ISOLATION AND CHARACTERIZATION OF HALOTOLERANT BACTERIA FROM SALINE SOIL OF KHAMBHAT, AND INVESTIGATION OF METAL TOLERANCE / RESISTANCE IN THEM

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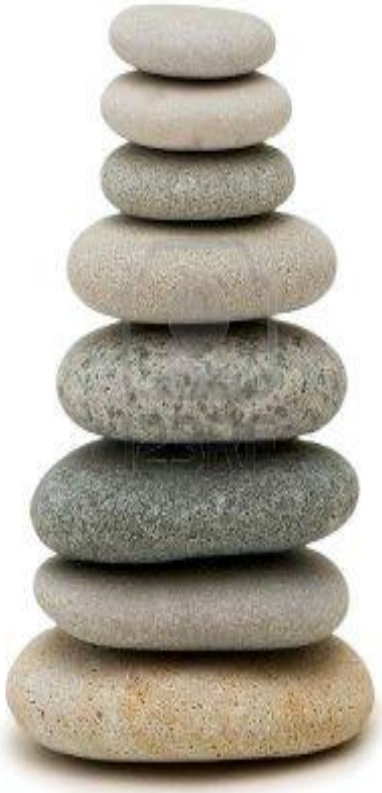

Dedicated to all those people whose little contributions made my success a bigger one!!!

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Only as high as I reach can I grow, Only as far as I seek can I go,

Only as deep as I look can I see, Only as much as I dream can I be.

-Karen Ravns

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Date:

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Prelude

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them *-W.L.Bragg*

A vast variety of organisms belonging to different domains of life (Archaea, Bacteria and Eukarya) reside in extreme habitats i.e. those that lie outside the range of conditions in which most of organisms live. MacElroy in 1974 coined the term extremophiles. Such organisms were described by Kristjansson and Hreggvidsson (1995) as those which thrive beyond '*normal*' environmental parameters. They may thrive either in physical extremes, like temperature, radiation, pressure, or geochemical extremes, like desiccation, salinity, pH, depletion of oxygen, extreme redox potential, or more than one extreme condition simultaneously. By studying such extremophiles and their characteristic environment new insights may be gained that are important for the study of evolutionary relationships, emergence of new species and various ecological relationships among organisms which compensate certain environmental externalities. Extremophiles isolated from diverse habitats can be studied for certain novel metabolic pathway/metabolites (enzymes) characteristic to them that may have certain bioremediation potential (Oarga, 2009).

They also produce biocatalysts with unique properties that function under extreme conditions comparable to those prevailing in various industrial processes (Niehaus *et al.*, 1999; Kumar *et al.*, 2011). Extremophilic bacteria follow different metabolic pathway and produce novel substances under stress (Kanekar *et al.*, 2008).

Extremophiles are of following types: psychrophiles (thrive at low temperatures), thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), piezophiles (under extremes of pressure), xerophiles (desiccation), and halophiles (salinity).

Halophiles are a class of extremophiles that grow at high salt conc. The greatest part of the biosphere is saline which inhabits a large diversity of organisms. Oceans and sea water contain 35 g/L dissolved salts. Halophiles are present in all three domains of life (Archaea, Bacteria and Eukarya). Pigmentation is also seen in numerous halophiles. They even impart color to their habitat where they thrive, eg., Bacterioruberin pigment in the membrane of red halophilic archaea

belonging to the family *Halobacteriaceae* are responsible for pigmentation of brines; presence of bacteriorhodopsin may contribute a purple color etc (Oren, 2002).

Halophiles have been classified by Kushner on the basis of their salt requirement into following categories (Table 1)

Table 1. Classification of halophiles based on salt requirement

(Nieto 1989; Oren, 2006)

Category	**Optimum salt requirement (M)**	**Examples**
Non halophilic	< 0.2	Most freshwater bacteria
Slight halophilic	0.2-0.5	*Demequina aestuarii* (Yi *et al.*, 2007), *Shewanella gaetbuli* (Yoon *et al.*, 2004)
Moderate halophilic	0.5-2.5	*Saccharomonospora saliphila* (Dastager *et al.*, 2008), *Salinivibrio costicola*
Borderline extreme halophilic	1.5-4.0	*Streptomyces tritolerans* (Syed *et al.*, 2007), *Halorhodospira halophila*
Extreme halophilic	2.5-5.2	*Actinopolyspora halophila* (Kokare *et al.*, 2003), *Halobacterium salinarum*
Halotolerant	Non-halophile which can tolerate salt; if the growth range exceeds above 2.5 M salt, it may be considered *extremely halotolerant*.	*Staphylococcus aureus*

Halophiles are being explored for the following (Margesin and Schinner, 2001).

- Bacteriorhodopsin (pigment from halophiles) has applications in holography, spatial light modulators, optical computing, and optical memories.
- Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, and stress-protective agents.
- Biopolymers (biosurfactants and exopolysaccharides) are of interest for microbially enhanced oil recovery.
- Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements

These products can be obtained from non-halophiles, but halophilic microorganisms may present advantages over the use of non-halophilic counterparts (Oren, 2002).

Few microorganisms can adapt to life over whole salt conc. range from fresh water to halite saturation like *Halomonas elongate*. Among the halophilic prokaryotes some are adapted to tolerate other forms of stress in addition to salt stress i.e. thermophilic, psychrophilic, and alkaliphilic halophiles are known. Acidophilic halophiles have not been reported (Oren, 2006).

1.1 Isolation of halophiles

Halophilic microorganisms can be isolated from different saline environments and different strains even belonging to the same genus have various applications (Zhuang *et al.*, 2010). It is difficult to generalize and describe media and conditions suitable for all or even a group of halophiles. They largely depend on the source from where the isolation is attempted. Most halophilic bacteria generally grow best on slightly alkaline pH. The media is thus modified accordingly like seawater is being added to the media for isolation of halophiles from tidal flats. Similarly, optimum temperature for isolation and cultivation also depends on source of sample (Flannery, 1956).

Halophiles are a diverse class with respect to metabolic diversity also. They include oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens. This metabolic diversity decreases with salinity (Oren, 2002).

Some studies have shown the dependence of salt requirement and tolerance on temperature as in the case of *Haloferax volcanii* (halophilic archaea) whose minimum and optimum salt concentrations shifted to higher values with increasing temperature. Another example is that of *Halomonas halophila* which requires 7.5% salt as optimum conc. for growth at 32 and 42º C ; whereas 5% salt as optimum at 22º C (Ventosa *et al.*, 1998).

A large no. of halophilic actinomycetes have been discovered from saline soils of different locations (Solanki and Kothari, 2011). Studies concerning their biological characterization have also been done indicating that halotolerant and halophilic actinomycetes have extensive adaptability to Na^+, K^+ and Mg^{2+} (Tang *et al.*, 2002). Their potential applications like enzyme production, antimicrobial action, bioremediate heavy metals, solvent tolerance, biosurfactant production etc. have also been explored.

Greater biocatalytic potential have been reported in halotolerant/halophilic organisms, among the various groups of extremophiles. They produce extracellular enzymes, some of which are shown to work optimally at alkaline pH, eg., *Streptomyces clavuligerus* strain MIT-1 is a salt tolerant and alkaliphilic organism which secretes alkaline protease. Its growth and protease production was optimum at pH 9 (Thumar and Singh, 2007; Solanki and Kothari, 2011).

1.2 Strategies to tolerate salt

All halophiles must maintain their cytoplasm isoosmotic with their surrounding medium. Biological membranes are permeable to water, and active energy-dependent inward transport of water to compensate for water lost by osmotic processes is energetically not feasible. Moreover, cells that keep a turgor need

even to maintain their intracellular osmotic pressure higher than that of their environment.

There are two fundamentally different strategies used by halophilic microorganisms to balance their cytoplasm osmotically with their medium (Galinski and Trüper, 1994; Zahran, 1997).

(1) Accumulation of molar concentrations of potassium and chloride. This strategy requires extensive adaptation of the intracellular enzymatic machinery to the presence of salt, as the proteins should maintain their proper conformation and activity at near-saturating salt concentrations. The proteome of such organisms is highly acidic, and most proteins denature when suspended in low salt. It is called the ***'high-salt-in strategy'***. Microorganisms employing such strategy generally cannot survive in low salt media. This strategy is energetically less costly to the cell, but still not widely used among the different phylogenetic and physiological groups of halophiles. Examples of organisms employing this strategy are *Halobacterium salinarum, Haloarcula marismortui* etc.

(2) Second strategy is based on the biosynthesis and/or accumulation of organic osmotic solutes. Cells utilizing this strategy exclude salt from their cytoplasm as much as possible. The high concentrations of organic 'compatible' solutes do not greatly interfere with normal enzymatic activity. Few adaptations of the cells' proteome are therefore needed. Organisms employing this strategy can often adapt to a surprisingly broad salt concentration range. Many organic compounds (amino acids, glycine betaine, ectoine, hydroxyectoine etc.) serve as osmotic solutes in halophilic microorganisms, in both prokaryotes and eukaryotes. Most compatible solutes are based on amino acids and amino acid derivatives, sugars, or sugar alcohols, and are either uncharged or zwitterionic (Kurz, 2008; Vreeland, 1987). This ***'low-salt-in strategy'*** of haloadaptation with accumulation of organic osmotic solutes is widespread in the small subunit rRNA sequence-based phylogenetic tree of life.

