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Isolation of Manganese Tolerant Strains from Effluents of Talcher Thermal Power Station, Orissa, India

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Abstract

With the continuous discharge of untreated industrial effluents into the inland surface water such as river, lakes, reservoir etc. depletion of the dissolved oxygen concentration in water occurs resulting in aquatic life hazards and when it's used to discharge in raw land, the post-effects will be serious groundwater contamination and also degradation of soil fertility of that region. Samples were collected from six different sites of TTPS such as Brahmani river, Ash pond (Clear water), Ash pond (Irrigation), Sewage treatment plant, Ash pond (Ash) and Effluent pipe. Out of 25 isolates fifteen were studied for Manganese tolerance out of which five strains showed better tolerance such as 50 ppm, 15 ppm, 40 ppm, 50 ppm and 30 ppm respectively in comparison with others. These five isolates were characterized morphologically, microscopically and biochemically and were identified as *Bacillus* sp., *Micrococcus* sp., *Pseudomonas fluorescens*, *Bacillus* sp., *Alteromonas* sp., following Bergey's manual of systematic Bacteriology. Two strains such as TMB-1 and TMB-8 belongs to *Bacillus* sp., showed a maximum tolerance i.e. upto 50ppm whereas TMB-4 i.e. *Micrococcus* sp., showed a minimum tolerance i.e. upto 15ppm. Other ten strains showed resistance to manganese in the range 1 ppm up to 5 ppm. Though Manganese concentration in the sample was low but tolerance level of the isolates was higher i.e. upto 50ppm. These isolates were found to have a great potential in utilizing Mn (II) in the media supplemented with the metal. Study for these manganese tolerant strains is to develop a safety strain by recombinant DNA technology which will be able to serve the purpose in a more efficient way eliminating 100% of the metal and allowing the waste effluents to be reused once again thereby solving some problem of water scarcity. Hence, these strains may serve as "eco-friendly bioremediation tools".

Key words: TTPS, Manganese tolerance, bioremediation, waste effluents, eco-friendly bioremediation tools

Introduction

Industrialization and globalization are the new trends of modern century inviting with them a lot of environmental stresses. In the process, it's the water resources which are under maximum exploitation. The water quality is degraded on a daily basis with the discharge of toxic chemicals and heavy metals in varied concentrations. The bio-chemical characteristics of effluents of some distilleries and sugar industries have shown the presence of high organic content[1]. One of the rising concerns is the heavy metal pollution of the water resources and the constant bioaccumulation of these

Wastewater from electroplating, battery manufacturing, ceramics, dyeing, textiles, mining industries, thermal power stations, steel plants, metal soldering industries are the one discharging the major portion of heavy toxic metals in their effluents[2]. Even the indiscriminate use of heavy metal containing fertilizers and chemical pesticides in agricultural fields are leading to deterioration of water quality posing a high threat to human beings as well as aquatic lives[3]. The deadlier effects of the metals such as Pb, Cd, Mn, Cu etc. are gastrointestinal, muscular, reproductive, genetic and neurological malfunctions, edema of eyelids, tumours, congestion of nasal mucous membranes and pharynx, stiffness of head and many others[4-6].

The metal microbe interaction is widespread in nature, causing a diversification in environment. Amongst all naturally occurring metals, several are indispensable for growth, differentiation, metabolism and proper biological functioning of various prokaryotic and eukaryotic cells[7]. Living as well as dead microbial cells, as well as products derived from microbial transformation are capable of heavy metal removal along with metalloids, organometals and even organometalloids from industrial effluents[8]. Natural tolerance to one or more of these high density metals occurs in many bacterial species with a sufficiently high frequency, even in the absence of frequent exposure to the metal source, which helps in the selection of some good heavy metal tolerating strains from the complex population[9]. As a case, it has been reported in some gram-negative, mesophilic, acidophilic, aerobic *Acidiphilium* strains which have an extremely high natural tolerance to Cd, and resistance to Cu, Ni and Zn even after culturing for several years in-vitro, in a simple growth medium devoid of any heavy metal[7]. The theoretical and practical evidence for micro-remediation as a feasible alternative to the existing system of removal of heavy toxic metals from liquid effluents emphasizes on the maximum usefulness and utilization of microbes in metal accumulation. Various species of *Bacillus, Pseudomonas, Desulphovibrio,Desulphotomaculum, Streptomyces,* have proved to eliminate a major portion of heavy metals supplemented in the growth media; optimal pH, growth conditions, nutrient supplementations, are some of the driving factors for the maximum metal uptake varying from species to species[10]. An attempt was made here, to propose a natural remediation system which will serve as a powerful strategy in solving the problem of heavy metal pollution. The present study was undertaken to find suitable means for reducing the threatening effects of the effluents discharged from Talcher Thermal Power Station (TTPS) in Talcher district of Orissa, by identifying the scavenging potentials of various bacterial strains that grow in the polluted regions and effectively utilizing them as a biotechnological weapon for waste-water treatment. Hence, the study was aimed at the isolation of manganese tolerant strains from effluents discharged from a long term thermal power station and the nearby contaminated soil and water bodies.

