International Journal of Advanced Research and Review

www.ijarr.in

TAXONOMIC ASSESSMENT OF ALKALI TOLERANT METALLOPHILES FROM SOIL OF M.I.D.C. PARBHANI

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ABSTRACT

Five metallophilic, alkali tolerant, motile, non spore forming, catalase positive and Gram negative bacteria were isolated from sediment soil samples collected from effluent canal of M.I.D.C. Parbhani district in Maharashtra and further identified based on their morphological, microscopic and physiological characters, enzyme profile and sugar-use pattern as *Cupriavidus* sp. MRC1, *Ralstonia eutropha* MRC2, *Pseudomonas stutzeri* MRC3, *Aeromonas media* MRC4 and *Ralstonia metallidurans* MRC5. Taxonomic assessment of the isolates revealed that most of the isolates were belonged to the class betaproteobacteria and gammaproteobacteria. MIC of these isolates was determined against Cu, Cd, Pb, Hg and Zn. *Ralstonia metallidurans* MRC5 showed highest resistance against Zn and 0.40 mg/mL MIC was recorded. The metallophiles reported by us can be used as a bioremediation tool for the treatment of effluents from industries handling heavy metals.

Keywords: Bioremediation, M.I.D.C. Parbhani, Metal resistant, Metallophiles, MIC, *Ralstonia* sp., Sucrose minimal medium

INTRODUCTION

From the last 50-80 decades, uncontrolled urbanization and increased industrialization have caused metal pollution which is one of the serious issues in environment. This was noticed due to the release of sewage and industrial effluents directly into water bodies. A large quantity of metal is therefore concentrated in food chain where the man is ultimately victimized (Selvi et al. 2012). Heavy metals are absorbed in vegetation due to irrigation with waste water that can cause serious issues related to human health. Various effects and diseases were observed in human population viz. reduced and damaged central nervous system functions, undesirable change in blood composition, liver, lungs, kidney and in other important organs. Exposure to heavy metals for long duration results in neurological degeneration that mimics illness, allergies, Alzheimer's disease, Parkinson's disease, muscular dystrophy and multiple sclerosis (Gupta and Kumar, 2012).

Therefore, the bioremediation is an alternatively tool to solve the soil and water pollution problem of heavy metals. Bioremediation is the use of microorganisms to remove pollutants. The bacteria utilize metal ions as their inorganic energy source in nature. They acquire their metal resistance characteristic due to extrachromosomal circular covalently closed metal resistant plasmid DNA. Metallophilic and metal tolerant microbes can act as highly efficient bioaccumulators of soluble and particulate forms of metals. Metallophiles are adopted to metal-rich biotopes and therefore considered as extremophiles (Meargey, 1999). Interactions between metals and microbes may be extracellular, exocellular and intracellular. Metal resistant microorganisms remove metals from polluted samples by various mechanisms viz. adsorption, complexation, precipitation, and volatilization. Metal detoxification in polluted samples is also possible by physical and chemical methods but these methods have some limitations. In drawbacks, physical methods require large input of energy and expansion of materials and in chemical methods, use of certain chemicals cause secondary pollution (Dubey, 2007; Rathod, 2009).

The present study was planned with objectives as isolation and identification of metal resistant bacteria from industrial area of Parbhani district in Maharashtra (India).

MATERIALS AND METHODS

Enrichment, screening and isolation of metallophilic bacteria

Sediment soil samples were collected in presterilized plastic bags from the effluent canal of M.I.D.C. area, Parbhani. pH of the soil sample was recorded. For the selective enrichment of metallophiles, 1 g of collected soil sample was added into 5 different flasks containing sucrose minimal salt (SMS) broth each supplemented respectively with 0.1 mg/mL of copper chloride, cadmium chloride, zinc chloride, mercuric chloride and lead chloride. The flasks were incubated at 30 ^{o}C and 120 rpm for 48 h. After incubation, 100 µL of sample from each flask was spread individually on SMS agar plates containing the same concentration of each selected metal chloride to screen for metal resistance. The plates were incubated at 30 ^{o}C for 24 h. Morphologically distinct colonies were selected and cultivated on agar slants of the same media containing same concentrations of selected metal chlorides. Selected isolates were purified by repeated subculturing on agar plates of same media.