All groups of halophiles have not yet been examined for the occurrence and distribution of organic solutes (Oren, 2008). The osmotic solutes described earlier may be either produced by *de novo* synthesis or the organism may accumulate it from the environment (medium). The latter mechanism is preferred over *de novo* synthesis (Galinski and Trüper, 1994; Oren, 1999; Irwin and Baird, 2004). The intracellular accumulation of compatible solutes as an adaptive strategy to high-osmolality environments is evolutionarily well-conserved in Bacteria, Archaea, and Eukarya (Kempf and Bremer, 1998).
Ectoines constitute predominant class of osmolytes accumulated by halophiles. Eg. *Halomonas elongata* strain KS3 have been shown to accumulate ectoine and hydroxyectoine as compatible solutes (Ventosa *et al.*, 2008).

Occurrence of certain solutes in halophilic/halotolerant prokaryotes is often correlated with their position in the phylogenetic tree of life, i.e. a few solutes in the domain Archaea have not yet detected elsewhere within the tree.
Halophilic methanogens like *Methanohalophilus* species contain, in addition to glycine betaine found widespread in nature, β-amino acids and derivatives that are rarely found in other groups: β-glutamine, β-glutamate, and Nε-acetyl-β-lysine. Sulfotrehalose has thus far been found only in a few alkaliphilic members of the *Halobacteriaceae*; it is accumulated in substantial concentrations (up to 1 M) in addition to KCl which serves as the main osmotic solute like in their neutrophilic relatives (Oren, 2008).

Salt-tolerant bacteria usually exhibit structural modifications to cope with salt stress. One important aspect of structural adaptations is the change in composition of the cell envelope and membranes. The stretched state of the wall and the internal osmotic pressure of bacteria are usually affected by the biophysical properties of the stress bearing peptidoglycan. Changes in composition of bacterial membranes which might be caused by environmental factors are thought to act as an adaptive response to maintain membrane stability and function. In fact, structural adaptations of membranes mainly involve alterations in the composition and synthesis of proteins, lipids and fatty acids (Zahran, 1997).

Paul *et al.* (2008) from India compared the genomes (genes) and proteomes (protein patterns) of halophiles and non-halophiles. From their experiments it was concluded that halophilic proteins have following features:

1. Low hydrophobicity, i.e. they are more hydrophilic than hydrophobic
2. Lots of acidic amino acids – especially aspartic acid; under-representation of cystine
3. Limited helix formation and higher occurrence of coiled structures

At the DNA level, the dinucleotide abundance profiles of halophilic genomes have some distinctive and common characteristics, i.e., specific DNA salt-adaptation signatures. Thus, it may halophiles have special membrane proteins and internal proteins that enable them to survive salty environments (Reinhardt, 2010).

1.3 Haloalkaliphiles

The group of bacteria able to grow under alkaline conditions in the presence of salt are referred as haloalkaliphiles. They have twin extremities of pH and salinity (Singh *et al.*, 2010). These organisms require high concentrations of NaCl, high pH (8.5 - 11), and low Mg2+ (<10mM) (Jones and Grant, 1999). Tindall in 1984 first mentioned the occurrence of haloalkaliphiles. Such organisms possess special adaptation mechanisms for survival in high salinity and alkaline pH that make them interesting not only for fundamental research but also towards exploration for applications (Horikoshi, 1999; Singh, 2010). Mostly they have been isolated from alkaline soda lakes but some are being isolated from natural saline habitats as well. Limited attempts have been made to explore their enzymatic potential. Haloalkaliphilic organisms isolated from natural saline habitats of coastal Gujarat have been studied for amylase and protease production. All the isolates secreted protease in haloalkaline medium, but none secreted amylase. Higher salt was required for the enzyme secretion (Dodia *et al.*, 2006). Salinity is also an important defining factor in the alkaline lakes.

Study of haloalkaliphilic bacteria isolated from sea water, saline soil and other saline habitats (coastal Gujarat) have indicated extracellular secretion of proteases, amylases, chitinase and lipases. The patterns revealed that the secretion

and properties of extracellular enzymes could also be useful as marker to judge the microbial heterogeneity among the haloalkaliphilic bacteria. Majority of these enzymes were also thermostable in nature where salt acted as positive effectors. Further, majority of the enzymes displayed salt-dependent resistance against chemical denaturation, a rare feature among the proteins. Therefore, these enzymes could provide a unique model to study protein folding and stability under set of extreme conditions (Singh *et al.*, 2010).

Novel members belonging to this category of organisms have also been discovered from lake in California, eg., *Spirochaeta americana sp.* nov., that grows at 37^0C, 3% NaCl w/v and pH 9.5 (Hoover *et al.*, 2003).

Moderately halophilic and alkalitolerant *Halomonas campisalis,* isolated from alkaline lonar lake (India), was able to produce enzymes amylase and lipase at alkaline pH. It showed the presence of PHA (Polyhydroxyalkanoate) within 24 h of incubation with a good yield of 75%. It is the first report on production of PHA by *H. campisalis* (Kanekar *et al.*, 2008).

1.4 How they tolerate metals?

Certain transition metals like manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) serve as essential cofactors in the physiology of all organisms. Recently it has been estimated that over half of all proteins in every organism are metalloproteins. They are essential in trace amounts, but are toxic at higher levels as they directly/indirectly compromise DNA, protein, and membrane integrity and function thereby proving lethal to the cell. This is evident from the eg. cycling in redox states of metals such as Fe and Cu and antioxidant scavenging by redoxinactive metals such as Zn can both cause oxidative damage to cell components through increased production of reactive oxygen species (Kaur *et al.,* 2006).

Metal toxicity is being avoided/resisted by organisms by the following processes: selective uptake, trafficking, and efflux of metal ions, enzymatic conversion of

metals into non- or less-toxic redox states, or sequestering toxic metal ions with ferritins and metallothioneins. These mechanisms are often regulated by free metal-ion concentration. Therefore all the other factors such as salinity, pH, temperature, and growth-medium components that can alter effective free metal ion concentration in the cell influence the metal stress response (Kaur *et al.*, 2006).

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. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interaction. 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 (Rathnayake *et al.*, 2009).

In *Pseudomonas* sp. strain 40, salinity-dependent cadmium tolerance has been reported. Poor growth at 2 mg/mL and no growth at 2.5 mg/mL of $CdCl_2$ were observed at 1 M NaCl, whereas moderate growth was observed at 2.5 mg/mL of $CdCl_2$ in 2-4 M NaCl. Cadmium toxicity was enhanced in presence of $NaNO_3$ and Na_2SO_4. Cadmium ions react with chloride ions to form complexes whose nature depends on the chloride concentration (Ventosa *et al.*, 1998).

Metal tolerance level of a gram-negative moderately halophilic eubacteria, *Halomonas elongata* and the genes responsible for the tolerance have been reported. It showed the highest resistance to cadmium (8 mM). It was found that the presence of other metals with Cd, increase Cd toxicity, and the optimum salt concentration for high Cd resistance was 10% NaCl. Characterization of genes involved in metal resistance in halophilic bacteria has been reported for the first time. Multiple resistance systems rather than a single are possibly involved in cadmium tolerance (Amer *et al.*, 2005).

Effect of salinity and media constituents (yeast extract) on toxicity of heavy metals for certain strains has also been reported. The general trends that were observed are: on lowering the salinity the sensitivity to cadmium was enhanced and sometimes to cobalt and copper, whereas on increasing the salinity only a slight decrease in the cadmium, copper, and nickel toxicities was observed. Reducing the concentration of yeast extract (0.01% w/v) resulted in an increased sensitivity to all metals; whereas on increasing the concentration, the toxicities of nickel and zinc were only slightly lessened (Nieto *et al.*, 1989).

Intracellular contents of magnesium and manganese contents were measured in bacteria of several halophilic levels, in *Vibrio costicola*, a moderately halophilic eubacterium growing in 1 M NaCI, *Halobacterium volcanii*, a halophilic archaebacterium growing in 2.5 M NaCl, *Halobacterium cutirubrum*, an extremely halophilic archaebacterium growing in 4 M NaCl, and *E. coli*, a non halophilic eubacterium growing in 0.17 M NaCl. Their contents varied with the growth phase, being maximal at the early log phase. The increase of magnesium and manganese contents associated with the halophilic character of the bacteria suggests that manganese and magnesium play a role in haloadaptation (Medicis *et al.*, 1986).

The mechanism by which microorganisms resist to various metals can be either accumulation in the form of particular protein-metal association, or blockage at the level of the cell wall and the systems of membrane transportation. In experiments related to determination of MIC (minimum inhibitory concentration) of heavy metals, certain factors like, metal-binding capacity of the microorganisms, chelation to various components of the media, and formation of complexes cause a reduction in the activities of free metals thereby posing problems related to determination of actual concentration of metals that inhibits the microbial growth. Such metal resistant bacteria can be used as bio-indicators of pollution (Hassen et al., 1998)

Apart from reports studying the tolerance to toxic heavy metals and oxyanions, effect of salinity on tolerance to toxic oxyanions has also been reported in spore

forming bacilli. It was shown that increase in salinity from 5% (w/v) to 15% (w/v) enhanced tolerance. One of the reasons for the high tolerance to oxyanions was the presence of Na/K in chemical structure of the oxyanions. Sodium and potassium are necessary elements for the activity of enzymes and pumps in halophiles; therefore it seems that these elements enhanced the toxic metal tolerance (Ventosa *et al.*, 1998; Amoozegar *et al.*, 2005).

Studies have also shown that megaplasmids of unknown function are harbored by the majority of halobacteria (Gutiérrez *et al.*, 1986). An insight of metal tolerance may reveal some functions for some of these plasmids and, in addition, the possible heavy metal resistances could be used in halobacteria as genetic markers. Additionally, metal tolerance could be relevant to the ecology and physiology of halobacteria, since they usually grow in habitats such as solar salterns or hypersaline lake that may be polluted with heavy metals (Nieto *et al.*, 1987).

