Journal of **N**onlinear **A**nalysis and **O**ptimization
Vol. 15, Issue. 1, No.2 : 2024
ISSN : **1906-9685**

IDENTIFICATION AND DETERMINATION OF MIC VALUE OF HEAVY METAL TOLERANCE BACTERIA FROM COAL MINE OF CHHATTISGARH AND THEIR MOLECULAR CHARACTERIZATION

Virendra Kumar Vaishnav PhD Scholar, Depaertment of Biotechnology Raipur Institute of Technology Raipur, INDIA virendravaishnav7@gmail.com
Tanushree Chatterjee Associate Professor, Raipur Institute of Technology Raipur, INDIA tanushree52004@yahoo.com
Manisha Agrawal Professor Rungta College of Engineering and Technology Bhilai, INDIA dr.manisha.9000@gmail.com

Abstract—
In current years, coal mining is highly contaminating agricultural land, Environment, Soil as well as water source through heavy metals. Through survey recorded that Chhattisgarh's coal mines polluted heavy metals that affects people's health which affecting muscles, bones and skin. This study mainly focused to isolate, screening and identify bacteria able to degrade heavy metals (Lead, Arsenic and Mercury) from soil of coal mine. Five isolates showed different MICs against the Pb, As and Hg at different levels. Two isolates were recorded highest resistant ability against Lead, Arsenic and Mercury and identified up to genus level based on their morphological and biochemical characteristics as Bacillus sp. and Micrococcus sp. After 16S rRNA sequencing both bacterial isolates was identified as Bacillus cereus and Micrococcus luteus. Phylogenetic tree of isolates were constructed through Clustal W and Bacillus cereus (0.8 kb) and Micrococcus luteus (1.3 kb) size of DNA was measured through Agarose gel. Therefore, identified these two bacteria for their heavy metal tolerance capacity and biodegradation ability may be a primary step to develop the bioremediation agents for heavy metal contaminated soil and water.
Keywords—Bioremediation, Heavy Metal, Coal Mine, Phylogenetic Tree, 16S rRNA

I. INTRODUCTION

Chhattisgarh has enormous coal treasury and 2nd position on coal production in India. Gevra coal mine and Mand coal field Raigarh both is open cast mine and continuous coal mining impacted on environment (Department of Mineral Resources, C.G. 2017). Coal mining increases the level of toxic element such as Cadmium, Mercury, Chromium, Copper and Arsenic which is contaminated air, soil and water (Das et al., 2018). These heavy metals impacted on human health and can cause serious diseases. Arsenic and Cadmium are carcinogenic for human being whereas Lead affects nervous system (Pande V et al., 2022).

One of the major problems is fly ash combustion which has produce high volume of solid wastes contain heavy metal to contaminate soil. Zinc and Copper are necessary for plant growth whereas higher concentration of Zinc and Copper turn out to be toxic for organism and plant.(R.K. Goswami et al., 2021)

Recent years Bioremediation through indigenous bacteria is useful way to take away toxic elements from soil. In this method organism break down the heavy metals from environment (Jaishankar M et al., 2014).

The main aim of this study is to find out bacteria isolated from coal mine of Chhattisgarh for heavy metal resistance, Minimal Inhibitory Concentration against Pd, As and Hg as well as molecular characterization of heavy metal tolerance bacteria for bioremediation of heavy metals.

II. MATERIALS AND METHODS

A. Sampling Area

Soil samples were collected from opencast mines Gevra Coal Field Korba (22.336312, 82.545748) (Fig.1) and Mand Coalfield Raigarh (22°16′6″N 83°20′38″). Soil were collected from the top 20 cm soil layer and stored in pre labeled polythene bags and preserved at laboratory condition.