Experimental

Sampling site

Talcher Thermal Power Station is India's one of the coal based power stations situated in the Talcher Township in Angul district of Orissa. It has been generating a high energy of 460 MW from its various working units. The coal is extracted from the Talcher mining zone and a good supply of water is supported by Brahmani river. Earlier the raw materials were loaded in trucks to reach the power station, but from a long time the concept of conveyor belt is being used. A 24 hour system is running from the coal fields up to the power plant which is a boosting factor in high efficiency power production by eliminating the time cost burden. The water from Brahmani river is stored in a big reservoir alongside the power plant. The effluents are allowed to discharge in a far away area near the mining fields to utilize as landfill.

Collection of Sample

Samples were collected from six different sites such as Brahmani river, Ash pond (Clear water), Ash pond (Irrigation), Sewage treatment plant, Ash pond (Ash) and Effluent pipe in the outskirts of the thermal power station (Fig 1). Effluent samples were collected in one litre high density polythene (HDPE) bottles. The bottles were cleaned with 6M nitric acid (HNO_3) and milli-Q water prior to sampling. While collecting the sample, the HDPE bottle was first rinsed with the sample so that the inner surface of the bottle get surface adsorbed with the sample to be filled in. Two bottles of sample were collected from each site. After collection each bottle was capped tightly, marked well and kept in ice box for carrying to the laboratory. In the laboratory the samples were stored at a temperature of 4^0C till the time of analyses/study.

Analysis of Physico - Chemical Parameters

Electrical conductivity and pH of effluent samples were measured at the collection site by using a conductivity tester (HI-98303) and a digital pH meter (HI-98107) respectively.

Heavy Metal Analysis

As the main aim of the present study is to isolate various heavy metal tolerating bacterial species from the polluted regions, the concentration of various heavy metals in samples collected were analysed by using atomic absorption spectroscopy (Shimadzu, AA-6300). Each sample was shaken well and filtered through a membrane filter of pore size 0.45µ and transferred to an acid cleaned volumetric flask. The filtrate was made 2% acidic with addition of concentrated nitric acid (HNO_3). The samples along with blank were analysed by AAS after calibrating the instrument with different standards such as Cr (0.5, 1 & 2.5 ppm), Pb (2, 4 & 10 ppm), Fe (1, 2 & 4 ppm), Zn (0.5, 1 & 2 ppm) and Mn (1, 2 & 5 ppm).

Isolation of Bacteria

Separate sets of samples were taken for microbiological studies. Before performing any biochemical experiments, the glass wares and petriplates were sterilized properly. Nutrient agar medium was used for cultivation of total viable count of bacteria by spread plate method. Then the plates were incubated at 35^0 C for 24 hours. The same procedure was repeated for all the six samples and the total colony forming unit CFU/ml was calculated (Table-1). Single colonies were pricked from the mixed culture plate and pure culture of each colony was obtained by repeated subculturing. The pure cultures were transferred to slants and were maintained in each 15 days interval and were stored at 4^0C.