1.5 Scope

Microorganisms play a major role in bioremediation or biotransformation processes of toxic elements, converting them to less toxic or non-toxic elements. Determining the potential of microorganisms and their tolerance against high concentrations of toxic metals will assist the selection of suitable species for bioremediation and biotransformation of toxic metals (Amoozegar *et al.*, 2005). Metal resistant bacteria have potential application in toxic metal control in waste water treatment. As certain microorganisms are responsible for environmental metal transformation, they may serve as bioassay indicator organisms in polluted and non polluted environments (Trevors *et al.*, 1985)

Bacterial population from natural environment can be exploited for treating heavy metal containing wastes (Sannasi *et al.*, 2009). Treatment of industrial waste containing heavy metals by artificially mutated bacteria may be a solution for effluent disposal problems. Use of mutation in metal resistant bacteria enhances the bioremediation of heavy metals from effluents of the factories and improves the disposal problems of the waste with little expense (Shakibaie *et al.*, 2008)

By studying metal tolerance among bacteria, those that seem to be highly sensitive to a particular metal ion can thus serve as biosensor to detect that particular metal ion in environmental sample (Rathnayake *et al.*, 2009).

Due to the above mentioned attributes of halophiles, they can serve as model for stress response in bacteria as they can even tolerate multiple stresses of metals, alkalinity, etc.

1.6 Aims and Objectives

- Isolation, identification and characterization of halotolerant bacteria from saline soil of Khambhat (Gujarat)
- Characterization of isolates with respect to metal tolerance
    - Study of organism's response to single metal challenge
    - Study of organism's response to two or more metals at a time

Materials and Methods

Materials: Sodium chloride (Merck, Mumbai), Dextrose (Merck), Agar agar (HiMedia, Mumbai RM666), Casein peptone (tryptic digest) (HiMedia), Yeast extract (HiMedia, RM 026), Cobaltous chloride hexahydrate ($CoCl_2.6H_2O$; S d Fine Chem. Ltd., Mumbai), Cobaltous nitrate ($Co(NO_3)_2$; HiMedia), Cadmium chloride monohydrate ($CdCl_2.H_2O$; S d Fine Chem. Ltd.), Lead acetate ($C_4H_6O_4Pb.3H_2O$; S d Fine Chem. Ltd.), Nickel chloride hexahydrate ($NiCl_2.6H_2O$; RFCL Ltd., New Delhi), Silver nitrate ($AgNO_3$; S d Fine Chem. Ltd.), Cupric chloride dehydrate ($CuCl_2.2H_2O$; RFCL Ltd.), Ferric sulphate ($Fe(SO_4)_3.xH_2O$; S d Fine Chem. Ltd.), Barium chloride ($BaCl_2$, Merck), Potassium chromate (K_2CrO_4, Merck), Sulphuric acid (H_2SO_4, Merck).

2.1 Sample collection:

Sample was collected from salt pans of Khambhat located at $22^0 18' 05.33$" N and $72^0 37' 10.78$" E at an elevation of 23 ft. Sample was collected in October 2010. Sample was collected in plastic bags and brought to the laboratory. It was immediately refrigerated.

Plate 1. Sample collection site

2.2 Characterization of soil:

The soil sample was subjected to physico-chemical characterization. Three properties viz. salinity, pH and chloride content were determined.

2.2.1 Salinity testing: 20 g of soil sample was weighed on a weighing balance (Shimadzu, BL 620S) and mixed with sterile distilled water (50 mL) in a 250 mL conical flask (Borosil). The soil suspension was kept on rotary shaker (Remi) for 1 hour. After this, the suspension was allowed to stand still for 30 min. This allowed the soil to settle down with supernatant above. This supernatant was then carefully transferred to another 250 mL flask. The contents were then centrifuged in a centrifuge (Remi, BZCI-8729) at 10,000 rpm for 10 min. The contents were poured into a pre-weighed 150 mm Petriplate (Borosil). The empty Petriplate was weighed till constant reading came successively 3 times. The Petriplate containing the suspension was kept into the hot air oven (EIE, Ahmedabad) set at 60^{o} C for overnight. The following day the Petriplate was again weighed in a similar manner as empty Petriplate was weighed and difference was calculated which was used to determine the salinity of soil sample.

2.2.2 pH measurement: Soil suspension was prepared as above and the supernatant was used to measure the pH of the soil sample by using digital pH meter (EI products, Model 111). The pH meter was calibrated with buffers (Merck) of known pH (4 and 9.2).

2.2.3 Chloride estimation: Mohr's argentometric titration method was used to determine the chloride ion conc. In this method, the chloride ion conc. of soil sample was measured by titration with silver nitrate. 50 mL supernatant was taken in a 250 mL conical flask and titrated against silver nitrate. Before titration, 1 mL of potassium chromate (as indicator) was added. End point was marked by the first appearance red-brown colour in the flask. The chloride content was then calculated by the formula (Eaton *et al.*, 2005)

$$\text{Chloride ion conc. (g/L)} = \frac{(A-B)\times N \times 35.450}{Vol.\ of\ sample\ (mL)}$$

where, A: Vol. of $AgNO_3$ used for sample (mL)

B: Vol. of $AgNO_3$ used for blank i.e. water (mL)

N: Normality of $AgNO_3$ used

2.3 Isolation of organisms from soil sample:

Halophilic nutrient agar (Appendix 6.2) was used for the isolation and cultivation of organisms from the soil sample.

20 g of soil sample was weighed on a weighing balance and mixed with sterile distilled water (50 mL) in a 250 mL conical flask. The soil suspension was kept on rotary shaker for 1 hour. After this, the suspension was allowed to stand still for 30 min. This allowed the soil to settle down with supernatant above. This supernatant was then inoculated on solid media by spreading a constant vol. (100 µL) onto plates consisting of three different salt conc. i.e. 5%, 10% and 15%. In preparing plates of different salt conc. only the quantity of salt (NaCl) was adjusted and all other media components were kept constant. Three plates were inoculated of each salt conc. The plates were then incubated in incubator (EIE, KCE-419-HAG) maintained at temperature 35º C. After 24 h of incubation the plates were examined for growth. Multiple colonies were present on plates containing 5% and 10% salt, and few colonies were observed on 15% plates.

Now to obtain pure culture, a single colony was picked up from plate containing 5% salt and streaked onto another plate containing 5% salt. This was done with every colony that appeared different in the plate. The above method of isolating pure colonies was also done with the colonies on 10% and 15% plates. The plates were then incubated at 35º C. The plates were examined for growth after 24 h of incubation. Pure culture was obtained on plates. 3 different colonies from 10% plate (that were previously confirmed that they are pure culture by streaking a single colony onto a 10% plate) were cultivated and used for further experiments. Similarly, 2 different colonies from 15% plates were also used for further experiments i.e. total 5 isolates different from each other were selected for further experimentation.

The isolates growing at high salt conc. (10% or 15%) were selected for further investigation. The isolates were labelled as VJP 1, VJP 2, VJP 3, VJP 4, and VJP 5.

2.4 Characterization of the isolated organisms:

The isolated organisms (VJP 1-5) were subjected to Gram staining and their colony characteristics were noted after growth on halophilic nutrient agar.

2.5 Media sterilisation

2.5.1 Autoclave treatment:

Autoclaving was effected at 121°C, 15 lbs/square inch pressure for 15-20 min.

2.5.2 Microwave treatment:

For the metal tolerance experiments in broth, the medium (200 mL) was sterilised in a 500 mL screw capped bottle (Borosil) by microwave (Electrolux; 2450 MHz) treatment (Patadia and Trivedi, 2011) for 10 min at 900 W. The cap of the glass bottle was kept slightly loose during the process. After sterilisation, 140 mL of media remained in the bottle. Medium composition (Appendix 6.3) was adjusted according to this final volume (140 mL) before microwave treatment.

2.6 Determination of salt range and pH range:

2.6.1 Salt range:

All the 5 isolates i.e. VJP 1, VJP 2, VJP 3, VJP 4, and VJP 5 were grown on plates containing salt conc. 0.5-20%. The pH was adjusted with NaOH to that of the soil sample (8.3). This was done to determine the salt range over which the isolates could grow. Suspension of the organism was made in sterile distilled water tubes by picking isolated colonies from working slants prepared from master plates of the isolates. The suspension was then compared with 0.5 McFarland standard (Appendix 6.1). From this suspension 100 µL was dispensed onto the plate by using micropipette (Eppendorf) and then spread by using a glass

spreader. The plates were then incubated at 37º C upto 8 days. Optimum salt conc. was also determined by this experiment, which was based on amount of growth and first appearance of growth on plate of a particular salt conc.

2.6.2 pH range:

After the determination of optimum salt conc. for each organism, their pH range was tested. In this, the organisms were grown on plates containing their optimum salt conc. with varying pH being adjusted by adding sodium hydroxide solution (NaOH) or diluted hydrochloric acid (HCl) to the medium. pH was set in the range 4-10. The pH was adjusted prior to media sterilization i.e. autoclaving. The plates were then spread with respective organism as mentioned above and incubated at 37º C upto 8 days.

2.7 Temperature optimization:

For all the 5 isolates, plates with their optimum salt and pH were prepared in duplicate. The plates were spread with respective organism and incubated at two different temperatures i.e. one plate was kept in incubator set at 35ºC and another at 37ºC. The plates were then observed for growth.

2.8 Metal tolerance:

Incubation during all the metal tolerance experiments was carried out at 37 ºC under static condition. Intermittent shaking (once every 24 h) was provided. Experiments were done in triplicate i.e. for each combination 3 plates/tubes were prepared so as to ensure reproducibility in results.

