Impact of Mutation on Metal Resistance in *Pseudomonas Spp.*

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ABSTRACT

The toxic heavy metal pollution of the environment is a serious environmental concern. The bacterial resistance to heavy metals is of practical significance and can be useful for remediation of heavy metal polluted sites. In this research, the impact of mutation in the metal resistant bacteria isolated from wastewater was investigated. The Polluted water sample was collected from Waldhuni river near Ulhasnagar, District Thane, Maharashtra, India. Pseudomonas spp. were isolated from contaminated wastewater using cetrimide agar medium and confirmed on the basis of morphological, cultural and biochemical characteristics. Pseudomonas spp. showing tolerance to the metals (0.5mM) were selected and Minimum inhibitory concentrations for copper (Cu) and zinc (Zn) were determined. 5 different Pseudomonas spp. showing the highest tolerance to the metals were exposed to Sublethal concentrations of the two mutagenic agents, acridine orange and ethidium bromide and the minimum inhibitory concentration values for copper and zinc metal ions were again determined for the mutant strains. Two Pseudomonas spp. showed the prominent increase in minimum inhibitory concentrations value. Thus, the results concluded the effectiveness of the artificially mutated bacteria as an appropriate solution for the treatment of contaminated wastewater containing heavy metals.

Keywords: Bioremediation, Mutation, Pollution, Wastewater.

INTRODUCTION

Water pollution due to heavy metals is a global problem. Indiscriminate disposal of wastes from industries, housing areas, agricultural fills is one of the major causes of gross pollution of many rivers in India and in many other countries. (Moore et al., 1998). There are microscopic organisms in the environment like bacteria that have ability to grow and absorb effluents containing heavy metals (Lovely and Coates, 1997). The heavy metal resistant bacteria are capable of bioaccumulation of high concentrations of metals like Ag, Cu, Pb or Cd can play an important role in clean up or bioremediation of the effluents from heavy metal industries (Shakibaieet al., 1999; Wood and Wang, 1985). Humans are exposed to heavy metals since long back, but rapid industrial development and urbanization have augmented the issue.

Therefore, bioremediation of heavy metals is very important for environmental health. Resistance to heavy metals is observed in a wide variety of bacteria, especially in Gram negative bacteria, such as Pseudomonas, Alcaligenes, Ralstonia and Burkholderia (Lovely, D. R., 1993; Diels et al., 1999). Bacteria of the genus Pseudomonas are well-studied and are of great interest not only because of their high resistance to heavy metals and other toxic substances but also due to their simple nutritional requirements and rapid growth on standard laboratory media. Hifelieet al., (1984) used Pseudomonas stutzeriisolated from silver mines for the removal of heavy metals from industrial wastes.

The mutagenic agents like acridine orange and ethidium bromide have ability of intercalating with DNA and thus cause frameshift mutation. Induced mutations in resistant strains have been reported to increase the tolerance limit and thus enhance the potential of such strains in the management of metal contaminated sites. Future research on induced mutations would also be important in the functional genomics of stable bacterial strains for bioremediation studies. Thus, the objective of this research was to study the impact of the mutation on metal resistance in Pseudomonas spp. for bioremediation of metal contaminated sites.

MATERIALS AND METHODS

A. Sample collection

Water sample was collected from the polluted Waldhuni river near Ulhasnagar, District Thane, Maharashtra, India.

B. Determination of concentrations of Cu and Zn in the polluted water

The pH of the collected sample was determined by using pH paper. For measurement of concentrations of Cu and Zn, 300 mL of polluted water was filtered using blotting papers. The concentration of the heavy metals in the effluents was then determined by atomic absorption spectrophotometer attached to a graphite analyzer.

C. Isolation of Pseudomonas SPP from wastewater

For isolation of Pseudomonas spp, the collected water sample was streak inoculated on the selective media, cetrimide agar and incubated at 35°C for 24 h. The plates were observed for bluish green colonies and isolated colonies were further confirmed by the biochemical tests (shakibaieet al., 1999).

D. Determination of MIC

All the selected colonies were then studied for their tolerance towards heavy metals. The minimum inhibitory concentration (MIC) of heavy metals for isolated strains was determined by broth dilution technique (Xin Cai et al., 2006). For MIC experiment all the selected organisms were grown for 8 h in 20 mL sterile Muller Hinton broth separately and 0.1 mL log phase (108cells/mL) cultures were inoculated into serially diluted Muller-Hinton broth containing 0.5, 1.0, 5.0, 10, and 20 mM concentrations of copper and zinc. The sensitivity of the isolates was determined by observing growth in the form of turbidity after 24-48 h at 35°C.

