to absorb 29.9 % of mercury ion Saccharomyces This indicates that *Bacillus subtilis* uses high amount of mercury ions for its metabolism and accumulates mercury in high rate Saccharomyces.

4. Conclusion

This study was focused to determine metal tolerating capacity and bio-sorption capacity of two microbial isolates i.e. *S cerevisiae* and *Bacillus subtilis* the results of biosorption studies demonstrated that Bacillus subtilis was most effective in the removal of Hg^{2+} and Cd^{2+} from the contaminated soil. *Bacillus subtilis* absorbed 75.76% while *Saccharomyces cerevisiae* absorbed 69.56% Cd^{2+} from contaminated soil after 5 days. *Saccharomyces cerevisiae* was able to accumulate 19.5 % while *Bacillus subtilis* was able to absorb 29.9 % Hg^{2+} from contaminated soil after 5 days. After 21 day of incubation *Saccharomyces cerevisiae* was able to accumulate 92.68% of Cd^{2+} and 90.48% of Hg^{2+} from contaminated soil.

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