Metal solutions were filter sterilised with disposable (HiMedia) or autoclavable syringe filters (Axiva) of pore size 0.22 µm. Such filter sterilized metal solutions were added to autoclaved medium. After filtration, the metal solutions were stored in a sterile test tube (at 4-8°C) for no longer than 4 days. The metal solution to be filtered was made of highest required concentration which was used as stock solution. Appropriate controls were set for all experiments.

2.8.1 **Single metal tolerance**:

The isolates were challenged with different heavy metals at different concentrations. Metal compounds used were: $Co(NO_3)_2$, $CoCl_2.6H_2O$, $CdCl_2.H_2O$, $C_4H_6O_4Pb.3H_2O$, $NiCl_2.6H_2O$, $CuCl_2.2H_2O$, $AgNO_3$, $Fe_2(SO_4)_3.xH_2O$.

Dilutions were made from stock solution. 333 µL of metal solution was added to 19.666 mL media i.e. the metal solution gets 60 times diluted in the system, therefore to achieve the desired conc. in the system, metal solution at 60X concentration of that desired was added. The isolates were challenged with metal solutions in the range of 0.005-12[#] mM. Irrespective of whether single metal or multi metal experiments, the system volume was kept constant i.e. 5 mL for liquid media 20 mL for solid media.

- Experiments on solid media:

Positive control: media (20 mL) + inoculum (100 µL was spread on plate)

Experimental: media (19.666 mL) + metal (333 µL) + inoculum (100 µL was spread on plate)

- Experiment on liquid media: Some of the above experiments were repeated on liquid media.

Sterility control: sterile media (5 mL)

Positive control: media (4.75 mL) + inoculum (250 µL) (5% v/v)

Abiotic control: media (4.666 mL) + metal solution (83.25 µL) + sterile distilled water (250 µL)

Experimental: media (4.666 mL) + metal solution (83.25 µL) + inoculum (250 µL).

Following inoculation, incubation was effected at 37º C for upto 7 days.

[#] 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0 mM.

2.8.2 Two metal combinations:

- For experiments on solid media:

Positive control: media (20 mL) + inoculum (100 µL was spread on plate)

Experimental: media (19.334 mL) + metal solution 1 (333 µL) + metal solution 2 (333 µL) + inoculum (100 µL was spread on plate)

- For experiments on liquid media:

Sterility control: sterile media (5 mL)

Positive control: media (4.75 mL) + inoculum (250 µL)

Abiotic control: media (4.583 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + sterile distilled water (250 µL)

Experimental: media (4.583 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + inoculum (250 µL).

The plates/tubes were incubated at 37º C upto 12 days and discarded thereafter if no growth was observed.

2.8.3 Three metal combinations:

For the three metal system, experiments only in liquid media were set.

Sterility control: sterile media (5 mL)

Positive control: media (4.75 mL) + inoculum (250 µL)

Abiotic control: media (4.5 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + metal solution III (83.25 µL) + sterile distilled water (250 µL)

Experimental: media (4.5 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + metal solution III (83.25 µL) + inoculum (250 µL).

The tubes were incubated at 37º C upto 8 days and discarded thereafter if no growth was observed.

2.8.4 Four metal combinations:

For the four metal system, experiments only in liquid media were set:

Sterility control: sterile media (5 mL)

Positive control: media (4.75 mL) + inoculum (250 µL)

Abiotic control: media (4.417 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + metal solution III (83.25 µL) + metal solution IV (83.25 µL) + sterile distilled water (250 µL)

Experimental: media (4.417 mL) + metal I (83.25 µL) + metal II (83.25 µL) + metal III (83.25 µL) + metal IV (83.25 µL) + inoculum (250 µL).

The tubes were incubated at 37º C upto 12 days and discarded thereafter if no growth was observed.

2.8.5 Five metal combination:

A single five metal combination was tried with the consortium of all the five isolates i.e. in this experiment all the 5 isolates were collectively challenged with five metals simultaneously. The five metals were selected on the observation that all the 5 isolates were able to tolerate all those metals when present individually (atleast 1mM).

Sterility control: sterile media (5 mL)

Positive control: media (4.75 mL) + inoculum (250 µL)

Abiotic control: media (4.333 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + metal solution III (83.25 µL) + metal solution IV (83.25 µL) + metal solution V (83.25 µL) + sterile distilled water (250 µL)

Experimental: media (4.333 mL) + metal solution I (83.25 µL) + metal solution II (83.25 µL) + metal solution III (83.25 µL) + metal solution IV (83.25 µL) + metal solution V (83.25 µL) + inoculum (250 µL).

The tubes were incubated at 37º C upto 12 days and discarded thereafter if no growth was observed.

2.9 Identification of the isolates

The isolates have been sent for 16s rRNA sequencing for identification purpose to GSBTM (Gujarat State Biotechnology Mission), Gandhinagar.

2.10 Preservation

All the 5 isolates were maintained on halophilic nutrient agar slants and stored at 4-8º C. They were subcultured every month on the same media. Paraffin stocks were prepared by overlaying agar slants with it thereby preventing the exposure of slants to air. Glycerol stocks were prepared by adding glycerol at concentration of 10% v/v to the broth inoculated with organisms. Paraffin stocks were preserved in refrigerator at 4-8°C and glycerol stocks were stored at -20°C in deep freezer.

Results and Discussion

3.1 Characterization of soil:

3.1.1 <u>Salinity testing</u>: Salinity is a measure of amount of salt present in the soil sample.

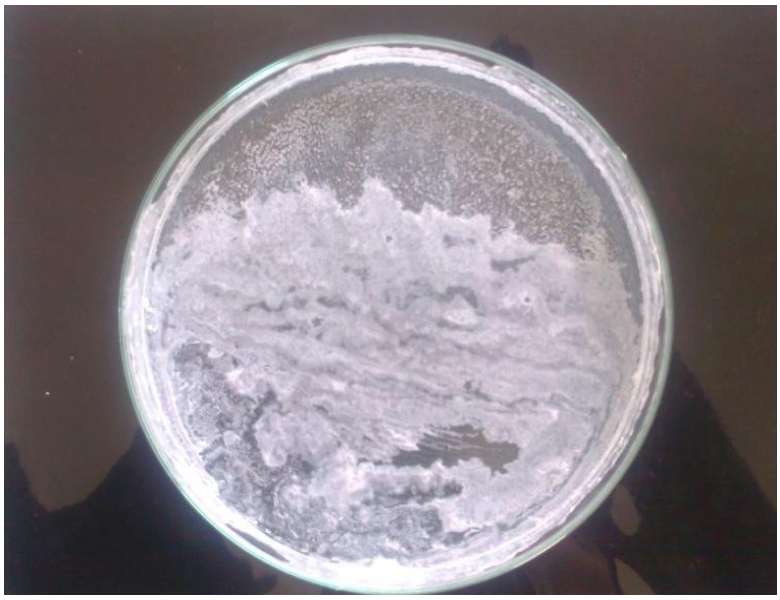


Plate 2. Petriplate with salt after evaporation of soil suspension

The weight of empty dried Petriplate was 108.314 g. The weight of Petriplate containing salt was 109.730 g. The weight of empty Petriplate was subtracted from the weight of salt containing plate.

The difference was 1.416 g i.e., 1.416 g salt is present in 20 g of soil sample. Thus, salinity of the soil sample is:

- 70.8 mg/g of soil;
- 7.08%

3.1.2 <u>pH</u>: pH of the soil sample was found to be 8.3.

3.1.3 <u>Chloride estimation</u>: Soil sample contained 21.625 g/L chloride.

Table 2. Physico-chemical characteristics of soil sample

Parameter	Value
Salinity	7.08%
pH	8.3
Chloride content	21.625 g/L

3.2 Gram staining:

Gram staining was performed with all the 5 isolates. Table 3 illustrates the results of gram staining and colony characteristics of all the isolates on halophilic nutrient agar.

Table 3. Results of gram staining and colony characteristics

Property	Isolate				
	VJP 1	**VJP 2**	**VJP 3**	**VJP 4**	**VJP 5**
Gram reaction	Positive	Positive	Negative	Negative	Positive
Shape	Cocci	Rods	Rods	Rods	Rods
Colony characteristics	Pin point, spherical, raised, white colonies with even margin.	Translucent, mucoid, irregular colonies that tend to merge. Upon incubation, develop wrinkles on their surface.	Regular, raised colonies. Slight orange color with a dark center develops on longer incubation.	Round, raised colonies with a distinctive center and even margin.	Flat colonies with uneven margins. Distinctive zones are formed upon longer incubation.

After isolation of all the 5 isolates, they were inoculated in autoclaved liquid media. But all the 5 isolates were unable to grow in liquid media. Thus all the experiments were performed on solid media, till microwave sterilized media was tried to grow the organisms. Growth was observed for all 5 isolates in microwave treated media. Therefore, initially the experiments were done on solid media. The experiments of salt range, pH range, single metal tolerance, and some experiments of two metal combination were done on solid media. Later, all the experiments were done in liquid media. Certain experiments were performed in both solid and liquid media.

