Copper accumulation potential of bacterial isolates recovered from Bhanpur landfill, area

R K Tenguria[1] Ragini Gothalwal[1] & Yogeshwari Yadav[2]

Division of Microbiology, Department of Botany, Govt. M. V. M., Bhopal

Department of Biotechnology Barkatullah, University, Bhopal, M.P. – 462026

ABSTRACT

Soil pollution by the heavy metals is one of the most significant environmental problems and has its negative impact on human health, agriculture and needs to be remediated by biological approach. Biosorption is a process that utilizes inexpensive biomass to sequester toxic heavy metal, which is mediated by Extracellular and Intracellular sequestration. This approach is particularly useful for the removal of contaminants from landfill site. The aim of this study was to screen the bacterial isolates for their potency to accumulate heavy metal (Cu^{2+}). Bacterial isolates (n=49) were isolated from soil collected from different zone of Bhanpur landfill area, Bhopal (M.P.) and screened for their capacity to tolerate in the presence of different concentration (100 to 700 µg/ml) of Cu^{2+}. All the isolates showed tolerance against Cu^{2+} (100 µg-ml). However the most promising results appeared in isolates CRSY3 and CRSY4. The strain were identified as on the basis of morphological and biochemical characterization *Bacillus sp* and *Pseudomonas sp.* In addition, bioaccumulation potential of bacterial isolates and their consortium were also determined by atomic absorption spectrophotometer (AAS). The capability of bacteria's alone or consortia (*Bacillus sp* + *Pseudomonas sp*) displayed maximum accumulation (51.2%) after 72 hrs compared to both cultures. The study revealed metal accumulating bacteria with metal removal ability which could further be utilized as a potential tool for detoxification of the polluted sites.

Key words: Soil, pollution, metal, Bisorption,Bacteria

INTRODUCTION

Soil is natural resource which plays vital role in the growth of microbes useful for the nutrient cycling and is essential for plant growth, nourishment and crop production. Municipal Solid Waste Management (MSW) is a real challenge for developing countries due to rapid urbanization and lack of public awareness (Prasad *et al*., 2015). In India, MSW is mostly food wastage and the average production rate is 300-600 gm/capita which is disposed on land without any scientific method (Joshi *et al*., 2016). Therefore, open dumps causing soil pollution which makes soil unfit for irrigation purposes and reduces crop yield.

Soil pollution by the heavy metals is one of the most significant environmental problems has its negative impact on human health and agriculture. Copper is metallic chemical element with atomic number 26. Environmental contamination due to copper is caused by mining, printed circuits, metallurgical, fiber production, pipe corrosion, metal plating, paper and pulp, petroleum refining and wood preserving industries (Barrell *et al.*, 1975). Agricultural sources such as fertilizers, fungicidal sprays and animal wastes also lead to water pollution due to copper. In some instances, exposure to copper has resulted in jaundice and enlarged liver. It is suspected to be responsible for one form of metal fume fever (Wagoner e*t al*., 1976).

Among all various sources, both living and inactivated biomass of bacteria exhibit interesting metal binding capabilities (Veglio, 1997). In general, two mechanisms have been proposed for heavy metal tolerance in bacteria. In extracellular mechanisms, different organic molecule such as oxalic acid and in particular di and tricarboxylic acids that do not belong to the matrix of the cell wall are excreted by the bacteria cell to chelate metal ions and binds onto cell wall component (Green and Clausen, 2003). In the intracellular mechanism, metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into vacuolar compartment.

This study was conducted with an aim to obtain metal tolerant bacteria with metal removal ability which could further be utilized as a potential tool for detoxification of the polluted sites.

MATERIALS AND METHODS

Collection of samples

Composite soil samples (n=4) were collected from the Bhanpur ladfill area from differen zones i.s.core zone which is closest to municipal solid waste activity (CRS1), buffer zone which is 2 km away from the MSW activity (BFS2), midpheriphery zone, 4 km away from the MSW activity (MPS3) and surrounding zone, 6 km away from the MSW activity (SRS4) from study site into clean polythene air-tight bags to prevent any further changes in moisture and volatile matter. The soil samples were brought to the laboratory was air- dried and the lumps of the air dried samples were broken down. The samples were sieved using a 2mm sieve and analyzed for pH, electric conductivity (EC) and metal (Cu^{2+}) concentration in sample.

