Research in Biotechnology, 3(3): 01-11, 2012 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Metal tolerance in halotolerant bacteria isolated from saline soil of Khambhat

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Five halotolerant bacteria were isolated from saline soil of Gujarat. Their identification and characterization with respect to optimum pH and salt concentration, and metal tolerance was carried out. Among all isolates *Virgibacillus salarius* exhibited better metal tolerance/resistance. In certain cases stimulatory effect of metal ions on growth was also observed. Such organisms can serve as a good model for study of stress response among prokaryotes, and can also be explored for their potential of bioremediation of metal polluted saline sites with alkaline pH.

Keywords: Halotolerant**;** Metal resistance; Molecular identification

In the present study, we report natural metal tolerance levels of certain halotolerant bacteria isolated from salt pan soil of Khambhat, Gujarat, India. Such information may prove useful, as metal-resistant halotolerant bacteria could be used as bioassay indicator organisms in polluted saline environments (Nieto *et al.*, 1989). Heavy metals are often defined as a group of metals whose atomic density is greater than 5 g/cm^3. Metals play a vital role in the metabolic processes of all the living forms including microorganisms. Some of them are required by the organisms as essential micronutrients (cobalt, chromium, nickel, iron, manganese, zinc, etc.) and are known as 'trace elements'. On the other hand, some heavy metals are detrimental to the organism even at low concentration (mercury, lead, etc.). However, at high levels both the essential and non-essential metals become toxic to the organisms (Rathnayake, 2009).

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of metals (especially cadmium, copper, and zinc) to the environment. Natural as well as anthropogenic sources account for this contamination, which poses threat to public health. High concentrations of toxic metals have caused environmental pollution in agricultural soils, surface and underground water. Microorganisms play a major role in bioremediation or biotransformation processes of these toxic elements, converting them to less toxic or non-toxic elements. Determining the potential of microorganisms and their tolerance against high concentration of toxic metals will assist the selection of suitable species for bioremediation and biotransformation of toxic metals (Trevors, 1985). Halophilic and halotolerant microorganisms are suitable candidates for these processes as they have exceptional properties, that is high concentrations of anions and cations are necessary for the growth of halophilic microorganisms (Ventosa, 1998), hence they are not only naturally tolerant to some elements that are toxic to other microorganisms, but also they have a requirement for these elements (Amoozegar, 2005).

Received : April 10, 2012; Accepted : May 14, 2012

Many approaches have been used to assess the risk posed by the contaminating metals in soil, water bodies, etc. The tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota, which has led to an increased interest on studying the interaction of heavy metals with micro-organisms. The preferred approach is to select the organisms that can be used to develop tools to assess the metal levels in the environment (Rathnayake, 2009). Metals may be present in saline soils of alkaline pH in bioavailable and non-bioavailable forms. To simulate organisms response to one or more metals present in either of these forms, it may be useful to perform experiments at a pH and salinity characteristic of that particular soil from which isolation is attempted. Objective of our study was to isolate and identify halotolerant bacteria from uncontaminated saline soil, and to investigate their natural tolerance to different metals.

Materials and Methods

***Sample collection and physicochemical characterisation*:** Soil sample was collected from salt pan of Khambhat located at 22°18′05.33″ N and 72°37′10.78″ E in October 2010. 20 g of this soil sample was mixed with 50 mL of distilled water. This soil suspension was put on rotary shaker for 1 h, and then allowed to settle for 30 min. Supernatant was then subjected to centrifugation at 10,000 rpm for 10 min to remove any residual soil particles. Suspension prepared in this way was then used for measurement of pH (with a pH electrode; EI-111) and salinity (with salinity meter; Eutech, Singapore). Same suspension was used for chloride estimation by argentometric titration method (Eaton et al., 2005).

Isolation and identification: Soil suspension (0.1 mL) was spread onto halophilic nutrient agar (LMG Medium 220; Atlas, 2010) plates with 3 different salt concentrations viz. 5%, 10%, and 15% followed by incubation at 35°C for 24 h. Growth medium (pH 8.3) contained casein peptone (10 g/L; HiMedia, Mumbai), glucose (5 g/L; Merck, Mumbai), yeast extract (5 g/L; HiMedia), and agar agar (15 g/L; HiMedia). Sodium chloride (Merck) was added according to required salt concentration. Among different colonies developed on these plates 5 colonies were selected from the plates with 10% and 15% salt concentration for further experiments. These isolates were named as VJP 1, VJP 2, VJP 3, VJP 4, and VJP 5, and subjected to 16s r-RNA sequencing for molecular identification.