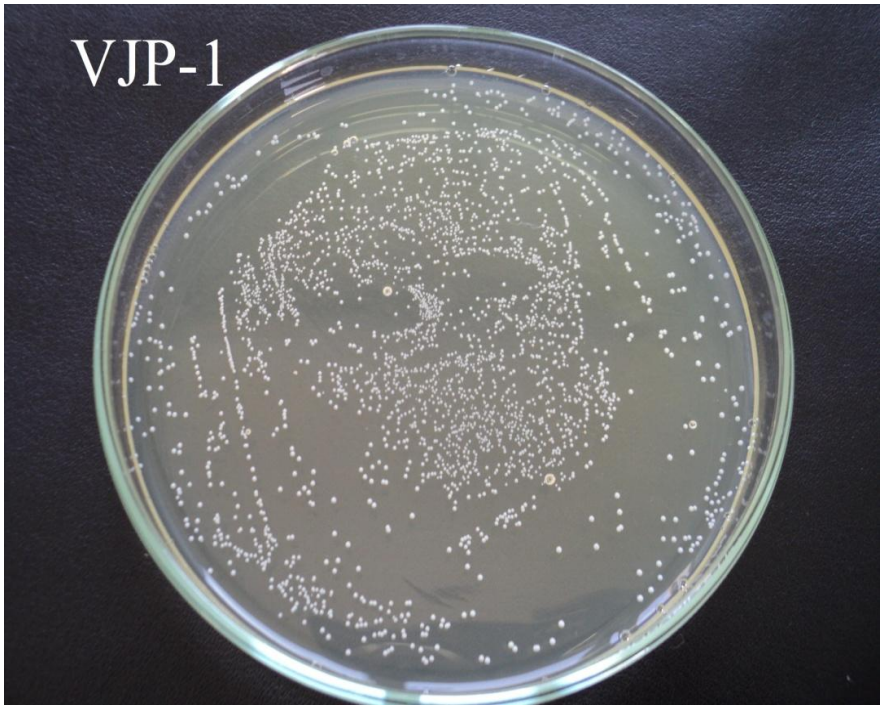


Plate 3. Colonies of VJP 1 on halophilic nutrient agar

Plate 4. Colonies of VJP 2 on halophilic nutrient agar

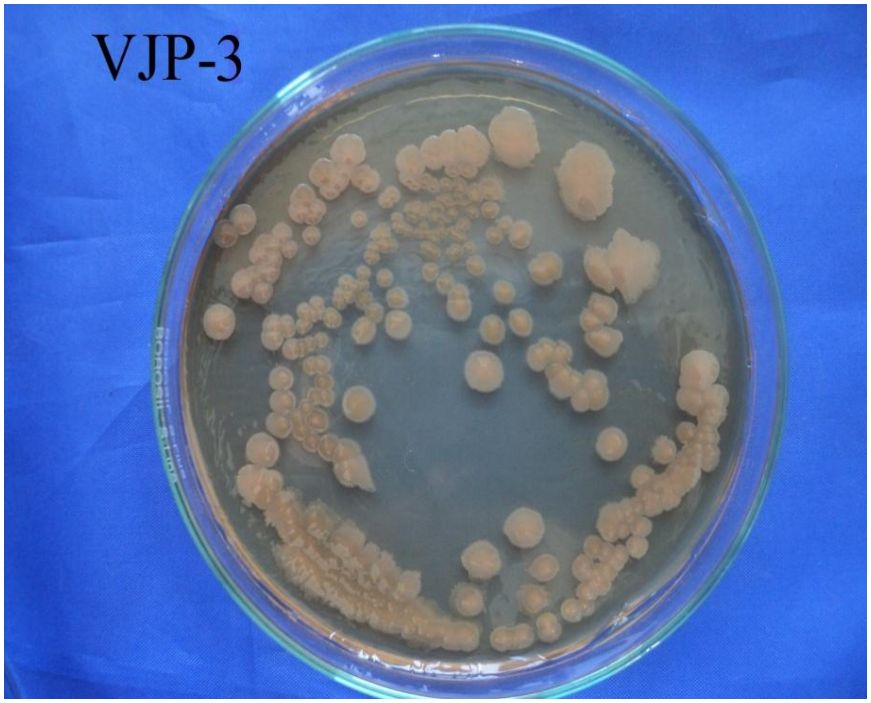


Plate 5. Colonies of VJP 3 on halophilic nutrient agar

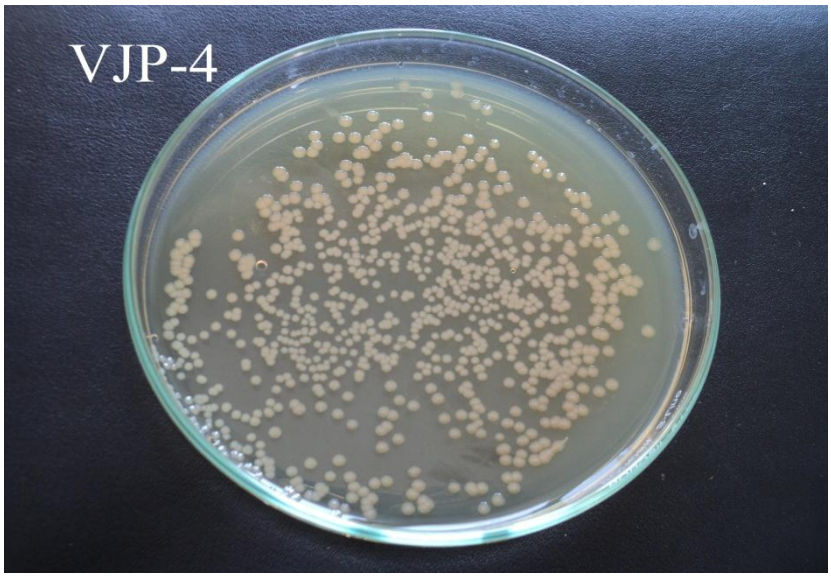


Plate 6. Colonies of VJP 4 on halophilic nutrient agar

Plate 7. Colonies of VJP 1 on halophilic nutrient agar

3.3 Salt and pH range:

By growing the isolates over a wide range of salt conc. (0.5-20%), the salt range over which each isolate could grow was determined as shown in table 4.

Table 4. Salt range and pH range of the isolates

Property		**Isolate**				
		VJP 1	**VJP 2**	**VJP 3**	**VJP 4**	**VJP 5**
Salt range (%)		0.5-16	0.5-16	0.5-16	0.5-16	0.5-16
Optimum salt conc.	**(%)**	6	7	6	6	6
	(M)	1.03	1.2	1.03	1.03	1.03
pH range		6-8	7-9	8-10	5-8	6-10
Optimum pH		8	8	9	8	9

All the isolates can grow over a wide salt range (0.5-16%). All the isolates except VJP 2 grow best in media containing 6% salt i.e. 1.03 M salt. VJP 2 grows best on 7% salt i.e. 1.2 M salt. Thus according to Kushner's classification, all the isolates fall in the '*Extremely Halotolerant'* category. Among all the 5 isolates, VJP 5 could tolerate widest pH range. Three of the isolates viz. VJP 2, VJP 3, and VJP 5 can be labelled as '*Haloalkaliphiles'* as all three of them are capable of growing above pH 9 (Horikoshi, 1999; Singh *et al.*, 2010).

3.4 Temperature optimization:

37º C was found to be favourable for growth of the isolates i.e. growth was observed faster at this temp as compared to that at 35º C. Accordingly all the experiments were performed at 37º C.

3.5 Metal tolerance:

3.5.1 <u>Single metal tolerance</u>

The isolates were challenged with various conc. of 8 different heavy metal compounds. The response of the isolates towards them has been reported in table 5 which represents the range of heavy metals tolerable by isolates. Response exhibited by different isolates to different metals was heterogenous (Table 6;

Figure 1). In all the experiments, a positive control was kept in order to observe the difference in growth on positive control and metal containing plates (experimental) and also to ensure that growth is absent/less on experimental plates only due to metal and not due to any other reason. In certain cases, growth was quantitatively more or appeared earlier than positive control indicating growth stimulation in presence of a particular heavy metal. Such experiments were repeated to confirm the observation.

Table 5. Range of heavy metal concentrations tolerable by all isolates.

Metal	Range of tolerance (mM)				
	Isolate				
	VJP 1	**VJP 2**	**VJP 3**	**VJP 4**	**VJP 5**
Co $[Co(NO_3)_2]$	0.005-3	0.005-3[##]	0.005-4	No growth at 0.005	0.01-6
CO $[CoCl_2]$	0.005-4	0.005-5[##]	0.005-4	0.005-3	0.005-3
Cd	0.005-0.5	0.005-0.5	0.005-0.5[#]	No growth at 0.005	0.005-1
Pb	0.005-4	0.005-4	0.005-1	0.005-6	0.005-3
Ni	0.005-8	0.005-7	0.005-4	0.005-3	0.005-7
Cu	0.005-7	0.005-7	0.005-2	0.005-3	0.005-9
Ag	0.005-0.1	0.005-0.1	0.005-0.05	0.005-0.05	0.005-0.05
Fe	0.005-4	0.005-5	0.005-4[*]	0.005-1	0.005-6

[#] Cadmium had a stimulatory effect on growth of VJP 3 at 0.1 mM
[##] Cobalt had a stimulatory effect on growth of VJP 2 at 2 mM
[*]Iron had a stimulatory effect on growth of VJP 3 at 2 mM

All the above reported experiments were observed for minimum 5 days, and in some cases where growth was seen in lower conc. the incubation time was increased accordingly. Two compounds of cobalt were tested against all the isolates. In case of VJP 2 growth promotion was confirmed by testing the isolate against both the compounds. The experiments were repeated to confirm the results. For quantification the experiment was even repeated in liquid with $Co(NO_3)_2$ only (section 3.5.1.1).

After challenging the isolates with 2 compounds of cobalt ($Co(NO_3)_2$ and $CoCl_2$) it was observed that four of the isolates responded differently to both compounds. This indicates that the anion which is different in both compounds plays a role in determining the response of isolates to cobalt. VJP 5 could tolerate maximum conc. of $Co(NO_3)_2$ (6 mM), while VJP 2 tolerated maximum conc. of $CoCl_2$ (5 mM). Co^{2+} is rapidly accumulated by the CorA system in most bacterial cells (Nies, 1999).