Fig. 1 Sampling Site (A) Gevra Coal Mine Korba Chhattisgarh (B) Mand Raigarh Coalfied Chhattisgarh

B. Screening of heavy metal resistant bacteria

To isolate bacteria soil samples were serially diluted (10-7) and Spread plate method was performed (Azad et al. 2013). Different morphological characteristics colonies were elected and pure cultured. Primary screening of heavy metal resistant bacteria were screened on Luria Bertani agar plates supplemented with 300 µg/mL of heavy metal and streaked plates were incubated at 37oC for 24 hours and Colonies were observed after.

C. Multiple metal resistance capacity

Screened isolates (GK1, GK2, GK3, MR1 and MR2) were individually inoculated on LB broth supplemented with Lead, Arsenic and Mercury (300 µg/mL) at pH 7.0 and incubated at 37oC for 24 hours. Over the incubation multiple metal resistance capacity was assessed against multiple heavy metals (Malik A and Jaiswal R., 2010).

D. Minimum inhibitory concentration (MIC)

The MIC of selected isolates were tested in heavy metals (Pb, As, and Hg) containing LB agar plates and the concentrations of heavy metals on LB agar plates were increased until isolated bacteria failed to develop colony. The initial concentration of heavy metals was 50 µg/mL. MIC was determined through standard protocol of European food safety authority (EFSA), Parma, Italy, 2012.

E. Biochemical characterization of bacterial isolates

The bacterial isolates were characterized up to genus level through Bergey's Manual. Based on activities of bacterial isolates following biochemical tests Catalase, Indole, Motility, Methyl Red, Citrate Utilization, Glucose, Lactose, Casein Hydrolysis, Maltose, Oxidase and Gelatin Hydrolysis were performed.(Claus and Berkeley., 1986).

F. Molecular characterization of Heavy Metal Resistant Bacteria

DNA was extracted from bacterial isolates as per developed procedure via Sambrook and Rissell (2001) and Agarose Gel Electrophoresis was performed. PCR amplification was performed of 1.5kb long 16S rRNA gene using forward primer (5'AGAGTTTGATCCTGGCTCAG 3') and reverse primer (5´GGTTACCTTACGACTT 3´). (Gandhi V.P. et al., 2015)

G. Phylogenetic analysis

PCR amplified 1.5 kb 16S rRNA was used for DNA sequencing. NCBI Blast program was used to compare sequence of the DNA fragments. Phylogenetic tree was constructed of 16S rRNA sequences using clustal W (Virender S et al., 2010).

H. Statistical Analysis

All the experiments were performed in triplicate as well as mean or standard deviation was calculated on Microsoft Excel Software, version 2010 and showed as mean ± SD.

III. RESULTS AND DISCUSSION

A. Isolation and Screening of heavy metal resistant bacteria

Sixty colonies were isolated from coal mine soil sample and screened against heavy metal supplemented LB medium (Fig 2). Five isolates (GK1, GK2, GK3, MR1 and MR2) were developed colonies on heavy metal supplemented media and showed highest heavy metal resistances. (Table 1)

B. Analysis for multiple heavy metal resistance ability

Growth curve analysis method was used to analysis the Multiple Heavy metal resistance capacity. We find out that GK2 and MR2 showed good tolerance ability against Lead, Arsenic and Mercury (Fig. 3). On other hand GK1 and GK3 showed tolerance against Pb and As. Whereas MR1 not showed growth against Pb, As and Hg. (Graph 1,2,3)