Determination of soil pH, electrical conductivity and metal (Cu^{2+}) concentration in sample

The pH was measured using pH meter (Kalra, 1995) with fewer modifications. The conductivity of each sample was recorded using a conductivity meter (APHA, 2006). Total amount of metal (Cu^{2+}) present in each sample was determined by using a Perkin Elmer Analyst 800 Atomic Absorption Spectrophotometer fitted with a hollow cathode lamp at 324.9 nm. (Zhelijazkov and Nielson (1996)) with some modification.

Isolation of copper tolerant bacteria

Bacterial isolates were isolated by using standard isolation technique employing spread plate method on Nutrient Agar Medium in triplicate with containing 25 μg^{-l} Cu^{2+} as $CuSO_4.5H_2O$ and control plates were set up without the metals (Verma and Bisen, 1996). After incubation total numbers of bacterial isolates were counted and the percentage of bacteria tolerant to Cu^{2+} was calculated as follows. (Prescott and Harley, 2002).

% Metal tolerant Bacteria = Metal tolerant bacteria /Total bacteria x 100

Evalution of Bacterial tolerance to heavy metals

Bacterial isolates (n = 49) were screened for tolerance to copper on NAM using agar dilution method (Malik and Jaiswal (2000). NAM amended with different concentrations of Cu^{2+} (100 to 700 μg^{-ml}) were used. Each isolate was streaked individually on petridishes containing media and then incubated at 37°C ± 2 for 72 hr. The highest concentration of the metal ions beyond which no visible growth occurred was recorded as the maximum tolerable concentration (MTC) in which bacterial isolates were able to grow (Banerjee *et al*., 2015).

Characterization of Bacterial isolates

Isolated colony of purified strains grown on NAM were observed and data were recorded for the size, shape, color, elevation and margins. In order to determine the cellular morphology, bacterial colonies were gram stained using standard procedure. Slides were observed under a light microscope (oil immersion at 100 X magnification) to determine the shape, type and arrangement of cells. The biochemical test were included as citrate test , urease test , amylase test, hydrogen sulpide production test, gelatin test, catalase test, TSI and MR test (Bisen and Verma, 1996; Sherman and Cappuccino, 2006).

Bioaccumulation efficacy of copper tolerant isolate

Selected isolates were determined for copper accumulation in a M-9 minimal salt medium supplemented with 100 μg^{-ml} of Cu^{2+}. The ability of the isolates to accumulate metal ion (Cu^{2+}) was determined by inoculating 1 ml of the exponential phase of bacterial culture (10^6 to 10^7 cells/ml) in to 250 ml of sterilize flask with 99 ml of M-9 minimal medium and incubated on shaker at 100 rpm at 35 ± 2° C for 96 hrs. After that bacterial dry weight was determined by harvesting the cells by centrifugation at 5,000 rpm for 20 min at 4°C. Harvested cells were washed twice with copper free phosphate buffer. Each of the cell pellets and supernatant was digested with HNO_3 at 100°C for measuring the ability of microorganisms in accumulating and biosorbing copper respectively. The copper content was determined by using an Atomic Absorption Spectrophotometer at 324.9 nm. Copper biosorption were calculated as the difference in total heavy metals added to the medium and remaining total heavy metals in the medium after bacterial growth. All the experiments were done in triplicates to ascertain the accuracy of the results (Aka *et al*., 2017).

Results and Discussion

Soil characteristics

Soil pH, conductivity and presence of nutrients are important parameters that strongly influence the chemical behavior of metal ions in environment. They have a direct/indirect effect on the solubility and mobility of metal ions including their potential to form chelates with other soil constituent.(Jan *et al*., 2013).

Physicochemical properties of the soil sample showed. pH value in range of 4.73 to 6.79 i.e., acidic to slightly neutral whereas, the electrical conductivity (EC) ranged from 1.0 to 1.9 μS^{-cm}. Heavy metals are typically released by acidic pH. Usually this heavy metal was found moderate concentration levels in municipal landfill site (Jensen *et al.*, 1999), due to disposal of bottle caps, blades, and pharmaceuticals, galvanizing, paints, pigments, insecticides and cosmetics along with

garbage etc (Barrell *et al.*, 1975). Heavy metal concentration in the soil sample indicates the appreciable contamination of the soil by leachate migration. This is indicated that the migration and distribution of the contaminants are still localized and not diffused with a wide area. However, these pollutant species continuously migrate and percolate through the soil strata and after certain period of time might contaminate the groundwater system if no action is taken to prevent this phenomenon (Table 1).