Silver (Ag) had the most toxic effect on all isolates and all were able to tolerate very less conc. of the metal, maximum being 0.1 mM tolerated by VJP 1 and 2. However later two tolerated Ag at a concentration higher than that reported by Nieto *et al*. (1989) for different moderately halophilic eubacteria. Silver resistance may be based on RND-driven transenvelope efflux in gram-negative bacteria, efflux by P-type ATPases in gram-positive organisms, and additional complexation by intracellular compounds (Nies, 1999).

Our isolates were also not capable of tolerating higher concentration of cadmium. VJP 4 could not grow even in the presence of 0.005 mM cadmium after an incubation period of 5 days. VJP 5 could tolerate a maximum of 1 mM cadmium. 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).

VJP 4 showed max. tolerance to Pb (6 mM), being relatively more sensitive to other metal compounds at lesser concentrations. Lead-tolerant bacteria were isolated, and precipitation of lead phosphate within the cells of these bacteria was reported. Resistance to lead in *Ralstonia* sp. CH34 was shown to be mediated by a P-type ATPase, CadA P-type ATPase, and may be based predominantly on metal ion efflux (Nies, 1999).

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 2 and 3 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 showed highest tolerance to iron (6 mM) followed by VJP 2 (5 mM). One of the possible reasons for this may be the fact that Fe^{3+} is not toxic to aerobic bacteria because of its low solubility (Nies, 1999).

Table 6. Maximum metal conc. tolerable by the isolates

Metal	Maximum metal conc. tolerable (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
Pb	4	4	1	6	3
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

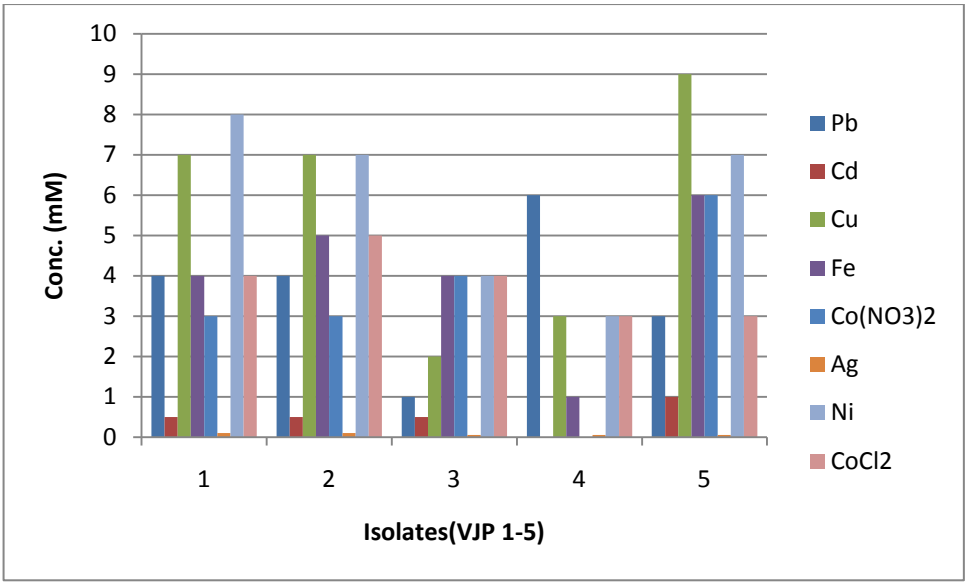


Fig 1 Maximum metal concentration tolerated by all isolates

VJP 5 showed highest tolerance to 4 metals, i.e. Cu (9 mM), Fe (6 mM), Co (6 mM), and Cd (1 mM) and 2nd highest tolerance to Ni (7 mM) after VJP 1 which showed highest tolerance to Ni (8 mM). VJP 4 was highly sensitive to Cd, but on the other hand displayed highest tolerance to Pb (6 mM). VJP 4 showed high sensitivity to Co when corresponding anion was nitrate, but was able to grow when challenged with $CoCl_2$. This indicates the significance of anion associated with a particular metal cation.

As each of the isolates exhibited considerable tolerance to one or more metal compounds, they were then subjected to different two, three, and four metal combinations thereby increasing metal stress; with higher interest in tolerance of multimetal combination in VJP 5 as it showed highest tolerance to 4 metals out of the 8 tested against it.

3.5.1.1 Stimulatory effect of certain metal ions:

The growth of VJP 2 was stimulated in the presence of cobalt [$Co(NO_3)_2$] at 1 mM. This experiment was done earlier on solid media with both compounds of cobalt and then later confirmed by repeating in liquid media (only with $Co(NO_3)_2$) (Table 7). 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 Co. 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 new class of cobalt-containing enzymes, nitrile hydratases, has been described (Nies, 1999).

In a study done by Kaur *et al.* to study haloarchaeal strategies to withstand stress from transition metals taking *Halobacterium NRC-1* (an archaeal halophile) as model organism, similar results were being 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 7. Growth of VJP 2 in presence of $Co(NO_3)_2$

Time (h)	Positive 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	0.151	1.5	0.351	3.1	-	-
22	0.183	1.7	0.320	2.8	-	-
42	-	-	-	-	0.178	1.6
51	-	-	-	-	0.161	1.5

Cadmium was found to have stimulatory effect on the growth of VJP 3 at 0.1 mM. This experiment was done earlier on solid media and later confirmed by repeating in liquid media (Table 8). 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.

Table 8. Growth of VJP 3 in presence of $CdCl_2$

Time (h)	Positive control		$CdCl_2$			
			0.05 mM		0.1 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	0.106	1.1	0.120	1.2	0.194	1.8
21	0.223	2	-	-	0.196	1.8
25	0.244	2.2	0.194	1.8	0.225	2

Iron also had a stimulatory effect on growth of VJP 3 at 2 mM (Table 9). 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. 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 9. Growth of VJP 3 in presence of $Fe(SO_4)_3.xH_2O$

Time (h)	Positive control		$Fe(SO_4)_3.xH_2O$			
			2 mM		3 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	0.110	1.1	0.117	1.1	0.092	1
20	0.121	1.2	0.225	2	0.066	0.8
24	0.151	1.5	-	-	-	-

3.5.2 Two metal combination:

Based on the results of single metal exposure, different two metal combinations were tried with all the isolates. Initially we challenged the isolates with metals at their maximum tolerable concentration (Table 6) e.g., VJP 1 was challenged with Cu (7 mM) and Ni (8 mM) simultaneously, because these were the highest concentrations of respective metals tolerated by VJP 1 (as deciphered from single metal exposure experiments). Other isolates were also challenged with two metals, at a time, in a similar manner. But none of the isolates was able to grow at such high metal concentration. These experiments were done on solid media.

Then metal combinations with lower conc. were tried with all the 5 isolates starting from 1 mM of each metal (Table 10). Now experiments were done in liquid media sterilized by microwave treatment. They were then challenged with higher concentration if growth was observed at lower conc. The turbidity was measured in tubes showing growth at 625 nm (Table 11-17).

In presence of lead, there seems to be a stimulatory effect of $Co(NO_3)_2$ on growth of VJP 2 (Table 11), as the growth in experimental tube was higher (200% higher cell density after 21 h of incubation) and faster than in control. VJP 2 registered a generation time of 38.10 h when challenged simultaneously with Pb and Co at 1 mM each. $Co(NO_3)_2$ was also shown to stimulate growth of VJP 2 at 1 mM when tested in absence of any other metal (Table 7). Maximum cell density achieved by organism in presence of Co does not seem to be affected much by Pb.

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 12). Generation time in control and experimental was 13.68 h, and 23.7 h respectively.

When VJP 4 was challenged with Co and Ni (1 mM each), it was able to achieve lesser cell density at a slower growth rate (g= 13.68 h for experimental tube). 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 upto 3 mM of chloride salt of Co (Table 6). Interestingly,

VJP 4 is not inhibited by $Co(NO_3)_2$ in presence of $NiCl_2$ (Table 13). This may be due to possible positive effect of chloride as an anion.

[*#] Table 10. Response of isolates towards two metal combinations

Isolate				
VJP 1	**VJP 2**	**VJP 3**	**VJP 4**	**VJP 5**
Cu + Ni (1:1)* (2:2) (3:4) (4:5) (5:6) (6:7) (7:8)	Cu + Ni (1:1)* (2:2) (4:4) (5:5) (6:6) (7:7)	Cu + Ni (1:1)* (2:2)	Cu + Ni (1:1)* (2:2) (3:3)	Cu + Ni (1:1)* (2:2)* (3:3)* (4:4) (7:5) (8:6) (9:7)
Cu + Fe (7:4)	Cu + Fe (7:5)	Fe + Ni (1:1)* (2:2)	Pb + Ni (6:3)	Cu + Fe (9:6)
	Pb + Co (1:1)*		Pb + Cu (1:1)* (6:3)	Pb + Cu (3:9)
			Fe + Co (1:1)* (2:2)	Pb + Ni (3:7)
			Co + Ni (1:1)* (2:2)	Fe + Co (1:1)* (2:2)* (3:3)* (4:4)
				Co + Ni (1:1)* (2:2)*
				Cu + Co (1:1)* (2:2)* (3:3)* (4:4) (5:5)
				Pb + Cd (1:1)

[*] At these conc. organism was able to grow
[#] All figures expressed are in mM

Table 11. Growth of VJP 2 in presence of Pb+Co (1:1)

Time (h)	OD_{625}	
	Positive control	Experimental (with metal)
18	0.101	0.297
21	0.106	0.318
24	-	0.335

Table 12. Growth of VJP 3 in presence of Cu+Ni (1:1)