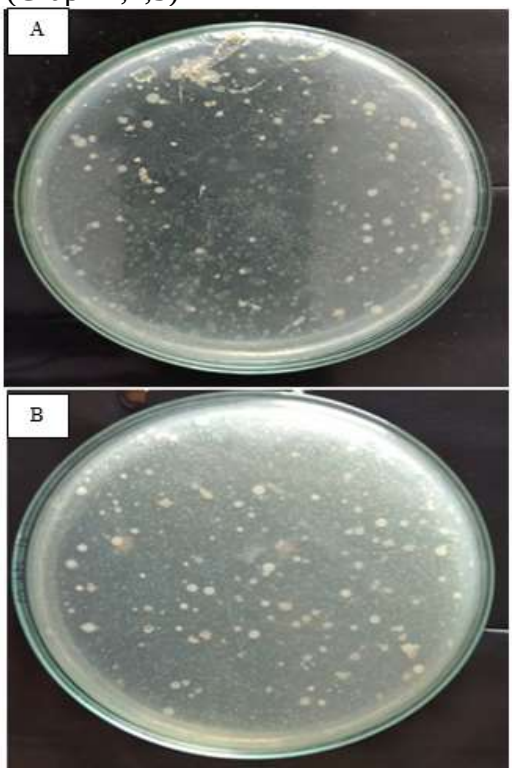


Fig. 2 Culture plate (A) with Heavy Metal Culture Plate (B) without Heavy metal

Table 1 Morphological Characteristic of Bacterial Isolates

Bacterial Isolates	**GK1**	**GK2**	**GK3**	**MR1**	**MR2**
Colony Morphology					
Colony Color	Whitish	Milky White	Milky White	Whitish	Yellowish
Cell Shape	Coccus	Rod Shaped	Rod Shaped	Rod Shaped	Coccus
Gram Nature	Gram Positive	Gram Positive	Gram Positive	Gram Positive	Gram Positive

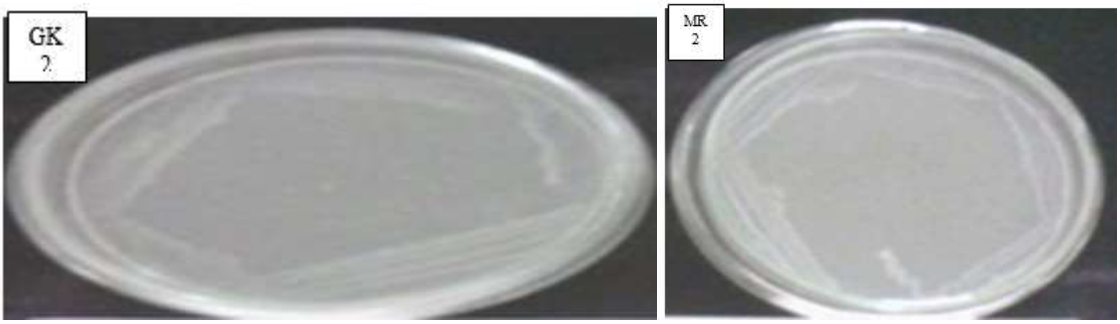
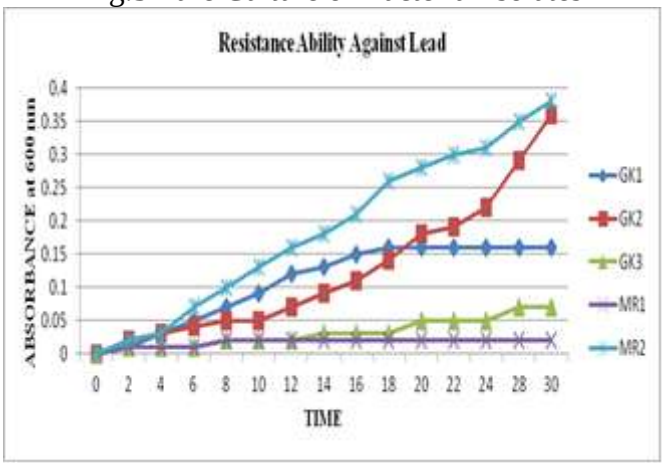
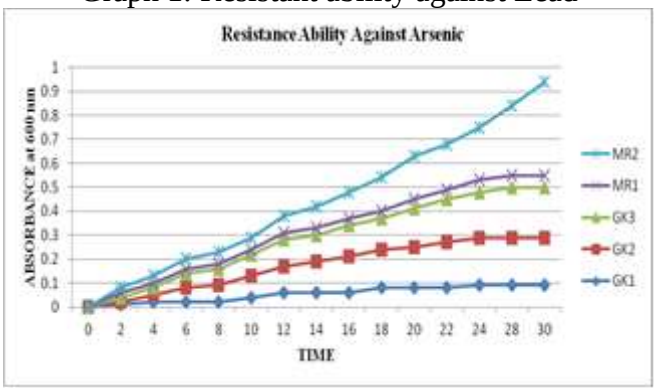