Selection of Manganese tolerant strains

From the six samples 15 different types of colonies were isolated. These strains were then named as "TMB" (Talcher Metal tolerating Bacteria) according to their collection place and characteristics. For the Manganese tolerance of individual strains Manganese (II) sulphate monohydrate purified ($MnSO_4.H_2O$) was used as the source of manganese. After preparation of stock solution (100ppm) nutrient broth supplemented with different concentrations of manganese such as 5 to 50ppm was prepared and sterilized. The test cultures were inoculated in liquid medium and incubated for 24 hours. Then measured amount was transferred to the metal supplemented broth and incubated. AAS analysis data for manganese concentration was recorded at 0 hour and after 96 hours. For each experiment a control was taken in which no innoculum was added. Control reading was also taken after 96 hours to detect the change of concentration of manganese.

Identification of the Selected Manganese tolerant strains

The morphological characteristics of the bacterial colonies were studies following the standard microbiological methods. The shape, size, colour, margin, opacity, elevation and surface were recorded from the isolated colonies, after streaking on nutrient agar plates and incubating for 24 hours at 28-30 ^{0}C. Microscopic study has been carried only by Gram's staining methodology. After morphological and microscopic study, different biochemical tests were carried out such as growth of the organisms in different NaCl concentrations, catalase, urease, indole, methyl red, nitrate reduction, citrate utilization, phenylalanine deami--nation, ornithine utilization, lysine utilization, H_2S production, starch hydrolysis, casein hydrolysis, sugar utilisation (mannitol, sorbitol, glucose, sucrose, lactose, starch) and activity against different antibiotics such as streptomycin, gentamycin and ampicillin.

Results and Discussion

In all samples pH was more than 7.0 whereas in case of the sample-6 i.e. from effluent pipe, pH was 6.57 making it a little acidic. Sample-1 and sample-5 showed neutral pH such as 7.1 and 7.2 respectively. Samples- 2, 3 & 4 were in a higher side, with sample-4 being the most alkaline i.e. 8.29. However pH of all samples were well within the permissible limits for discharge of effluent[11]. The electrical conductivity of the samples measured with a digital conductivity meter recorded the lowest EC i.e. 120µS in Sample 1 and highest i.e. 284µS in sample-6. The total colony forming unit (CFU/ml) of different samples were calculated and it was higher i.e. 8.5×10^7 in case of the sample collected from sewage treatment plant (Sample 4) and least i.e. 4.6×10^3 in the sample collected from Brahmani river (Sample 1). In other samples the total count was between 5.7×10^5 to 7.4×10^6 CFU/ml (Table 1).

The six samples collected from different regions showed varied concentrations of heavy metals (Table 2). Among the five heavy metals, manganese showed highest concentration i.e. 6.398 ppm in the sample collected directly from the effluent pipe. Other metals such as Cr, Pb, Fe and Zn the concentrations were 0.343ppm, 0.350ppm, 1.612ppm and 1.684ppm respectively. Sample 6 collected from the effluent pipe showed the highest concentration of manganese as compared to other samples. Sample-1 collected from the Brahmani river showed the presence of lowest amount of manganese in it, but the iron concentration was comparatively higher. Sample-2 contains manganese and iron in a concentration of 1.8 ppm and 1.0 ppm respectively, with other three metal concentrations below 1.0 ppm. Sample-3 was relatively less metal contaminated, witnessing a higher manganese concentration among other metals. Sample-4 has all the metals in the range 0.2 to 3.0 ppm. Sample - 5 has iron, zinc and manganese in a sufficient concentration whereas chromium and lead in an amount below 1.0 ppm. Sample-6 has chromium concentration more than 6 ppm and others in the range 0.3 to 1.6 ppm. Summarizing all the data, it has been found that the concentration of chromium, iron, lead, manganese and zinc in the range 0.09 to 0.3 ppm, 0.7 to 1.6 ppm, 0.08 to 0.3 ppm, 0.6 to 6.3 ppm and 0.02 to 1.6 ppm respectively in the six different samples collected; hence revealing a wide spectrum for manganese contamination. Manganese content of all samples were above the permissible limits of drinking water quality[12]. Likewise concentrations of other metals such as Cr, Pb and Fe remained higher than the permissible limits of drinking water in all samples. Lead (Pb) content of all samples except Sample-1 (Bramhani river) showed higher than the standards for discharge of effluents to public water body. From the six different samples collected, a total of 15 isolates were obtained out of which five more potent strains were selected for further study basing on their Mn tolerance activity (Table-5). Identification of those strains such as TMB -1, 4, 5, 8 & 9 was done by different biochemical tests (Table-4). Antibiotic sensitivity of those isolates were done using different antibiotics such as Streptomycin (S), Ampicillin (A) and Gentamycin (G) and the inhibition zone was recorded. All the five isolates were sensitive to Gentamycin (22mm). All the strains were sensitive to Streptomycin except TMB–5 which is resistant