Time (h)	OD_{625}	
	Positive control	Experimental (with metal)
18	0.110	0.092
20	0.121	0.112
24	0.151	0.128

Table 13. Growth of VJP 4 in presence of Co+Ni (1:1)

Time (h)	OD_{625}	
	Positive control	Experimental (with metal)
18	0.328	-
42	0.334	0.148
48	-	0.212

Table 14. Growth of VJP 5 in presence of Fe+Co

Time (h)	OD_{625}		
	Positive control	Experimental (with metal) Fe+Co (1:1)	Experimental (with metal) Fe+Co (2:2)
18	0.362	0.540	0.250
24	0.651	0.420	0.201

Table 15. Growth of VJP 5 in presence of Cu+Ni

Time (h)	OD_{625}					
	PC*	Exp. Cu+Ni (1:1)	PC*	Exp. Cu+Ni (2:2)	PC*	Exp. Cu+Ni (3:3)
18	0.362	0.088	0.182	-	-	-
22	-	-	-	-	0.319	-
23	-	-	0.542	0.162	-	-
24	0.651	0.197	-	-	0.363	-
42	-	0.289	0.742	0.239	-	-
72	-	-	-	0.248	-	0.210
96	-	-	-	-	-	0.251
120	-	-	-	-	-	0.280

*positive control

VJP 5 acheived higher cell density in absence of Fe and Co, however initial growth rate (upto 18 h) was higher in presence of Fe-Co (1 mM each) combination (Table 14). The maximum cell density achieved was 16.92% and 61.53% lesser than positive control in tubes containing iron and cobalt salts at 1 mM and 2 mM each, respectively.

When VJP 5 was grown in presence of Cu and Ni, its growth was slowed down with increase in metal concentration (Table 15). At all concentrations tested it registered a lesser cell density than positive control. Its generation time was 18.69 h, 43 h, and 150.5 h in presence of Cu:Ni at 1, 2, and 3 mM each, respectively. VJP 5 failed to grow in media containing higher concentrations of Cu and Ni (Table 10).

Table 16. Growth of VJP 5 in presence of Co+Ni

Time (h)	OD_{625}		
	Positive control	**Exp Co+Ni (1:1)**	**Exp Co+Ni (2:2)**
18	0.588	0.801	0.458
20	-	0.825	0.875
22	-	0.885	0.830
24	1.5	-	-

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 16). Generation time of the organism at former concentration was 33.44 h. The tube with 1 mM metal concentration had faster growth than tube with 2 mM metal concentration as well as positive control upto 18 h of incubation. Faster growth rate of VJP 5 during initial phase of incubation was also observed with Fe-Co (1 mM each) combination (Table 14). This may be due to some stimulatory effect of $Co(NO_3)_2$ (at 1 mM concentration) on growth of VJP 5.

Table 17. Growth of VJP 5 in presence of Co+Cu

Time (h)	OD_{625}				
	Positive control	**Exp. Co+Cu (1:1)**	**Exp. Co+Cu (2:2)**	**Positive control**	**Exp. Co+Cu (3:3)**
17	0.744	0.124	0.224		
18				0.588	
22	0.636[a]	0.175	0.219		
24				1.5	
65	-	0.540	0.315		
72					0.203
88					0.250
96					0.305

[a]2X dilution

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 upto initial 22 h of incubation. However higher cell density was attained at 1 mM concentration after longer incubation (Table 17). 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.

The stimulation of growth in presence of either single metal (as in the case of growth promotion experiments) or in presence of metal combinations, indicates that the metal ion plays an important role in some of its metabolic pathway that directly/indirectly promotes growth of the organism in its presence.

A summary of growth expression parameters for different isolates in presence of various metals is presented in table 18.

Table 18. Growth expression parameters of isolates in presence/absence of metals

Isolate	Growth parameters		
	Generation time'g' (h)	Division rate 'v' (h^{-1})	Specific growth rate '*k*'
VJP 3 (P.C.)	7.34	0.136	0.041
VJP 3 with Cu+Ni (1:1)	23.7	0.042	0.012
VJP 4 with Co+Ni (1:1)	13.68	0.07	0.022
VJP 5 (P.C.)	3.45	0.289	0.087
VJP 5 with Cu+Ni (1:1)	18.69	0.053	0.016
(2:2)	43	0.010	0.003
(3:3)	150.5	0.006	0.002
VJP 5 with Co+Cu (1:1)	27.36	0.036	0.011
(3:3)	48.54	0.020	0.006
VJP 5 with Co+Ni (1:1)	33.44	0.029	0.009

Growth retardation in presence of metals may be due to replacement of some essential native metal ion by the metal in present in the medium. Another possibility is that it may block some some important pathway thereby partially/completely inhibiting growth of the organism.

3.5.3 Multi metal tolerance:

Based on the results of two metal combinations, different three and four metal combinations were tried with VJP 5 (Table 19). It was selected because it showed tolerance to many metals at higher concentrations.

Table 19. Response of VJP 5 towards multi metal combinations

3 Metal combinations		4 Metal combinations		
Fe + Co + Cu	**Fe + Co + Ni**	**Fe + Co + Cu + Ni**	**Fe + Co + Cu + Pb**	**Fe + Cd + Cu + Pb**
(1:1:1)* (2:2:2)	(1:1:1)* (2:2:2)	(1:1:1:1)	(1:1:1:1)*	(1:1:1:1)

*At these conc. organism was able to grow
#All figures expressed are in mM

Maximum cell density of VJP 5 in simultaneous presence of Fe, Co, and Cu was 47.43% lesser than that of positive control (Table 20). Maximum cell density achieved by VJP 5 in simultaneous presence of Fe, Co, and Ni was 43.39% lesser than positive control (Table 21).

Table 20. VJP 5 in presence of Fe+Co+Cu (1:1:1)

Time (h)	Positive control		Experimental
	OD_{625}	Generation time (h)	OD_{625}
18	0.182	16.72	-
23	0.542		0.103
42	0.742		0.318
72	-		0.390

Table 21. Growth of VJP 5 in presence of Fe+Co+Ni (1:1:1)

Time (h)	Positive Control		Experimental
	OD_{625}	Generation time (h)	OD_{625}
18	0.182	16.72	-
23	0.542		0.319
42	0.742		0.420

From the comparison of table 20-21, 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 4 metals simultaneously (Table 22). However its growth was slowed down, it took 41 h to reach almost same cell density which it could achieve in positive control in just 17 h. Generation time in absence and presence of metals was 4.97 h, and 5.57 h respectively.

Table 22. Growth of VJP 5 in presence of Fe+Co+Cu+Pb (1:1:1:1)

Time (h)	OD_{625}	
	Positive control	Experimental
17	0.325	-
19	0.438	-
41	-	0.352
44	-	0.536
48	-	0.331

3.5.4 Multi metal tolerance in consortium of isolates:

After challenging single organism with multi metal combinations, an experiment to test the tolerance of these isolates in consortium was tried i.e. all the 5 isolates were coinoculated and collectively challenged with 5 metals (Pb, Cu, Co, Ni, Fe) at a conc. of 1 mM each. Following 4 days of incubation, a loopful from the

experimental tube was streaked on a halophilic nutrient agar plate. The plate was incubated at 37º C for 1 day. Colonies characteristic of VJP 2 and VJP 5 (1 colony each) were observed on the plate, indicating that the effect of metal combination on these two bacteria was bacteriostatic and not bactericidal. The tubes were incubated for 12 days at 37º C but no growth was observed. Similar experiments with bacterial consortium were reported by Sannasi *et al.* (2009).

Final Comments

- Five *extremely halotolerant* bacteria (VJP 1-5) were isolated from salt pan soil of Khambhat, Gujarat. All of them could tolerate upto 16% NaCl, however none was incapable of growing at low salt concentration. Three of them (VJP 2, 3, and 5) were able to grow at pH 9 or above and thus labelled as *haloalkaliphiles*.
- These isolates were challenged with different metal compounds to investigate their metal tolerance/resistance. Overall there was a heterogenous response towards different metal compounds by different isolates.
- VJP 2 and 5 each tolerated Co, Ni, Cu, and Fe at $\geq$ 5 mM concentration.
- Silver followed by cadmium proved to be most toxic. Because of its toxicity, but it has been used a long time as an antimicrobial agent in medicine. In some bacteria, cadmium enters the cell by some manganese uptake system (Nies, 1999).
- Effect of cobalt on four of the isolates (except VJP 3) seemed to be anion dependent. VJP 1, 2, and 4 tolerated higher concentrations of cobalt chloride than cobalt nitrate. However VJP 5 tolerated latter in concentration higher than former.

  It is important to point out that the availability of the respective metals can be influenced by chlorides or other kinds of salts contained in the culture medium used (Nieto *et al.*, 1987).
- Few metal ions were found to have a stimulatory effect on growth of test organism(s) (Table 7-9). Cadmium and iron stimulated growth of VJP 3 at 0.1 and 2 mM respectively. Growth of VJP 2 was stimulated by cobalt at 2 mM.

  Mild stimulation of growth of a haloarchaeal isolate at low concentrations of Co(II) was reported by Kaur *et al*.(2006). Inducing effect of cadmium on growth and physiology of halophilic phosphobacteria was reported by Ravikumar *et al*. (2009). 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).

- 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 turbidity/precipitates at the concentrations tested, indicating acceptable 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.

- VJP 5 was tested with maximum number of metal combinations (two, three, or four metals simultaneously), and exhibited notable tolerance on more than one occasions. Thus it 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).
- Such 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).