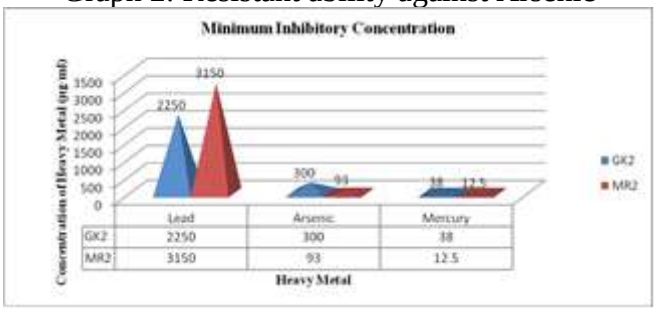
Fig.3 Pure Culture of Bacterial Isolates



Graph 1: Resistant ability against Lead



Graph 2: Resistant ability against Arsenic



Graph 3: Resistant ability against Mercury

C. Assessment of Minimum inhibitory concentration

Minimum inhibitory concentration was assessed ranging from 50 to 3500 µg/mL against Pb, As and Hg. Isolate GK2 was exhibited resistance 2250 ±2.3 µg/mL against Pd as well as Isolate MR2 tolerated 3150 ± 3.5 µg/mL. Whereas against Arsenic Isolate GK2 300 ± 1.6 µg/mL and Isolate MR2 93 ±1.2 µg/mL exhibited resistance. On other hand isolates GK2 and MR2 were recorded 38 ±0.7 µg/mL and 12.5 ± 0.5 µg/mL tolerance against Mercury. (Graph 4)

Graph 4: Minimum Inhibitory Concentration of Isolates GK2 and MR2

D. Biochemical characterization

Two bacterial isolates (GK2 and MR2) having potential heavy metal degrading capacity against Pd, As and Hg were characterized based on their biochemical characteristics (Table 2).(Bergey et al., 1974; Williams and Wilkins, 1994). The isolates were recognized up to genus level as Bacillus sp. (GK2) and Micrococcus sp.(MR2) through Bergey's Manual of determinative bacteriology (Claus and Berkeley, 1986).

Table 2 Biochemical Test of Bacterial Isolates

Biochemical Test	**GK2**	**MR2**
Catalase	+ve	+ve
Citrate	+ve	+ve
Indole	-ve	-ve
Motility	+ve	-ve
MR (Methyl Red)	-ve	-ve
Glucose	+ve	+ve
Maltose	+ve	-ve
Lactose	-ve	-ve
Casein Hydrolysis	+ve	-ve
Oxidase	-ve	+ve
Gelatin Hydrolysis	-ve	+ve

E. Molecular Characterization through 16S rRNA analysis

16S rRNA identification of isolates was determined as Bacillus cereus (GenBank accession No. OQ691647) and Micrococcus luteus (GenBank accession No. OQ691646). PCR amplified produced fragment of 16S rRNA gene size was analyzed through Agarose gel and Bacillus cereus (GK2) about 0.8 kb (Fig. 4) and Micrococcus luteus (MR2) 1.3 kb (Fig. 5) in size assessed by Agarose. A phylogenetic tree was constructed by aligning 16S rRNA sequences of bacterial isolates using Clustal W.(Fig. 6 and Fig. 7)