Characterization and identification of selected metallophiles

Morphological characters of selected metal resistant isolates viz. colony shape, size, margin, elevation, consistency, density, surface and pigmentation were recorded. Microscopic features of selected metal resistant isolates were determined by performing Gram staining, spore staining, flagella staining, micrometry and motility test. Standard Gram stain kit was purchased from HiMedia, Mumbai and used for Gram staining. 25 days old cultures of selected metal resistant isolates were subjected for spore staining by using malachite green (5 % aqueous). Flagella staining were performed by using carbol fuchsin. Hanging drop technique was used for testing motility of selected metal resistant isolates. Effect of pH (6, 7, 8 and 9), temperature (20, 25, 30, 35, 40, 41, 45 and 50 ^{o}C), incubation period (24, 48 and 72 h) and the selected respective metal chlorides used for enrichment (0, 0.05, 0.07, 0.10, 0.13, 0.15, 0.17 and 0.2 mg/mL) on growth of selected metal resistant isolates was assessed in SMS broth. Sugar utilization pattern of the selected isolates was recorded by performing carbohydrate fermentation tests by using base of SMS medium in presence of sucrose and by replacing sucrose with13 different types of sugars viz. cellobiose, dextrose, fructose, galactose, glycerol, lactose, maltose, mannitol, mannose, mellibiose, raffinose, ribose, salicin, sorbitol, sucrose and xylose at predetermined optimum pH, temperature, incubation period and metal chlorides. Extracellular enzyme profile of the selected isolates was screened by performing amylase, protease, cellulase, gelatinase, lipase and urease production tests using the starch, casein, carboxy methyl cellulose, gelatin, tributyrin and urea substrates respectively at predetermined optimum parameters. Catalase and oxidase tests were performed by using 3% hydrogen peroxide and 1% tetramethyl-p-phenylenediamine dihydrochloride respectively. Indole, methyl red, Voges-Proskauer, citrate utilization and H_2S production tests were performed as per the standard procedures. Morphological, microscopic and physiological characters, enzyme profile and sugar-use pattern of the selected isolates were compared with the standard reference strains as described in 'Bergey's manual of systematic bacteriology' and selected isolates were identified (Sneath et al., 1986; Sharma et al., 2009; Khan et al., 2009; Vos et al., 2009; Palsaniya et al., 2012; Pathak and Sardar, 2012; Pathak et al. 2012, 2014; Pathak et al., 2015a,b,c,d,e,; Khairnar et al., 2012; Kolekar et al., 2013; Hingole and Pathak, 2013; Pathak and Rathod 2013, Sardar and Pathak, 2014; Pathak and Sardar, 2014; Pathak and Rathod, 2014; Rathod and Pathak, 2014a,b; Polkade et al., 2015; Dastager et al. 2015; Sharma et al., 2015; Jadhav and Pathak, 2015; Pathak and Rathod, 2015, 2016a, b; Hingole and Pathak, 2016; Rathod and Pathak, 2016; Sardar and Pathak, 2016).

Determination of MIC

MIC of the selected metal resistant isolates grown on metal incorporated media, against respective metal was determined by constantly increasing the concentration of the each metal, 0.05 mg/mL each time in SMS broth until the isolates failed to grow. The starting concentration of each selected metal was 0.10 mg/mL. 1 mL volume of the each culture growing in the last concentration was transferred to the media containing higher concentration. MIC was noted when the isolates failed to show turbidity as recorded by using a spectrophotometer (Shimadzu Corporation) at 660 nm (Rathod, 2009; Singh et al. 2010; Selvi et al. 2012; Gupta and Kumar, 2012).

RESULTS AND DISCUSSION

Screening and isolation of metal resistant bacteria

pH of sediment soil sample was 7.2. Enriched culture of metallophiles in SMS broth containing selected metal chlorides has shown in Figure 1. Number of colonies appeared on metal containing SMS agar plates have shown in Table 1. Purified culture of MRC2 isolate has shown in Figure 2. Many researchers have used different types of media for isolation of metal resistant bacteria. Gupta and Kumar (2012) have used M9 minimal medium for isolation of metal resistant bacteria from industrial effluent samples. Francois et al., (2011) have mentioned 2.72 μg/mL of $HgCl_2$ is typical concentration for isolation of Hg^{2+} resistant bacteria.

Characterization and identification of selected metal resistant isolates

Table 2 reflects morphological and microscopic characters of the selected metal resistant isolates. Microscopic view of MRC3 isolate is shown in Figure 3. Table 3 shows physiological characters of the selected metal resistant isolates. Biochemical pattern of the selected metal resistant isolates has been shown in Table 4. Enzyme profile of the selected metal resistant isolates has given in Table 5. The isolates MRC1, MRC2, MRC3, MRC4 and MRC5 were identified as *Cupriavidus* sp., *Ralstonia eutropha*, *Pseudomonas stutzeri*, *Aeromonas media* and *Ralstonia metallidurans* respectively.