Determination of optimum pH and salt concentration: All the 5 isolates were inoculated onto halophilic nutrient agar (pH 8.3) plates with different salt concentrations in the range 0.5-20%. Similarly for determination of optimum pH they were grown on same media (salt concentration was set at optimum level for each organism as determined from previous experiments at different salt concentrations) with different pH in the range 4-10. Incubation was made at 37°C for up to 8 days. Though isolation was effected at 35°C, later experiments were performed at 37°C owing to better growth of all the isolates at latter temperature.

Metal tolerance: All the 5 isolates were challenged with different metal compounds at various concentrations in the range 0.005-12 mM. Metal compounds used were cobaltous chloride (S d fine chem, Mumbai), cobaltous nitrate (HiMedia), cadmium chloride (S d fine chem), nickel chloride (RFCL, New Delhi), silver nitrate (S d fine chem), cupric chloride (RFCL), and ferric sulphate (S d fine chem). Metal solutions were filter sterilized by passing them through membrane filter (HiMedia) of porosity 0.22 μm. Stock solutions prepared this way in sterile distilled water were stored at 4-8°C for no longer than 4 days.

For the determination of heavy metal tolerance of the isolates agar dilution method was practiced. Tubes containing 20

mL of melted halophilic nutrient agar containing different metal concentrations were poured into sterile Petriplates. After solidification, 0.1 mL inoculum (standardized to 0.5 McFarland turbidity standard) of respective organism was spread onto it, followed by incubation at 37°C for up to 7 days to determine maximum tolerable concentration of a particular metal for different isolates.

Similar experiments were done in liquid medium of same composition, whereby the organism(s) was challenged with one or more metals at a time. For these experiments total volume of the system was kept 5 mL. Tubes containing medium without metals and inoculated with the test organisms served as growth control. Medium with metal but without bacteria served as abiotic control. Inoculum standardized to the OD equivalent to that of 0.5 McFarland turbidity standard was added at 5% v/v. Incubation was made under static condition at 37°C for up to 7 days. Growth was quantified by measuring OD at 625 nm (Themoscientific).

Results and Discussion

Soil characteristics: The soil sample was found to have a pH value of 8.3, salinity 7.08%, and chloride ion concentration 21.62 g/L.

***Characterization and identification of isolates*:** All the 5 isolates were subjected to Gram staining, and their colony characteristics on halophilic nutrient agar were noted (Table 1). All the isolates were capable of growth in same range of salt concentrations (0.5-16%), with optimum being 6% except for VJP 2. According to Kushner's classification all these isolates can be labelled as *extremely halotolerant* (Oren, 2006) as their growth range exceeds 2.5 M salt. VJP 5 was capable of growth in the widest pH range among all isolates (Table 1). Based on 16s r-RNA sequencing VJP 1-5 were identified as *Staphylococcus epidermidis, Bacillus atrophaeus, Halomonas shengliensis*, *Halomonas koreensis*, and *Virgibacillus salarius,* respectively. They were deposited with culture collection of Gujarat State Biotechnology Mission (GSBTM), Gandhinagar (Strain accession code BAB 505-509). GenBank accession code for VJP 1 is JQ389654, and for VJP 3-5: JX081416, JX081417, and JX081418, respectively.

Table 1. Colony characteristics, salt and pH range of all the isolates

Isolate	Gram staining	Colony characteristics	Pigmentation	Salt range (%)	Optimum salt concentration		pH range	Optimum pH
					%	M		
VJP 1	Gram-positive cocci	Pin pointed spherical, raised colonies with even margin	White	0.5-16	6	1.03	6-8	8
VJP 2	Gram-positive rods	Mucoid colonies which tend to merge, develop wrinkles on longer incubation	-	0.5-16	7	1.2	7-9	8
VJP 3	Gram-negative rods	Raised colonies with even margin which develop a dark centre upon longer incubation	Light orange	0.5-16	6	1.03	8-10	9
VJP 4	Gram-negative rods	Raised colonies with distinct centre and uneven margin	-	0.5-16	6	1.03	5-8	8
VJP 5	Gram-positive rods	Flat colonies with uneven margin	White	0.5-16	6	1.03	6-10	9