Table:1 Analysis of physicochemical properties of soil sample from dumpsite at Bhanpur (Mean is ±S.D;n=3)

S.N.	Soil sample code	pH	EC (μS^{-cm}	Cu^{2+}(mg^{-kg})
1.	CRS	**4.73**±0.19	**1.9**±0.047	1.70 ±0.471
2.	BFS	**5.97** ±0.942	**1.4**±0.071	1.60 ±0.016
3.	MPS	**6.34**0.±471	**1.2**±0.004	1.49±0.012
4.	SRS	**6.79**±0.001	**1.0**±0.005	1.32±0.013

Isolation of Copper tolerant bacteria

Metal ions such as Cu^{2+} are involved in many crucial biological processes and are necessary for the survival of all living organism at lowest concentration but it can be toxic at high concentration as they perturb the cellular redox potential and produce highly reactive hydroxyl radicals.

Microbial systems for regulating trace-metal uptake by minimal cation selectivity and are in combination with efflux system (Nies and Silver, 1995) can be important factors in competition with other microbes when the metal ions are either limiting (Kloepper *et al.*, 1980) or present at toxic levels (Silver and Walderhuag, 1992). The understanding of microbial adaptation to the presence of metal in the soil is critical in determining the management and potential long-term effect of land receiving heavy metal contamination through wastewater/sludge.

During the isolation procedure n=49, metal tolerant bacterial isolates were observed on NAM containing 0.25 μg^{-ml} of Cu^{2+} and the number of colony and the percentages of bacterial tolerant were shown in (Table-2).

Table:2 Total Number of isolates and population of metal tolerant in a given sample

S.No.	Sample code	Total No. of isolates	Cu^{2+}	
			No. of the isolates	%T
1.	CRS	20×10^4	09.0×10^4	60%
2.	BFS	24×10^4	10.0×10^4	75%
3.	MPS	21×10^4	12.0×10^4	47%
4.	SRS	14×10^4	18.0×10^4	64%

* % T = Percentage tolerance

In the present study the the maximum bacterial isolates i.e. n = 24 were observed on NAM plates with no metal induction and recovered from buffer zone (BFS) of the study area. But varied from 9.0×10^4 to 18×10^{-4} on the Cu^{2+}(0.25 μg^{-l})supplemented plate (Table:2). The percentages of bacterial colonies tolerant to Cu^{2+} fluctuated from 60% to 75%. During the initial screening process 30 morphologically distinct colony were selected at random and tested for tolerance against gradient concentration of metal ranging from 0.50 to 100 μg^{-ml} of $CuSO_4.5H_2O$. Finally 15 bacterial isolats were selected for further study.

Evalution of Bacterial tolerance to copper

The MTCs of all 15 bacterial isolates analysed against the copper showed (Fig: 1). High bacterial metal tolerance is an important factor to be considered for remediation of heavy metals because it is directly related to the survival and growth of bacteria in metal-contaminated environment. To survive in the polluted environment, microbes usually change intrinsic biochemical and structural properties and may have physiological and genetic adapatation (Zeng *et al*., 2009). For instance, many bacteria can mitigate the toxicity of heavy metal ions such as Hg^{2+} by converting it to a less toxic form through enzymatic reduction. Bacterial tolerance to heavy metals is also attributed to a number of processes, including bioaccumulation by cell biomass, efflux systems, complexation, precipitation, and oxidation reactions. These mechanisms could be utilized for remediation of metal-contaminated environments (Ahmed *et al.*, 2005).

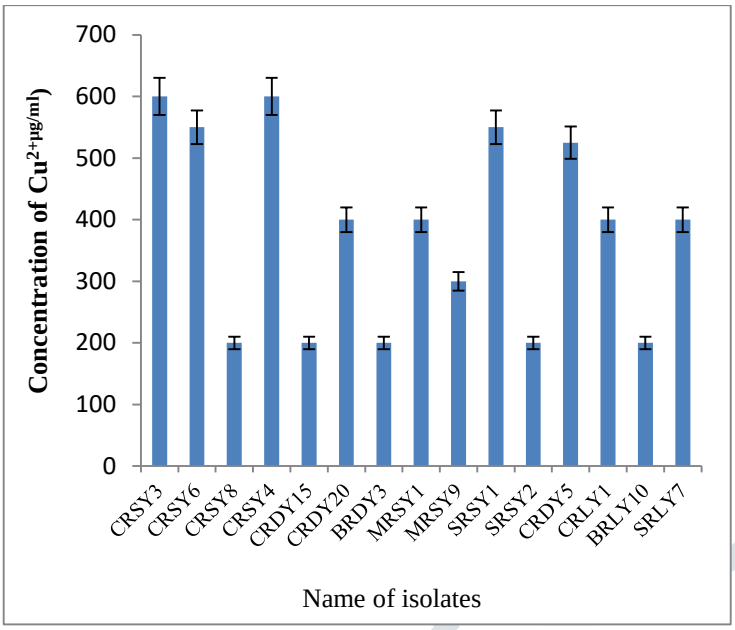

Fig: 1 Maximum Tolerable Concentration(MTC) of bacterial isolates(Cu^{2+})