Determination of MIC

Minimum inhibitory concentrations of selected metals for *Cupriavidus* sp. MRC1, *Ralstonia eutropha* MRC2, *Pseudomonas stutzeri* MRC3, *Aeromonas media* MRC4 and *Ralstonia metallidurans* MRC5 have given in Table 6. These isolates have shown very high degree of multi metal resistance. Choudhury and Srivastava (2001) have explained zinc resistance mechanism in bacteria. Raja et al. (2009) have isolated Pb resistant *Proteus* sp. and *Acinetobacter* sp. from sewage. Figueiredo et al. (2014) have isolated mercury-resistant *Bacillus* sp., *Aeromonas* sp. and *Vibrio* sp. from estuary sediments. Owolabi et al. (2015) have reported 8 mM minimum inhibitory concentration of $PbCl_2$ for *Serratia marcescens* HM1, *Aeromonas caviae*, *Micrococcus varians*, and *Citrobacter* sp. Pandit et al. (2013) have isolated Cu, Cr, Cd and Ni resistant 6 bacteria namely *Comamonas testosteroni*, *Bacillus* sp., *Aeromonas hdrophila*, *Exibacterium profundum*, *Bacillus cereus* and *Exiguobacterium* sp. from industrial effluent contaminated sites of Ankleshwar, Gujarat. Chovanova et al. (2004) have isolated Cd resistant 6 bacteria growing at 50 µg/mL Cd^{2+} namely *Alcaligenes xylosoxidans*, *Comamonas testosteroni*, *Klebsiella planticola*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Serratia liquefaciens* from sewage slude. The MICs recorded by us are superior as compared to Singh et al. (2010) and Kaur et al. (2015).

Fig. 1: Enrichment of metallophiles in SMS broth containing selected metal chlorides

Fig. 2: Purified culture of MRC2 isolate

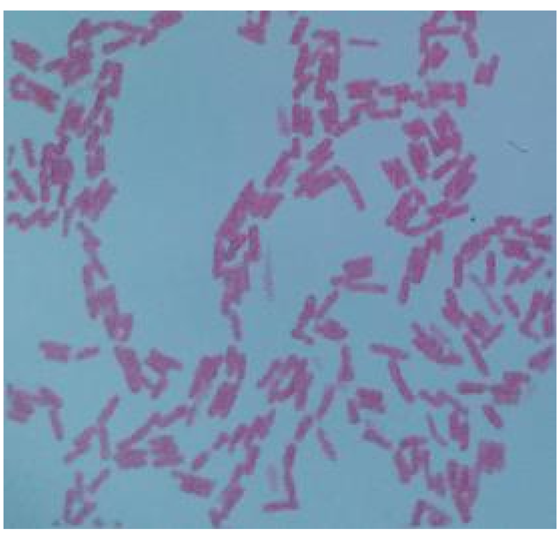

Fig. 3: Gram stained cells of MRC3 isolate

Table 1: Number of colonies appeared after enrichment on metal containing SMS agar plates

Metal chlorides (0.02 mg/mL)	Cu	Cd	Pb	Hg	Zn
Number of colonies appeared in 0.1 mL of enriched sample	98	112	109	121	110
Designation of selected morphologically distinct colonies	MRC1	MRC2	MRC3	MRC4	MRC5

Table 2: Morphological and microscopic characters of the selected metal resistant isolates

Characters	MRC1	MRC2	MRC3	MRC4	MRC5
Shape	Circular	Round	Round	Circular	Round
Size	4 mm	1.7 mm	1.3 mm	2.2 mm	1.5 mm
Margin	Entire	Entire	Wrinkled	Smooth	Smooth
Elevation	Convex	Raised	Semi raised	Convex	Convex
Consistency	Semi sticky	Semi sticky	Sticky	Semi sticky	Semi sticky
Density	Opaque	Opaque	Opaque	Semi-translucent	Opaque
Surface	Nucleoid	Glowing	Nucleoid	Nucleoid	Nucleoid
Pigmentation	Off-white	Dirty white	Beige	Pale white	Beige
Microscopic characters					
Gram stain reaction	Negative	Negative	Negative	Negative	Negative
Cell measurement	0.7× 0.9 µm	0.7× 2.0 µm	0.6× 1.9 µm	0.5× 1.0 µm	0.7× 1.4 µm
Cell shape	Coccoid rod	Rod	Rod	Coco-bacillary	Short rod
Spore formation	Non spore former	Non spore former	Non spore former	Non spore former	Non spore former
Motility	Motile	Motile	Motile	Motile	Motile
Arrangement of flagella	Peritrichous	Peritrichous	Single polar	Peritrichous	Peritrichous