E. Induction of mutation

The ability of the organisms to grow in higher concentrations of copper and zinc was augmented by exposing the isolates to different mutagenic agents like acridine orange and ethidium bromide. For induction of the mutation, the Gradient plate technique (GPM) was used. For GPM, 40 mg of mutagenic agents were dissolved in 100 mL D/W and mixed properly. To 10 mL of melted nutrient agar medium, 1 mL mutagenic agent solution was added and immediately plated into Petri plates and kept in a slanting position. After solidification of the medium, 10 mL Nutrient agar was poured onto the medium and allowed to solidify, by this method a gradient concentration of mutagenic agent was created. 0.1 mL of the bacterial culture was then spread into the medium and incubated for 24 h at 35°C. The colonies showing growth at the highest gradient concentration of the medium were selected and further studied for their tolerance to heavy metals by broth dilution technique.

RESULTS

The pH of the collected wastewater was found to be 5.2 indicating the acidic nature of the water. The concentration of Cu and Zn in the collected water was 50.525 ppm and 20.130 ppm respectively. The cetrimide agar is a selective medium for Pseudomonas which shows bluish green growth. 14 morphologically distinct colonies were observed on cetrimide agar and were found to be of Gram negative motile, aerobic non spore forming short rods. Further, all the isolates were spot inoculated on Muller-Hinton agar containing 0.5mM Cu and Zn separately. Only 5 Pseudomonas spp showed growth in presence of 0.5mM Cu and Zn hence were selected for further study. Pseudomonas spp were confirmed on the basis of their oxidase activity and ability to ferment glucose and mannitol by aerobic fermentation. The sensitivity of 5 different Pseudomonas strains isolated from polluted water was determined by the broth dilution technique (Table 1). Strains 3 and 4 showed the highest MIC value for Cu and Zn and showed growth up to 1.0 mM and 2.0 mM concentration respectively while the remaining strains showed growth till 0.5 mM concentration of Cu and 1:0 mM of Zn. After subjecting to the mutating agent acridine orange, all the strains showed an increase in their tolerance towards both the heavy metals (Table 2). Strains 3 and 4 showed a prominent increase in MIC value and both the strains showed growth up to 5mM for Cu and 15mM for Zn with $P<0.5$, while ethidium bromide did exert an increase in the MIC values but comparatively less than acridine orange (Table 3).

Table 1: The MIC values of Cu and Zn for the isolated Pseudomonas strain

Isolate No.	Growth in presence of Cu concentrations (mM)					Growth in presence of Zn concentrations (mM)				
	0.5	1:0	1:5	2:0	2:5	0.5	1:0	1:5	2:0	2:5
Isolate 1	+					+	+			
Isolate 2	+					+	+			
Isolate 3	+	+				+	+	+	+	
Isolate 4	+	+				+	+	+	+	
Isolate 5										

Table 2: The MIC values of Cu and Zn for the isolated Pseudomonas strain after exposure to acridine orange

Isolate No.	Growth in presence of Cu concentrations (mM)					Growth in presence of Zn concentrations (mM)				
	2.5	5	10	15	20	2.5	5	10	15	20
Isolate 1	+					+	+			
Isolate 2	+					+	+			
Isolate 3	+	+				+	+	+	+	
Isolate 4	+	+				+	+	+	+	
Isolate 5	+					+	+			

+ = growth, - = no growth

Table 3: The MIC values of Cu and Zn for the isolated Pseudomonas strain after exposure to ethidium bromide

Isolate No	Growth in presence of Cu concentrations (mM)					Growth in presence of Zn concentrations (mM)				
	2.5	5	10	15	20	2.5	5	10	15	20
Isolate 1	+					+				
Isolate 2	+					+				
Isolate 3	+					+	+			
Isolate 4	+					+	+			
Isolate 5	+					+				

+ = growth, - = no growth

DISCUSSION

In the present study, the impact of the mutational enhancement technique with respect to tolerance to heavy metals was studied in Pseudomonas spp. The polluted water sample was collected and 14 Pseudomonas strains were isolated. Pseudomonas strains were the predominant bacteria which could tolerate high concentrations of Cu and Zn. The isolated strains were subjected to different concentration of Cu and Zn, some could grow up to 2.5 mM Cu and 5 mM of Zn. Similar results have been reported by Shakibaieet al., (2008).

To enhance the accumulation of heavy metal ions, isolated bacterial cells were exposed to two mutagenic agents, acridine orange and ethidium bromide. These agents are intercalating dyes and are capable to bind to the DNA of bacteria and induce frame shift mutation, therefore they are strong mutagenic agents. The mutagenic agent, acridine orange had a profound effect on the ability of isolates to grow at very high concentrations of Cu (10 mM) and Zn (20 mM) compared to ethidium bromide.

A genetically engineered Acinetobacter baummannii has been reported to remove 2.5 mg/g biomass of Ag from effluents of the film industry (Shakibaieet al., 1999). Xin Cai et al., (2006) studied the tolerance and biosorption of copper and zinc by Pseudomonas. putida CZ1 isolated from metal-polluted soil and reported removal of about 8.72% of Cu and 9.8% of Zn during the active growth cycle.

CONCLUSION

The data presented in this paper indicated the use of mutation for the enhancement of metal resistance in bacteria. Such mutant bacterial with increased metal tolerance would considerably enhance the bioremediation of heavy metals from effluents of the factories and improve the disposal problems of the waste with little expense.

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