The highest MTC value was observed with the isolates CRSY4 followed by isolate CRSY3 which were isolated from core region soil of landfill site which is closed to the MSW activity at 600 μg^{-ml} of copper sulphate pentahydrate. Therefore these isolates were selected for further identification on the bases of morphological and biochemical characterization

Identification of the most copper tolerant isolate

Based on the morphological and biochemical examination the isolates CRSY4 was a Gram negative and rod shaped and isolate CRSY3 was a Gram positive and rod in shaped. Isolate CRSY4 belong to *Pseudomonas sp* showed positive result for catalase, oxidase, citrate and starch hydrolysis (Table:3) where as isolate CRSY3 belong to the *Bacillus sp* showed positive result for catalase,citrate,amylase and urease.

Table:3 Morphological and Biochemical characterization of metal tolerant Bacterial isolates

S.No.	Morphological & Biochemical Characteristics of Bacterial isolate	Name of the bacterial isolate	
		Bacillus spp **CRSY3**	***Pseudomonas spp*** **CRSY4**
1.	Nature of colony	Circular, raised and smooth	Irregular,rough,opaque and flat
2.	Gram reaction	+ve	-ve
3.	Shape	Short rod	rod
4.	Arrangement of cell	Streptobacillary	Descrete
5.	Catalase	+ve	+ve
6.	Amylase	-ve	+ve
7.	Oxidase	-ve	-ve
8.	Gelatinase	-ve	-ve
9.	Urease	-ve	-ve
10.	Citrate	+ve	-ve
11.	Indole	-ve	-ve
12.	Methyl red	-ve	-ve
13.	Vogus Proskuaer	-ve	-ve
14.	TSI	-ve	+ve
15.	H_2S	-ve	-ve
		Bacillus spp	*Pseudomonas spp*

Bioaccumulation efficacy of copper tolerant isolates

The potential capability of isolate CRSY4 (*Pseudomonas sp),* CRSY3 (*Bacillus sp)* and their consortia to biosorb copper showed in Fig: 3. The accumulation of Cu^{2+} by the bacterial isolates was characterized to evaluate their potential to remove heavy metals from solution. The amount (%) of metal uptake by each bacterial isolate was examined as a function of time, after having added the biomass to metal solution. Bacterial isolates also exhibited different accumulation capacities towards the metal ion. The maximum percentage of metal accumulation in both isolates and consortia were observed with in 72 hrs. After incubation extension upto 120 hrs did not improve the bioaccumulation. This can be attributed to intense saturation of bacterial cells with metal ions, metal toxicity or a decrease in viable cell count (Al-Garmi, 2005).

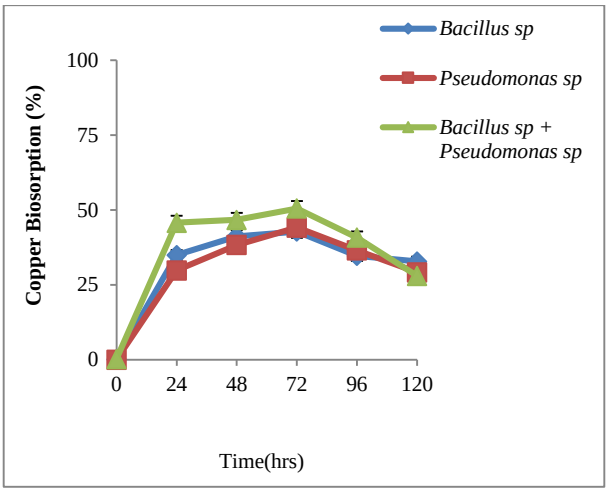

Fig:3 Comparison between the potency of pure culture and bacterial consortia to biosorp copper in medium containing 100 µg $^{-ml}$ of Cu^{2+}