Metal tolerance:

Single metal tolerance: After challenging the isolates with various metal compounds (0.005-12 mM) their maximum tolerable concentration for different metals were determined (Table 2). Response exhibited by the isolates to different metals was heterogenous. Silver had the most toxic effect on all isolates, none was capable of tolerating above 0.1 mM. Our isolates were also not capable of tolerating cadmium to any significant extent, VJP 5 being the most resistant which tolerated up to 1 mM. Cadmium toxicity in microorganisms may be due to thiol-binding and protein denaturation, interaction with calcium metabolism and membrane damage, interaction with zinc metabolism, or loss of a protective function (Nies, 1999). Cadmium ions may react with chloride ions to form complexes whose nature depends on the chloride concentration (Ventosa *et al*., 1998).

Table 2. Maximum metal concentrations tolerated by all isolates

Metal	Maximum tolerable metal conc. (mM)				
	VJP 1	**VJP 2**	**VJP 3**	**VJP 4**	**VJP 5**
Co $[Co(NO_3)_2]$	3	3	4	No growth at 0.05	6
Co $[CoCl_2]$	4	5	4	3	3
Cd	0.5	0.5	0.5	No growth at 0.05	1
Ni	8	7	4	3	7
Cu	7	7	2	3	9
Ag	0.1	0.1	0.05	0.05	0.05
Fe	4	5	4	1	6

VJP 1, 2 and 5 showed high tolerance to nickel. Nickel is known to be essential for functioning of most microbial hydrogenases and ureases (Madigan *et al*., 2009). The best-known nickel resistance in bacteria, in *Ralstonia* sp. CH34 and related bacteria, is based on nickel efflux driven by a RND transporter. Two systems have been described, a nickel/cobalt resistance Cnr and a nickel/cobalt/cadmium resistance Ncc. Both are closely related to the cobalt/zinc/cadmium resistance system Czc from strain CH34 (Nies, 1999).

VJP 3 and 4 tolerated relatively lesser concentration of copper. Copper toxicity is based on the production of hydroperoxide radicals membrane (Nies, 1999). VJP 1, 2, and 5 tolerated higher concentrations of copper. Besides copper/zinc superoxide dismutases, the most important function of copper is in the cytochrome c oxidase and related enzymes, which are oxygen-dependent terminal oxidases in the respiratory chain of many organisms (Madigan *et al*., 2009; Nies, 1999). VJP 5 displayed highest tolerance to copper (9 mM), followed by VJP 1 and 2 (7 mM). The concentrations tolerated by these bacteria are higher than those reported by Nieto *et al*. (1989).

VJP 5 and VJP 2 were able to grow on media containing up to 6 and 5 mM iron respectively. However one of the possible reasons for this may be the fact that ferric iron is not toxic to aerobic bacteria because of its low solubility (Nies, 1999). Since ferric iron at neutral or alkaline pH exists mainly in a water- insoluble form, its uptake under aerobic condition requires the microbial formation of ligands called siderophores, to render the ferric iron soluble (Ehrlich, 1997; Madigan et al., 2009).

VJP 5 showed highest tolerance to 4 metals, i.e. Cu (9 mM), Fe (6 mM), Co (6 mM), and Cd (1 mM), and second highest tolerance to Ni (7 mM) after VJP 1 which showed highest tolerance to Ni (8 mM). Interestingly, VJP 4 showed high sensitivity to cobalt when corresponding anion was nitrate, but was able to grow when it was

chloride. Except VJP 3 all organisms showed variation in their response to cobaltous nitrate and cobaltous chloride. Only VJP 5 was able to tolerate cobaltous nitrate at a concentration higher than that of cobaltous chloride (Table 2). This indicates that while evaluating microbial response to metal ions, one of the major influencing factors is the anion which is associated with a particular metal cation. In organisms growing at high salt concentrations, an increase in the amount of intracellular chloride is essential if the cells should increase their volume during growth and cell division. High concentrations of chloride were measured inside the cells of certain halophilic bacteria, high enough to be atleast isotonic with the medium (Oren, 2006).