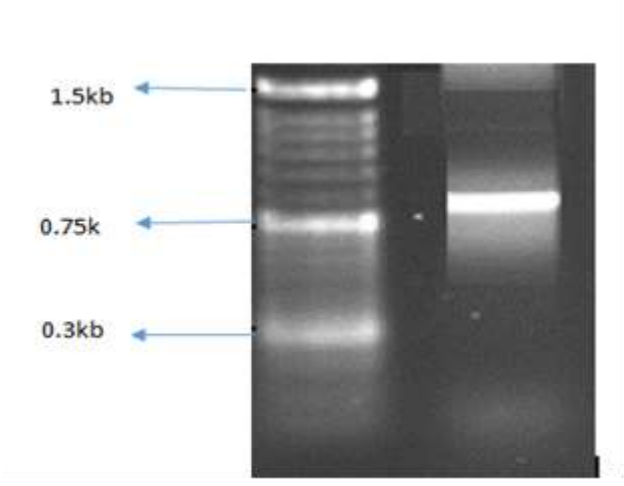


Fig. 4 (Bacillus cereus) Genomic DNA (0.8% Gel); Lane 1- 1.5kb ladder; Lane 2-Isolate 1

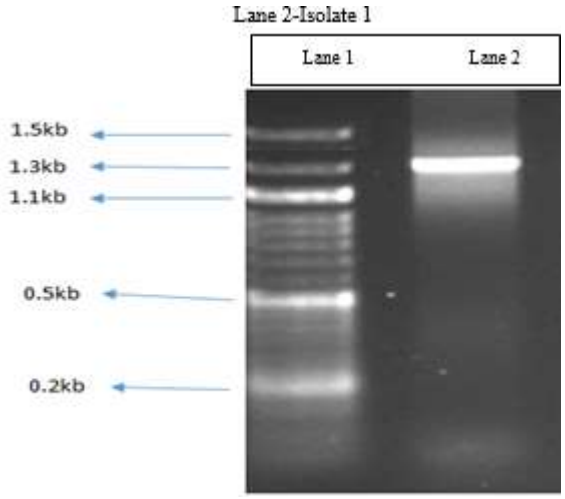


Fig. 5 (Micrococcus luteus) Genomic DNA (0.8% Gel); Lane 1- 1.5kb ladder; Lane 2-Isolate 1