Table 1. Physico-chemical and microbiological characteristics of the water samples collected from TTPS, Orissa

S.No.	Collection site	pH	Electrical conductivity (µS)	Colony forming unit per ml
Sample 1	Brahmani river	7.15	120	4.6×10^3
Sample2	Ash pond (Clear water)	7.81	236	7.4×10^6
Sample3	Ash pond(Irrigation)	7.70	185	5.7×10^5
Sample4	Sewage treatment plant	8.29	194	8.5×10^7
Sample5	Ash pond (Ash)	7.25	225	6.4×10^6
Sample6	Effluent pipe	6.57	284	5.6×10^6

Table 2. Metal ion concentrations (ppm) of water samples colleted from TTPS, Orissa

Sample	Concentration				
	Cr	Pb	Fe	Zn	Mn
1	0.094	0.087	1.612	0.034	0.639
2	0.187	0.350	1.038	0.051	1.874
3	0.187	0.175	0.718	0.028	4.370
4	0.249	0.175	0.814	0.135	3.945
5	0.343	0.175	1.533	1.342	4.616
6	0.343	0.087	0.782	1.684	6.398

(10mm). In case of ampicillin, four strains were resistant whereas TMB–5 was showing intermediate response (16mm) (Table-2). The strains were identified following Bergey's manual of Systematic Bacteriology upto genus level. TMB 1 was identified as *Bacillus* sp. TMB 4 as *Micrococcus* sp., TMB 5 as *Pseudomonas fluorescens*, TMB 8 as *Bacillus* sp., and TMB 9 as *Alteromonas sp.* (Table 5).

As per studies, microorganisms undergo various selection stress for survival in the presence of toxic substances and slowly develop a miraculous response to them[13, 14]. The most common resistance is to a metal which may be a response to essentiality of the metal for carrying out various biological functions by the microbe[14]. Because of stringent environment issues, the industries these days have become very particular about pollution regulation. Various boards and departments have been established as a part of the large scale projects to check the pollution status from time to time, whether its fly ash or slurry. The metal concentration level has been reduced to several low levels as a result of pre-treatment of the wastewaters before discharge. So, it has been found that the concentrations of the heavy metals in the samples in a much low concentration (Table1). Nowadays, the effluents are being utilized as multi-purpose source rather than disposing them in water bodies. Like as in Talcher Thermal Power Plant, the effluents discharged in the slurry state is used as landfill, and the residual water gradually gets collected at a lower region by continuous seepage. In this way the contamination of the water sources is reduced to a higher extent. Though the inhabiting source has a concentration around 6.5 ppm, but the strains could tolerate a much higher concentration of manganese metal when grown in-vitro. While selecting for the manganese tolerating strains, a negative control has been taken to obtain error free results. The control without inoculums showed a zero reduction of manganese concentration after 96 hours of inoculation; the culture broth showed high turbidity in 24 hours to 48 hours after inoculation. The culture broths showed a much reduced concentration of manganese after 96 hours in AAS, while some had attained their death phase still some others were still alive even after 96 hours (Fig 2 & 3). NaCl tolerance though is positive for all the five isolates, but *Bacillus* sp. showed the maximum turbidity after24 hours as compared to others. By performing all the above mentioned biochemical and physiological tests, the strains were identified upto the genus level, but for 100% identification many more confirmatory tests and molecular level study need to be done.