Stimulatory effect of certain metal ions: Certain metal ions exerted a stimulatory effect on growth of the test organism. Growth of VJP 2 was stimulated in presence of cobaltous nitrate as well as cobaltous chloride in solid media at 1 mM concentration. Experiments with cobaltous nitrate were repeated in liquid media too (Table 3). After 18 h of incubation, growth in presence of 1 mM Co was 132% more than positive control, indicating a faster growth rate (and higher cell density thereof) in presence of Co. VJP 2 registered a generation time of 23.15 h in absence of Co. In presence of 2 mM $Co(NO_3)_2$, there was no growth till initial 22 h of incubation. In this case, growth was delayed but maximum cell density achieved was almost similar (only 2.73% lesser) to that of positive control. There was no visible growth observed in presence of 3 mM cobalt. Cobalt is found mainly in the Co^{2+} form, Co^{3+} is only stable in complex compounds. Cobalt occurs mainly in the co-factor B12 (Madigan *et al*., 2009), which mostly catalyses C−C, C−O and C−N rearrangements. In addition, a class of cobalt-containing enzymes, nitrile hydratases, has also been described (Nies, 1999). In a study of haloarchaeal strategies to withstand stress from transition metals taking *Halobacterium NRC-1* (an archaeal halophile) as model organism, similar results were explained. It was shown that there was mild stimulation of growth with lower concentrations of Co(II). Putative metallochaperons have been speculated to have some role in regulating response of organisms to metal ions such as Cu^{+2} and Zn^{+2} (Kaur *et al*., 2006).

Table 3. Growth of VJP 2 in presence of $Co(NO_3)_2$

Time (h)	Growth control		$Co(NO_3)_2$			
			1 mM		2 mM	
	OD_{625}	Cell no. ($\times 10^8$/mL)	OD_{625}	Cell no. ($\times 10^8$/mL)	OD_{625}	Cell no. ($\times 10^8$/mL)
18	0.15	1.5	0.35	3.1	-	-
22	0.18	1.7	0.32	2.8	-	-
42	-	-	-	-	0.17	1.6
51	-	-	-	-	0.16	1.5

Cadmium stimulated growth of VJP 3 at 0.1 mM (Table 4). After 18 h of incubation, growth in presence of Cd both at 0.05 mM and 0.1 mM was higher than that of positive control, 83% higher cell density was recorded at 0.1 mM. However the maximum cell density achieved by organism after further incubation was lesser in presence of Cd. VJP 3 registered a generation time of 7.34 h in absence of Cd. Inducing effect of cadmium on growth and physiology of halophilic phosphobacteria was reported by Ravikumar *et al*. (2009).

Iron had a stimulatory effect on growth of VJP 3 at 2 mM concentration (Table 5). After 18 h, the growth in both positive control and experimental tube (2 mM Fe) was comparable, whereas growth

in presence of 3 mM Fe was 16.36% lesser than positive control. But after 20 h, there was 85.95% more growth in experimental tube (2 mM Fe) indicating that iron at 2 mM promotes the growth of the organism. VJP 3 registered a generation time of 13.68 h, and 2.33 h in absence and presence (2 mM) of Fe. Thus VJP 3 was able to achieve higher cell density at a faster growth rate in presence of Fe at 2 mM. However it should be noted that due to reduced solubility of ferric iron at alkaline pH, actual amount of bioavailable iron is likely to be lesser than that added into the system. Iron (Fe) is biologically the most important heavy metal cation. It is the only macro-bioelement of the heavy metals (Nies, 1999). Microbes need iron in greater amounts than other trace metals. It is essential for functioning of cytochromes, catalases, peroxidases, iron-sulfur proteins, and oxygenases (Madigan *et al*., 2009).