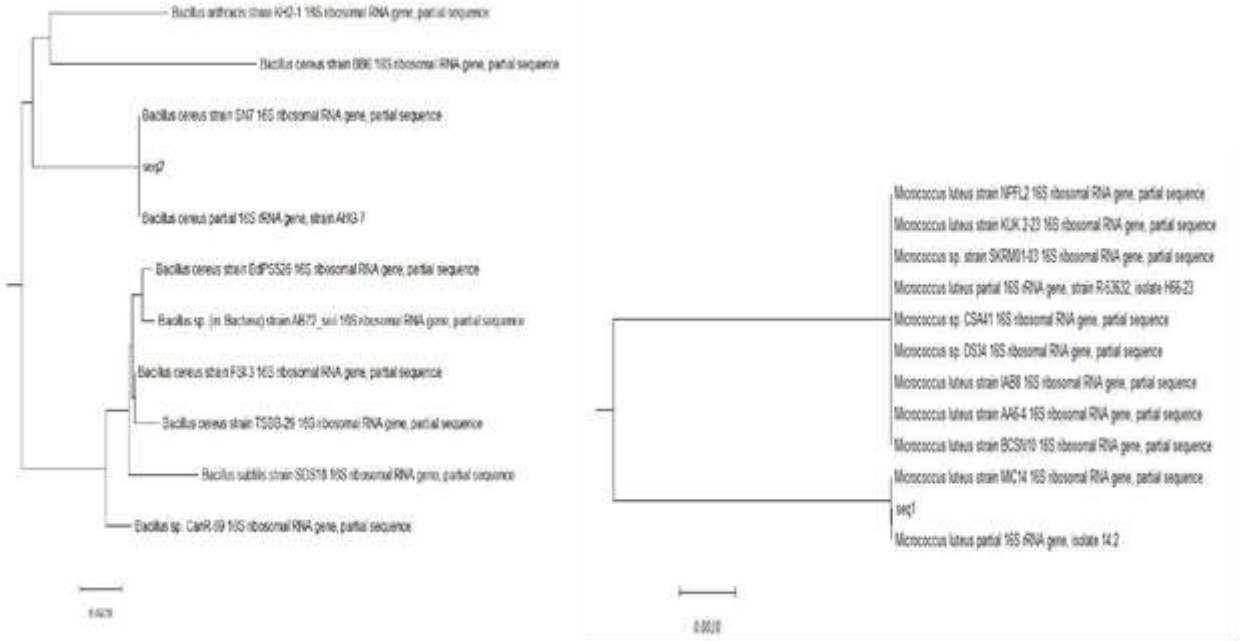


Fig. 6 Phylogenetic Tree of Bacillus cereus (GK2) and Micrococcus luteus (MR2)

IV. DISCUSSION

The main objective of this study is to isolate and identify the heavy metal degrading potential bacterial against Lead, Arsenic and Mercury that help the naturally detoxify agricultural land and environment. Also assess the maximum tolerance level of Lead, Arsenic and Mercury. Molecular Characterization of potential bacteria was performed as well.

Through Serial dilution method and spread plate method was used to isolate desired bacteria. Initial screening of the isolated bacteria's was positively grown on heavy metal containing culture plate. Initially screened bacterial isolates were characterized by colony morphology, biochemical tests, multiple heavy metal resistance capacity and Minimum inhibitory concentration capacity. Biochemical tests were performed to identify bacterial isolates upto genus level according to Bergey's Manual. (Bergey et al., 1974)

All five bacterial isolates (GK1, GK2, GK3, MR1 and MR2) were identified as gram-positive bacteria (Burke and Pister, 1986). The multi-metal resistance capacity of two bacterial isolates Bacillus sp. and Micrococcus sp. were highly tolerant against Pb, As and Hg.

In present study MIC for the GK2 isolates and MR2 was found as Pb >As >Hg. Bacillus sp. was assessed in our study through MIC against Pb 2250 ± 2.3 µg/ml, As 300 ± 1.6 µg/ml and Hg 38 ±0.7

µg/ml. where Micrococcus sp. Was found MIC against Pb 3150 ± 3.5 µg/ml, As 93 ±1.2 µg/ml and Hg 12.5 ± 0.5 µg/ml.

Biochemical tests catalase, Citrate, indole, Motility, Methyl Red, Glucose, Maltose, Lactose, Casein Hydrolysis, Oxidase and Gelatin Hydrolysis were performed and identify as Bacillus sp.(GK2) And Micrococcus sp.(MR2) (Table 4). Bacillus cereus (GK2) and Micrococcus luteus (MR2) identified through 16S rRNA gene sequencing. Bacillus cereus (GK2) (Fig. 6) and Micrococcus luteus (MR2) (Fig. 7) 16S rRNA gene 0.8 kb and 1.3 kb in size was measured through Agarose gel. (Fig. 4 and Fig. 5)

Further study of different heavy metals effect and optimization of growth is needed to identify their effectiveness as bioremediation agents. (Shivakumar et al., 2014) To formulate it use as derivative agent for contaminated land to remove heavy metal.

V. CONCLUSION

In this study, soil samples were collected from Gevra Coal mine Korba and Mand Coalfield Raigarh and their heavy metal degrading bacteria was assessed. Five bacterial isolates have been selected and subjected to multiple metal resistance capacity and MIC. The best result showing isolate selected for further use in bioremediation of heavy metal pollutants. Through 16S rRNA sequencing two isolates was identified as Bacillus cereus and Micrococcus luteus. The results of this study that two bacteria had significant bioremediation potentiality which might be used to formulate bioremediation agents to detoxify heavy metal contaminated soil in the natural environments.