The capability of bacterial consortia (*Bacillus spp + Pseudomonas spp)* showed maximum accumulation (51.2%) whereas minimum accumulation (42.8%) was observed in *Bacillus sp* after 72 hrs. It means that bacterial consortium enhanced their ability to accumulate copper than their pure culture. In this study each species in this consortium had different tolerant mechanism to copper. Copper tolerance mechanism of *Pseudomonas sp.* was facilitated through the intracellular sequestration (Irwati *et al.*, 2016) while, *Bacillus sp* accumulate copper ions through electrostatics interaction and secreting large amount of extracellular polymeric substances (EPS) such as proteins, polysaccharides and organic acids (Irawati *et al.*, 2012). According to Admas *et al.*, (2013). It was indicated that each species in this consortium have significant roles and may require the presence of another species to survive in an environment containing high concentration of copper. This observation was in similar with Sarkar *et al.*, (2013) who reported that bacterial culture in the consortium must be compatible with each other without any antagonism in order to concomitantly perform the entire metabolism required for higher biosorption.

Conclusion

It is concluded that native bacteria present in the soil sample have the property to tolerance and accumulate copper through the process of biosorption. Bacterial consortia (*Bacillus sp + Pseudomonas sp*) has the ability to accumulate high concentrations of heavy metal ions. This could be utilized as per the landfills, industries etc as a potential biotools for bioremediation of metal pollutants.

References

- American Wood Protection Association (AWPA). (2012).Annual Book of AWPA Standards. AWPA. Birmingham.Alabama.USA.*
- Baes, C. F. and Mesner, R. E. (1976).The hydrolysis of cations.Wiley.New York.
- Beavington, F.(1975). Some aspects of contamination of herbage with copper, zinc and iron. Environ. Pollut., 8: 65
- Bisen, P.S. and Verma K.(1996) .Characterization and cultivation of bacteria In Handbooks of Microbiology.2 nd Edition.pp.19-34.
- Brady, D. and Duncan, J .R. (1994a) Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 41:149–154.

- Bremner, J.M. (1960). Determination of nitrogen in soil by the Kjeldahl method. J. Agric. Sci. 55, 11-33.
- Cervantes, C., and Gutierrez-Corana, A.(1994) . Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev., 14:121– 137.
- Clausen, C. A. (2000). Isolating metal tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood. Waste Manage. Res. 18:264–268.
- Fourest, E. and Roux, J.C. (1992).Heavy metal biosorption by fungal mycelial by-products: mechanisms and influence of pH. Appl Microbiol Biotechnol 37:399–403.
- Gadd, G.M. (1993). Interactions of fungi with toxic metals. New Phytol., 124: 25-60. Gilman, J.C.(1959). A manual of Soil Fungi, 2nd Edition. Iowa State University, Iowa*
- Gilman, J.C. (1998).A manual of soil Fungi. Daya Publishing House, New Delhi*
- Jaishankar, M., Tseten, T., Anbalagan N., Mathew, B..B. and Beeregowda, N.K.(2014). Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol. 7(2): 60–72.
- Jan, F.A., M. Ishaq, S. Khan, I. Ihsanullah, I. Ahmad, and M. Shakirullah.(2010). A comparative study of human health risks via consumption of food crops grown on wastewater irrigated soil (Peshawar) and relatively clean water irrigated soil (Lower Dir). J. Hazard. Mater. 179:612–6215.
- MB,E., and Ellis, J.P. (1985). Microfungi on Land Plants. Biddles Ltd.,Guildford and Kings Lynn, Great Britain, pp 1–818
- Prescott, L. M., and J. P. Harley.(2002). Laboratory exercises in microbiology, 5th Edition. New York, USA: McGraw-Hill.
- Kumar R., Singh P., Dhir B., Sharma A.K. and Mehta D.(2014).Potential of Some Fungal and Bacterial Species in Bioremediation of Heavy Metals. J of Nuclear Ph.1(2):213-223.
- Sharman and Cappuccino.(2004). Biochemical activities of microorganism in Microbiology A Laboratory Manual.Pearson Education Publishers.6 th Edition.pp.133-195
- Siokwu, S. and Anyanwu, C. U. (2012). Tolerance for heavy metals by filamentous fungi isolated from a sewage oxidation pond.Afri. J. of Microbiol Rese .,6(9): 2038-2043.
- Stern, B.R. , Solioz, M. , Krewski, D., Aggett, P. , Ching, T., Baker, S. , Crump, K., Michael Dourson, M. , Haber, L., Hertzberg, R. , Keen, C. , Meek, B. , Rudenko, L.,Schoeny R.R. , Slob W. , Starr, T.(2007).Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships.Journal of Toxicology and Environmental Health, Part B, 10:157–222.