Table 4. Growth of VJP 3 in presence of $CdCl_2$

Time (h)	Growth control		$CdCl_2$			
			0.05 mM		0.1 mM	
	OD_{625}	Cell no. (× 10^8/mL)	OD_{625}	Cell no. (× 10^8/mL)	OD_{625}	Cell no. (× 10^8/mL)
18	0.10	1.1	0.12	1.2	0.19	1.8
21	0.22	2.0	-	-	0.19	1.8
25	0.24	2.2	0.19	1.8	0.22	2.0

Table 5. Growth of VJP 3 in presence of $Fe(SO_4)_3.xH_2O$

Time (h)	Growth control		$Fe(SO_4)_3.xH_2O$			
			2 mM		3 mM	
	OD_{625}	Cell no. (×10^8/mL)	OD_{625}	Cell no. (×10^8/mL)	OD_{625}	Cell no. (×10^8/mL)
18	0.11	1.1	0.11	1.1	0.09	1.0
20	0.12	1.2	0.22	2	0.06	0.8
24	0.15	1.5	-	-	-	-

Transition metals such as manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) are essential cofactors in the physiology of all organisms. Over half of all proteins in every organism are metalloproteins. Although essential in trace amounts, at higher levels these metals can be toxic to cells because they directly or indirectly compromise DNA, protein, and membrane integrity and function (Kaur *et al*., 2006). Uptake of trace metals and their subsequent incorporation into metalloenzymes or utilization in enzyme activation occurs in all microbes. Some examples of metalloenzymes are cytochromes (Fe), cytochrome oxidase aa_3 (Fe, Cu), and superoxide dismutases (Fe, Mn, Cu, Zn) (Ehlrich, 1997).

Challenge with two metals at a time: Based on the organism's response to individual metal ions, they were challenged with different combinations of two metal compounds at a time. Initially organisms were challenged with their maximum tolerable concentrations for those particular metal ions, as recorded in table 2. But none of them was capable of growth when challenged in this way. Then the isolates were challenged with different combinations of two metals at lower concentrations. During these experiments only nitrate salt of cobalt was used, and not the chloride salt.

VJP 1 and 2 were able to grow on solid medium containing both copper and nickel, each held at a concentration of 1mM, but failed to grow at higher concentrations

of these two metals put simultaneously. When VJP 3 was challenged with Cu and Ni (1 mM each), growth rate was somewhat slowed down. Experimental tube had 15.23% lesser cell density than that in control after 24 h of incubation (Table 6). Generation time in control and experimental was 13.68 h, and 23.7 h respectively. This organism was also able to tolerate simultaneous presence of iron and nickel (1 mM each) in solid medium, but was unable to do so when concentrations were raised to 2 mM.

Table 6. Growth of VJP 3 in presence of Cu and Ni (1 mM each)

Time (h)	OD_{625}	
	Growth control	Experimental
18	0.11	0.09
20	0.12	0.11
24	0.15	0.12

When VJP 4 was challenged with Co and Ni (1 mM each), it achieved lesser cell density at a slower growth rate. After 42 h of incubation experimental tube had 55.68% lesser cell density than control. Here nitrate salt of Co [$Co(NO_3)_2$] was used. Earlier when VJP 4 was challenged with 0.005 mM of $Co(NO_3)_2$, it was not able to grow, however it was capable of tolerating up to 3 mM of chloride salt of Co (Table 2). Interestingly, VJP 4 is not inhibited by $Co(NO_3)_2$ in presence of $NiCl_2$ (Table 7). This may be due to possible positive effect of chloride as an anion due to dependence of the organism on chloride for its growth. This organism was also able to grow on solid media containing iron and cobalt (1 mM each), as well as copper and nickel (1 mM each), but failed to tolerate the same metal combinations at higher concentrations.

Table 7. Growth of VJP 4 in presence of Co+Ni (1:1)

Time (h)	OD_{625}	
	Growth Control	Experimental
18	0.32	-
42	0.33	0.14
48	-	0.21

VJP 5 achieved higher cell density in absence of Fe and Co, however initial growth rate (up to 18 h) was higher in presence of Fe and Co (1 mM each) combination (Table 8). The maximum cell density achieved was 16.92% and 61.53% lesser than growth control in tubes containing iron and cobalt salts at 1 mM and 2 mM each, respectively. It took VJP 5, 72 h to reach a visible cell density in a medium containing iron and cobalt (3 mM each), which was achieved in the growth control in less than 24 h (data not shown). It failed to grow in presence of these two metals at 4 mM each. When VJP 5 was grown in presence of Cu and Ni, its growth was slowed down with increase in metal concentration (Table 9). At all concentrations tested it registered a lesser cell density than growth control. Its generation time was 18.69 h, 43 h, and 150.5 h in presence of Cu and Ni at 1, 2, and 3 mM each respectively, as compared to the average generation time of 3.45 h in growth control. VJP 5 failed to grow in media containing higher concentrations of Cu and Ni.