VI. REFERENCE

1. Azad, A.K., Nahar, A., Hasan, M.M., Islam, K., Azim, M.F., Hossain, M.S., Rahman, M.R., Ojha, R.K., Mahmud, G.M.S., Kayes, R., (2013). Fermentation of municipal solid wastes by bacterial isolates for production of raw protein degrading proteases. Asian J. Microbiol. Biotechnol. Environ. Sci. 15, 365–374.
2. Bergey, D.H., Buchanan, R.E., Gibbons, N.E., (1974). Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Co., Baltimore, pp 1246.
3. Burke, B.E., Pister, R.M., (1986). Cadmium transport by a Cd2+_ sensitive and a Cd2+ resistant strain of Bacillus subtilis. Can. J. Microbiol. 32, 539–542.
4. Claus, D., Berkeley, R.C.W. (1986). Genus Pseudomonas. In: Sneath, P. H.A., Mair, N.S., Sharpe, M.E. (Eds.), . In: Bergey's manual of systematic bacteriology, Vol 1. Williams and wilkins, Baltimore, pp. 140–219. 0-683-04108-8.
5. Das, A., Patel, S., Krishna, K. V. S. S., Kumar, R., Saha, M. C., Dutta, S., Sengupta, S., & Guha, D. (2018). Geochemical sources of metal contamination in a coal mining area in Chhattisgarh India using lead isotopic ratios. Chemosphere, 197, 152–167
6. Gandhi, V.P., Priya, A., Priya, S., Daiya, V.1, Kesari, J., Prakash, K., Kumar Jha, A., Kumar, K., and Kumar, N. (2015)" Isolation and molecular characterization of bacteria to heavy metals isolated from soil samples in Bokaro Coal Mines, India" Pollution,1(3): 287-295.
7. Islam, E.U., Yang, X., He, Z. and Mahnmood, Q. (2007). Assessing potential dietary toxicity of heavy metals in selected vegetables and food crocks. J. Zhejiang Univ. Sci., 8, 1–13.
8. Iwegbue, C.M.A., Nwajei, G.E., Ogala, J.E. and Overah, C.L. (2010). Deterermination of trace metal concentrations in soil profiles of municipal waste dumps in Nigeria. Environmental Geochemistry and Health, 32, 415-430.
9. Jaishankar M, Tseten T, Anbalagan N, Mathew BB, Beeregowda KN. Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol. 2014;7:60–72.
10. Malik, A., Jaiswal, R., 2010. Metal resistance in Pseudomonas strains isolated from soil treated with industrial waste water. World J. Microbiol. Biotechnol. 16, 177–182.
11. R.K. Goswami, K. Agrawal, M.P. Shah P. Verma,(2021) "Bioremediation of heavy metals from wastewater: acurrent perspective on microalgae-based future", Letters in Applied Microbiology 75, 701-717

12. Sambrook, J. and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, Third Ed. Cold Spring Harbor Laboratory Press.
13. Shivakumar, C.K., Thippeswamy, B., Krishnappa, M., (2014). Optimization of heavy metals bioaccumulation in Aspergillus niger and Aspergillus flavus. Int. J. Environ. Biol. 4 (2), 188–195
14. Pande V, Pandey SC, Sati D, Bhatt P and Samant M (2022) Microbial Interventions in Bioremediation of Heavy Metal Contaminants in Agroecosystem. Front. Microbiol. 13:824084. doi: 10.3389/fmicb.2022.824084
15. Virender, S., Chauhan, P.K., Kanta, R., Tejpal, D., Vinod, K., 2010. Isolation and characterization of Pseudomonas resistant to heavy metals contaminants. Int. J. Pharm. Sci. Rev. Res. 3 (2), 164–167.
16. Williams, Wilkins, 1994. Bergey's Manual of determinative bacteriology, nineth ed.