Conclusion

The abundance and activity of culturable manganese tolerant bacterial strains were assessed from the wastewater samples of Talcher Thermal Power Station. These strains can perform the bio–removal of the toxic heavy metal (Mn) from contaminated solutions by living as well as dead biomass as witnessed in the study. There is also a chance of metal transformations which may be associated with the decrease in toxicity level of the culture media after 4 to 5 days and therefore, may have a significant relevance with wastewater treatment. Most living cell systems exploited to date have been used for the detoxification of effluents below toxic levels but microbes have proved in a better way in the decontamination of wastewater containing various heavy metals above toxic limits[15,16]. The manganese reducing efficiencies in these strains was found to be more than 80%. These strains will prove highly efficient and effective in bioaccumulation of a major part of manganese from the effluents thereby reducing the toxicological effects of the pollutants on various water bodies nearby. Another study for these manganese tolerant strains is to develop a safety strain by recombinant DNA technology which will be able to serve the purpose in a more efficient way eliminating 100% of the metal and allowing the waste effluents to be reused once again thereby solving some problem of water scarcity. These isolates have proved to have a great potential in detoxifying Mn (II) in the media

Table 3. Colony morphology of the isolates showing maximum Mn tolerance

Type	Form	Elevation	Margin	Surface	Opacity	Size	Colour
TMB1	Regular	Flat	Wrinkled	Shiny & Slimy	Opaque	Medium	Pale yellow
TMB4	Regular	Flat	Round	Slender	Opaque	Tiny	Pale yellow
TMB5	Regular	Convex	Round	Shiny	Opaque	Small	Yellow
TMB8	Regular	Flat	Wrinkled	Dry	Translucent	Medium	Pale white
TMB9	Irregular	Flat	Wrinkled	Dry	Opaque	Large	Off- white