Table 3: Physiological characters of the selected metal resistant isolates

Characters	MRC1	MRC2	MRC3	MRC4	MRC5
Growth at pH	6-9	6-9	6-9	6-9	6-9
Optimum pH	7	8	7	8	7
Growth at temperature	20-45	20-41	20-41	20-45	20-45
Optimum temperature	35	30	35	35	30
Optimum incubation period	48	24	24	48	48
Optimum growth in presence of selected metal chloride	$CuCl_2$ 0.05-0.20 mg/mL	$CdCl_2$ 0.05-0.17 mg/mL	$PbCl_2$ 0.05-0.15 mg/mL	$HgCl_2$ 0.05-0.13 mg/mL	$ZnCl_2$ 0.05-0.17 mg/mL

Table 4: Biochemical pattern of the selected metal resistant isolates

Sugars	MRC1	MRC2	MRC3	MRC4	MRC5
Cellobiose	+	-	-	-	-
Dextrose	+	+	-	w+	-
Fructose	+	+	-	-	-
Galactose	+	-	-	-	-
Glycerol	+	+	-	+	-
Lactose	-	+	-	-	-
Maltose	+	-	+	-	-
Mannitol	-	-	+	+	-
Mannose	+	-	-	-	-
Salicin	-	+	-	-	-
Sorbitol	-	-	-	-	-
Sucrose	w+	+	+	+	+
Xylose	+	+	-	-	-
Indole test	-	-	+	+	-
Methyl red	-	-	w+	w+	-
Voges-Proskauer	-	+	-	+	-
Citrate utilization	+	+	+	-	-
H_2S production	-	-	-	-	-

Table 5: Enzyme profile of the selected metal resistant isolates

Enzymes	MRC1	MRC2	MRC3	MRC4	MRC5
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Amylase	+	-	+	+	+
Protease	-	-	-	+	+
Gelatinase	-	-	-	+	+
Lipase	-	+	+	+	-
Urease	-	-	+	-	-
Cellulase	-	-	-	-	-
Identified as	*Cupriavidus* sp. MRC1	*Ralstonia eutropha* MRC2	*Pseudomonas stutzeri* MRC3	*Aeromonas media* MRC4	*Ralstonia metallidurans* MRC5

Table 6: Minimum inhibitory concentrations of selected metals for the metallophiles isolated from soil of M.I.D.C. area, Parbhani.

Metallophiles	Resistant to	MIC
***Cupriavidus* sp. MRC1**	Cu	0.30 mg/mL
	Cd	0.25 mg/mL
	Pb	0.20 mg/mL
	Hg	0.15 mg/mL
	Zn	0.25 mg/mL
***Ralstonia eutropha* MRC2**	Cu	0.25 mg/mL
	Cd	0.35 mg/mL
	Pb	0.30 mg/mL
	Hg	0.20 mg/mL
	Zn	0.25 mg/mL
***Pseudomonas stutzeri* MRC3**	Cu	0.25 mg/mL
	Cd	0.20 mg/mL
	Pb	0.35 mg/mL
	Hg	0.15 mg/mL
	Zn	0.30 mg/mL
***Aeromonas media* MRC4**	Cu	0.15 mg/mL
	Cd	0.15 mg/mL
	Pb	0.30 mg/mL
	Hg	0.25 mg/mL
	Zn	0.20 mg/mL
***Ralstonia metallidurans* MRC5**	Cu	0.25 mg/mL
	Cd	0.20 mg/mL
	Pb	0.25 mg/mL
	Hg	0.10 mg/mL
	Zn	0.40 mg/mL

CONCLUSIONS

Five metallophilic bacteria were isolated from sediment soil samples collected from effluent canal of M.I.D.C. Parbhani. Taxonomic assessment of the isolates revealed that *Cupriavidus* sp. MRC1 and *Ralstonia metallidurans* MRC5 belonged to the class betaproteobacteria and family burkholderiaceae; *Ralstonia eutropha* MRC2 belonged to class betaproteobacteria and family ralstoniaceae; *Pseudomonas stutzeri* MRC3 belonged to class gamma proteobacteria and family pseudomonadaceae and *Aeromonas media* MRC4 belonged to class gamma proteobacteria and family aeromonadaceae. The isolated metallophiles may be useful as indicators of potential toxicity of heavy metals in industrial effluents and these isolates can be used as a bioremediation tool for the treatment of effluents from industries handling heavy metals. Beside their use in bioremediation, these isolates are producer of some industrially important enzymes like amylase, protease and lipase.

ACKNOWLEDGEMENT

The authors are thankful to the Hon. Vice-Chancellor, S.R.T.M. University, Nanded for providing infrastructure and necessary facilities.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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