Table 8. Growth of VJP 5 in presence of Fe and Co

Time (h)	OD_{625}		
	Growth control	Fe + Co (1 mM each)	Fe + Co (2 mM each)
18	0.36	0.54	0.25
24	0.65	0.42	0.20

When VJP5 was exposed to Co and Ni simultaneously, maximum cell density in tubes containing both these metals at 1 mM and 2 mM each did not differ much (Table 10). The tube with 1 mM metal concentration had faster growth than tube with 2 mM metal concentration as well as growth control up to 18 h of incubation. Faster growth rate of VJP 5 during initial phase of incubation was also observed with Fe and Co (1 mM each) combination (Table 8). This may be due to some stimulatory effect of $Co(NO_3)_2$ (at 1 mM concentration) on growth of VJP 5.

Co and Cu together at different concentrations were able to slow down the growth of VJP 5 in halophilic nutrient broth. Interestingly there was better growth in presence of Co and Cu at 2 mM than at 1 mM up to initial 22 h of incubation. However higher cell density was attained at 1 mM concentration after longer incubation (Table 11). VJP 5 registered a generation time of 27.36 h, and 48.54 h at 1 mM and 3 mM of these two metals respectively. At latter concentration organism was able to reach the same cell density in 96 h, which it achieved at 2 mM metal concentration in just 65 h. It could not grow when Cu and Co were both present in 4 mM concentration.

Table 9. Growth of VJP 5 in presence of Cu and Ni

Time (h)	OD_{625}					
	Growth control	Cu + Ni (1 mM each)	Growth control	Cu + Ni (2 mM each)	Growth control	Cu + Ni (3 mM each)
18	0.36	0.08	0.18	-	-	-
22	-	-	-	-	0.31	-
23	-	-	0.54	0.16	-	-
24	0.65	0.19	-	-	0.36	-
42	-	0.28	0.74	0.23	-	-
72	-	-	-	0.24	-	0.21
96	-	-	-	-	-	0.25
120	-	-	-	-	-	0.28

Tolerance to multiple metals at a time: VJP 5 was challenged with three or more metals at a time. Maximum cell density of VJP 5 in simultaneous presence of Fe, Co, and Cu was 47.43% lesser than that of positive control. Maximum cell density achieved by VJP 5 in simultaneous presence of Fe, Co, and Ni was 43.39% lesser than positive control (Table 12). Generation time of VJP 5 was found to be 32.02 h, and 50.16 h in medium containing (Fe + Co + Cu), and (Fe + Co + Ni) respectively, as compared to 16.72 h in growth control. It seems that Cu is more inhibitory to VJP 5 than Ni. When Cu is present along with Fe and Co, VJP 5 took 72 h to reach almost same cell density which it achieved in just 42 h when Ni (instead of Cu) is present along with Fe and Co. VJP 5 could tolerate neither (Fe + Co + Cu) nor (Fe + Co + Ni) when these metals were taken at 2 mM concentration each.

Table 10. Growth of VJP 5 in presence of Co+Ni

Time (h)	OD_{625}		
	Growth control	Co + Ni (1 mM each)	Co + Ni (2 mM each)
18	0.58	0.80	0.45
20	-	0.82	0.87
22	-	0.88	0.83
24	1.5	-	-

Table 11. Growth of VJP 5 in presence of Co and Cu

Time (h)	OD_{625}				
	Growth control	Co + Cu (1 mM each)	Co + Cu (2 mM each)	Growth control	Co + Cu (3 mM each)
17	0.74	0.12	0.22	-	-
18	-	-	-	0.58	-
22	0.63[a]	0.17	-	-	-
24	-	-	-	1.5	-
65	-	0.54	0.31	-	-
72	-	-	-	-	0.20
88	-	-	-	-	0.25
96	-	-	-	-	0.30

[a]2X dilution

The stimulation of growth by metal ions in certain cases (Table 3-5) indicates that they may play an important role in some metabolic pathway so as to promote growth of the organism. Some heavy metals have no biological role and are detrimental to the organisms even at very low concentration (cadmium, mercury, lead etc.). However, at high levels both of the essential and non- essential metals become toxic to the organisms. These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA (Kaur et al., 2006). Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions. Also, toxicity can occur as a result of alterations in the conformational structure of the nucleic acids and proteins, and interference with oxidative phosphorylation and osmotic balance. Microorganisms have evolved various mechanisms to resist the heavy metal stress. Several metal resistance mechanisms have been identified: exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions (Rathnayake, 2009).