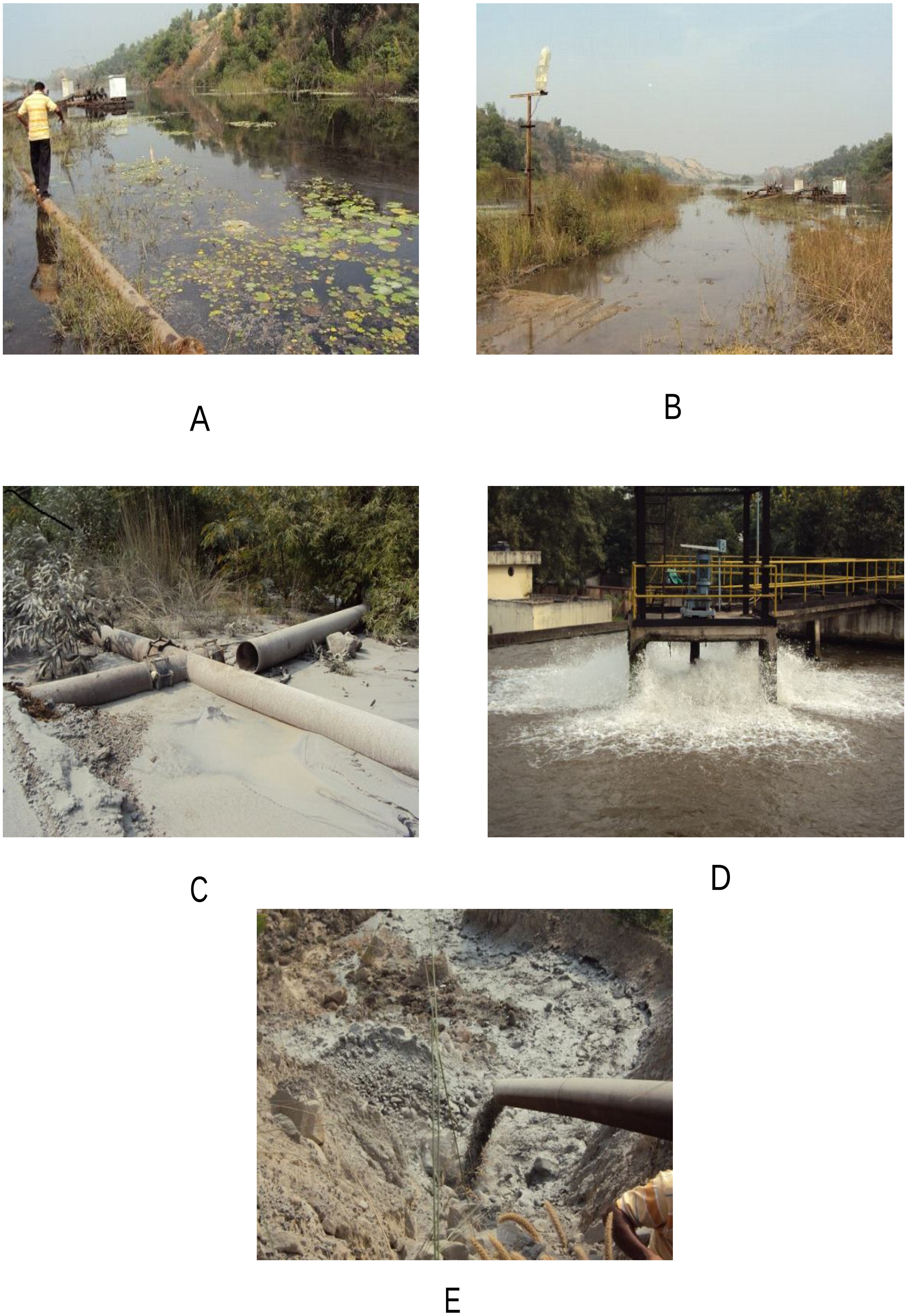

Fig. 1. Sampling sites of TTPS, Talcher

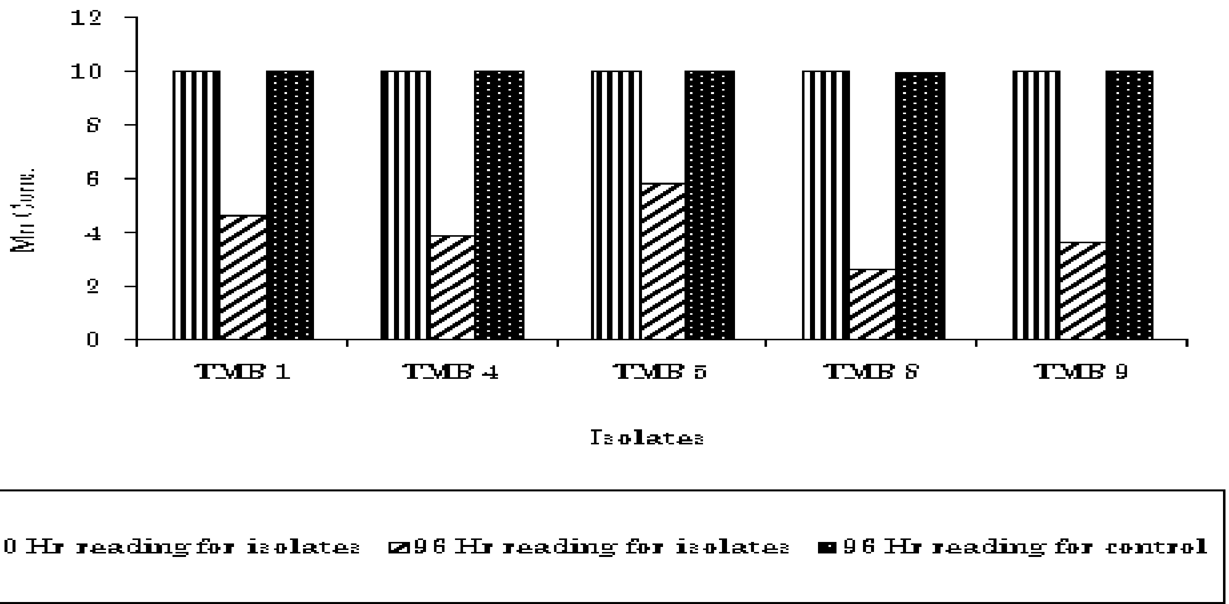

Fig 2. Activity of the manganese tolerant strains in 10 ppm source of Mn

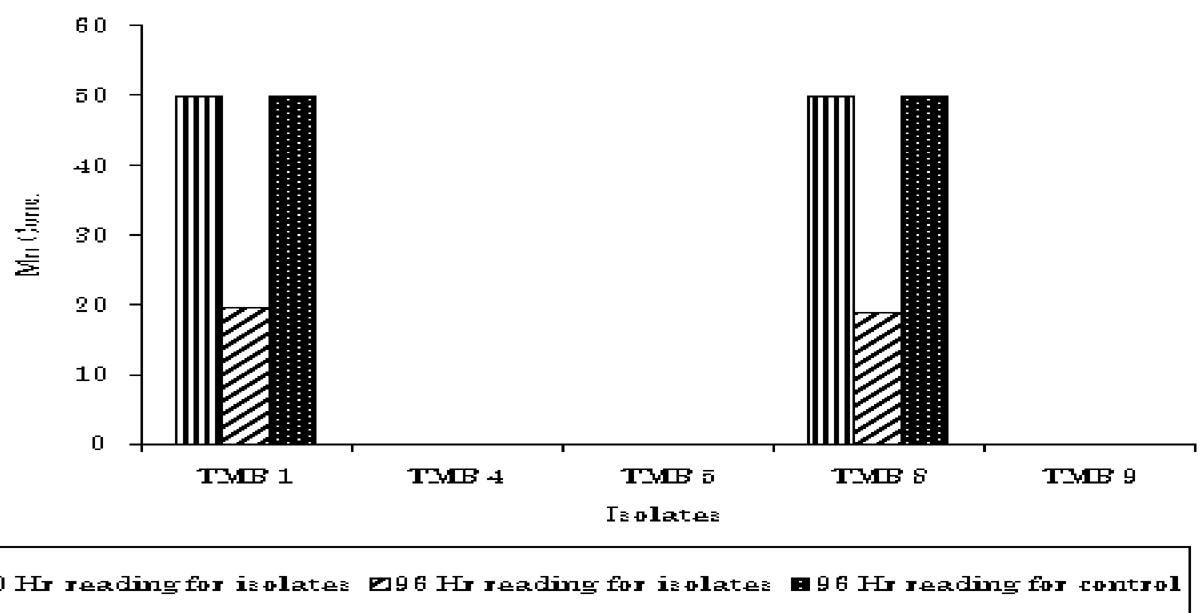

Fig 3 . Activity of the manganese tolerant strains in 50 ppm source of Mn

Table 4 . Biochemical test results for the five manganese tolerant strains

Biochemical tests	Results				
	TMB 1	TMB 4	TMB 5	TMB 8	TMB 9
Gram's Staining	+ve, Bacilli, Single rods	+ve,Cocci	-ve, Bacilli	+ve, Bacilli	-ve, Bacilli, Chain of 3-4 rods
Starch Hydrolysis	+	+	-	+	-
Casein Hydrolysis	+	+	-	+	+
Catalase	+	++	++	+	++
Indole Production	-	-	-	-	-
Methyl Red	-	-	-	-	-
Voges -Proskauer's	-	-	-	-	-
Citrate Utilization	+	+	-	+	+
Glucose	+	-	+	-	-
Adonitol	-	-	-	-	-
Arabinose	-	-	-	-	-
Lactose	-	-	-	-	-
Sorbitol	-	-	-	-	-
Mannitol	+	-	+	-	-
Rhamnose	-	-	+	-	-
Sucrose	+	-	+	+	-
Lysine	+	+	+	+	+
Ornithine	+	+	+	+	+
Urease-	-	-	-		-
Phenylalanine Deamination	+	+	-	-	-
Nitrate Reduction	-	-	-	-	-
H_2S Production	-	-	-	-	-
NaCl Tolerance(2%)	+	+	+	+	+
Antibody Sensitivity	G – Sensitive S - Sensitive A- Resistant	G – Sensitive S - Sensitive A- Resistant	G – Sensitive S - Resistant A- Int	G – Sensitive S - Sensitive A-Resistant	G –Sensitive S - Sensitive A-Resistant

G- Gentamycin, S- Streptomycin, A- Ampicillin

supplemented with the metal. Protein engineering is another aspect that can be used to improve enzymes that can reduce manganese more efficiently, from these strains that can minimize manganese toxicity and can also function in the presence of other pollutants. Hence, these strains may serve as "eco-friendly bioremediation tools".

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Table 5. Identification results of the Mn tolerant strains

Strains	Sample (isolated from)	Identification
TMB 1	1	*Bacillus sp.*
TMB 4	6	*Micrococcus sp.*
TMB 5	2	*Pseudomonasfluorescens*
TMB 8	6	*Bacillus sp.*
TMB 9	5	*Alteromonas sp.*

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