Table 12. Response of VJP 5 to multi-metal challenge

Time (h)	OD_{625}		
	Growth control	Fe + Co + Cu (1 mM each)	Fe + Co + Ni (1 mM each)
18	0.18	-	-
23	0.54	0.10	0.31
42	0.74	0.31	0.42
72	-	0.39	-

While evaluating metal tolerance /resistance, it is important to pay enough attention to bioavailability of metal ions. A bioavailable metal is one that can be taken up by a microbial cell. The total metal in a system does not necessarily reflect the degree of biological metal toxicity (Roane *et al.*, 2009). Factors such as salinity, pH, temperature, and growth-medium components can all influence the metal stress response because they can alter effective free metal ion concentration in the cell or influence metal state. Reduction in concentration of yeast extract was noted to result in increased microbial sensitivity to different metals (Nieto *et al.*, 1989). In addition, the formation of metal complexes in culture medium may determine the true soluble metal concentrations, and indeed, the toxicity of some metals could be attributed to a metal complex rather than a metal cation (Nieto *et al.*, 1987). Metal salts and microbiological media components can interact in ways which make data interpretation difficult. Some components of commonly used media such as peptone, tryptone, yeast extract, casamino acids share a high binding power to different metal ions and, hence, can prevent their toxicity. Copper is reported to get modified in the presence of agar (Nieto *et al.*, 1989).

The culturing of metal resistant microorganisms often occurs in either nutrient-rich or chemically defined media, which may contain yeast extract, phosphate buffers, and amino acids that bind metal ions. Neutral medium pH is an additional factor increasing metal binding in culture medium. Thus, depending on the growth medium metal toxicity will vary. pH strongly influences metal bioavailability. Metals readily precipitate as carbonic salts at pH > 7.0. Therefore, medium pH is suggested to be kept slightly acidic (~6.0) to maintain metal solubility (Roane *et al.*, 2009). Ravikumar *et al*. (2000) reported that, the higher pH levels are shown to enhance the toxicity of the heavy metals (Cd and Hg) whereas, the addition of NaCl is found to reduce the toxicity of Cd and Hg to the free-living nitrogen fixing *Azotobacter vinelandii* isolated from Pichavaram mangrove forest (South east coast of India).

Our studies on metal tolerance were performed at alkaline pH. Except iron, no metal compound developed any precipitates at the concentrations tested, indicating solubility of respective metal compounds in culture medium at alkaline pH. In case of iron, it may be that actual concentration of soluble iron available to organism could be lesser than that added in medium. However putting iron at alkaline pH in medium is a more realistic simulation of the organism's natural environment. As almost all halophiles are found in alkaline habitats, where solubility of iron (if present) is likely to be compromised. Our results clearly indicate that despite low solubility at alkaline pH, ferric iron does affect organism's growth and their interaction with other metal species.

Organisms such as VJP 5 which exhibit notable metal tolerance may serve as a useful model for study of stress (tolerance) response among halophiles. It will be interestingly useful to decipher the strategy by which such organisms tolerate multiple stresses (metal, high salt, alkalinity, etc.) and still maintain viability. How their metabolism differs from those growing in normal conditions needs to be explored which may lead to significant biotechnological applications (Solanki and Kothari, 2011). Microbial communities in natural alkaline environments have attracted attention because of possible biotechnological use of enzymes and metabolites from such organisms (Kanekar 2008). Metal tolerant/resistant halophilic bacteria should be tested for their potential for reclamation of metal-polluted saline sites (Trevors *et al*., 1985; Amoozegar *et al.,* 2005). Metal sensitive strains can be used to develop biosensor for detection of particular metal ion in environmental samples (Rathnayake *et al*., 2009).

Acknowledgment

NERF (Nirma Education & Research Foundation) for financial and infrastructural support.

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