Summary

Five *extremely halotolerant* bacteria (VJP 1-5) were isolated from salt pan soil of Khambhat, Gujarat. These isolates were challenged with different metal compounds to investigate their metal tolerance/resistance. Overall there was a heterogenous response towards different metal compounds by different isolates. VJP 5 proved to be a step ahead of the rest in terms of metal resistance/tolerance. Such organisms can serve as a good model for study of stress response among prokaryotes. Additionally, they may be explored for their potential of bioremediation of metal polluted saline sites characterized by alkaline pH.

Appendices

Appendix 6.1: Preperation of 0.5 McFarland standard

0.5 mL of 1.175% $BaCl_2.2H_2O$ was added to 99.5 mL of 1% H_2SO_4 with constant stirring. Absorbance was recorded at 625 nm; it was adjusted in the range of 0.08-0.1. It was stored in dark, away from sunlight not more than 3 months.

Appendix 6.2: Halophilic nutrient agar (Atlas, 2010).

Composition per litre:

- NaCl 60 g
- Agar 15 g
- Casein peptone (tryptic digest) 10 g
- Glucose 5 g
- Yeast extract 5 g

For initial experiments (salt, pH, single metal tolerance) Casamino acid and protease peptone were used, which were later replaced by Casein peptone for all experiments in liquid media.

Appendix 6.3: Media preparation for microwave treatment

Composition for 140 mL:

- NaCl 8.4 g
- Agar 2.1 g
- Casein peptone (tryptic digest 1.4 g
- Glucose 0.7 g
- Yeast extract 0.7 g

Appendix 6.4: Glossary

- Halophile: An organism requiring salt (NaCl) for growth (Solanki and Kothari, 2011).
- Haloalkaliphiles: Organisms that require high concentrations of NaCl, high pH (8.5 - 11), and low Mg2+ (<10mM) for their growth. (Jones and Grant, 1999)
- Halotolerant: Capable of growing in presence of NaCl, but not requiring it (Solanki and Kothari, 2011).

- Heavy metals: Chemical elements with high atomic weight, and specific gravity at least 5 times that of water. This group of metals include transition metals, lanthanides, metalloids, and actinides (Solanki and Kothari, 2011).

- Generation time: The time required by a cell population to double. The generation time of an exponentially growing culture can be determined from the slope of straight line function obtained in a semilogarithmic plot of exponential growth. It can be calculated as 0.301/slope (Madigan et al., 2009).

- Division rate: A useful growth expression, which is reciprocal of generation time. It is a measure of the no. of generations per unit time in an exponentially growing culture. It can be calculated as v=1/g**.** Its unit is h^{-1}(Madigan et al., 2009).

- Specific growth rate: A growth expression equal to the slope of the growth curve i.e. 0.301/g. It is denoted by *k* (Madigan et al., 2009).

Appendix 6.5: Concentration of NaCl in different units

Concentration		
%	**M**	**g/L**
5.8	1	58
5	0.86	50
6	1.03	60
7	1.2	70
16	2.75	160

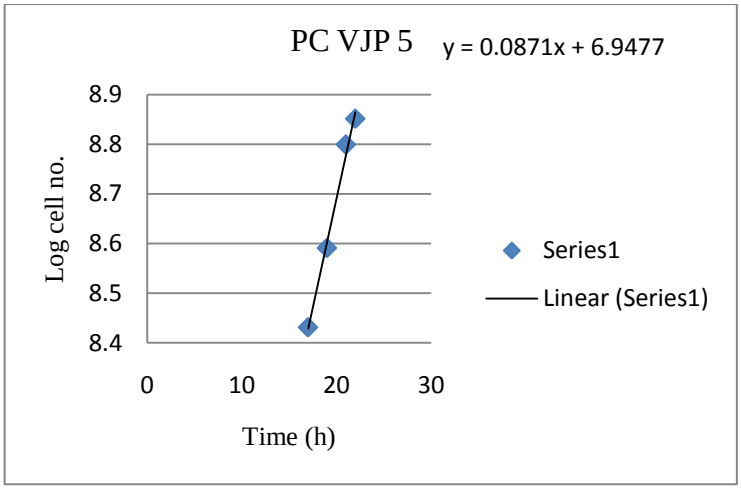
Appendix 6.6: Metal concentrations in terms of mM and mg/L

Metal compound	Concentration (mM)	Concentration (mg/L)
$Co(NO_3)_2$	1	291.03
$CoCl_2.6H_2O$	1	237.93
$CdCl_2.H_2O$	1	201.32
$C_4H_6O_4Pb.3H_2O$	1	379.33
$NiCl_2.6H_2O$	1	237.71
$CuCl_2.2H_2O$	1	170.48
$AgNO_3$	1	169.87
$Fe(SO_4)_3.xH_2O$	1	399.88

Appendix 6.7: Calculation of growth parameters

- Generation time (g) = 0.301/slope
- Division rate (v) =1/g
- Specific growth rate (k) = 0.301/g

Time (h)	log of cell no.
17	8.431364
19	8.591065
21	8.799341
22	8.851258



Calculation:

- Generation time (g) = 0.301/slope

  = 0.301/0.0871

  = 3.455 h

- Division rate (v) =1/g

  = 1/3.455

  = 0.289 h^{-1}

- Specific growth rate (k) = slope of graph
  = 0.0871

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Halophilic Actinomycetes: Salt-loving Filaments

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ABSTRACT:
A large number of halophilic organisms belonging to the group actinomycetes have been isolated and characterized from various locations around the world in recent times. A few have been reported from Indian soils as well. Certain species have been studied for their tolerance to organic solvents and metal ions, and also production of extracelluar enzymes in presence of solvents and/or metals. Potential applications of these organisms in bioremediation and industry have been elucidated. Such organisms may also serve as good model for studies on stress response among bacteria.

Key words: Actinomycetes, Bioremediation, Halophiles, Metal tolerance, Solvent tolerance

INTRODUCTION

Actinomycetes are a heterogeneous group of gram-positive bacteria with a high G+C content. They produce branching mycelium of two types- substrate and aerial. Actinomycetes are found in diverse habitats (marine, soil, saltern, mangroves) and diverse environmental condition (covering a wide range of pH, temperature, salinity, heavy metals, etc.). Quite a few of them are capable of growing in salt-rich habitats, and are thus halophilic or halotolerant (Box 1).

Box 1. Glossary

- **Actinomycete**: A group of filamentous gram-positive, aerobic bacteria with a high G+C content.
- **Halophile**: An organism requiring salt (NaCl) for growth.
- **Halotolerant:** Capable of growing in presence of NaCl, but not requiring it.
- **Heavy metals**: Chemical elements with high atomic weight, and specific gravity at least 5 times that of water. This group of metals include transition metals, lanthanides, metalloids, and actinides.

Halophiles have been classified by Kushner [1] on the basis of their salt requirement into 5 categories (Table 1). Strains of halophilic actinomycetes having the potential of secreting extracellular enzymes (protease, lipase, esterase, galactosidase, amylases, etc.), which work well in alkaline pH range, tolerating high concentrations of organic solvents in their environment have been reported in last few years [2-4]. Their ability of producing antibiotics has also been explored [5]. New species of halophilic actinomycetes have been isolated from saline soils of different locations (Table 2). Halophilic actinomycetes have also been isolated from sea anemone [6]. Some of the genera whose members include halophilic actinomyctes are listed below [11]:

Table 1. Classification of halophiles based on salt requirement [1]

Category	Optimum salt requirement	Representative actinomycete
Non halophilic	< 0.2 M	*Saccharopolyspora gloriosae* [7]
Slight halophilic	0.2-0.5 M	*Demequina aestuarii* [8]
Moderate halophilic	0.5-2.5 M	*Saccharomonospora saliphila* [9]
Borderline extreme halophilic	1.5-4.0 M	*Streptomyces tritolerans* [10]
Extreme halophilic	2.5-5.2 M	*Actinopolyspora spp*. [5]

Nesterenkonia: This genus of the family *Micrococcaceae* was first proposed by Stackebrandt et. al. in 1995 and later amended by Collins et. al. (2002), and Li et. al. (2005). Currently this genus has eleven species, out of them six are halophilic, with three being actinomycetes [12].

Prauserella: This genus proposed by Kim and Goodfellow (1999) has been named after Helmut Prauser, a German microbiologist who made notable contributions to actinomycete systematics. Seven of its species are halophilic actinomycete. *Prauserella halophila* and *Prauserella alba* are relatively novel examples.

Georgenia: This genus from family *Bogoriellaceae* was proposed by Altenburger et. al. (2002), and later amended by Li et. al. (2007). Currently it contains three species, among whom one (*G. halophila*) is a moderately halophilic actinomycete.

Isoptericola: It belongs to the family Promicromonosporaceae, and was proposed by Stackebrandt et. al. (2004). Among four members of this genus, one (*I. halotolerans*) is halophilic actinomycete.

Table 2. Halophilic actinomycetes discovered between 2000-2010

Name of organism	Isolated from	Reference(s)
Haloactinopolyspora alba	Salt lake, Xinjiang province, North-west China	[13]
Haloactinobacterium album		[14]
Georgenia halophila		[15]
Amycolatopsis halophila		[16]
Prauserella salsuginis, P. flava, P. aidigensis, P. sediminis, P. alba, P. halophila		[17,18]
Saccharopolyspora qujiaojingensis		[19]
Saccharopolyspora halophila		[20]
Streptomonospora amylolytica Streptomonospora flavalba		[21]
Nesterenkonia halophila		[22]
Nesterenkonia halotolerans		[23]
Nesterenkonia xinjiangensis		
Streptomonospora alba		[24]
Streptomonospora halophila		[25]
Haloactinospora alba		[26]
Nocardiopsis salina		[27]
Saccharomonospora paurometabolica		[28]
Nocardiopsis xinjiangensis		[29]
Nocardiopsis rosea		[30]
Nocardiopsis gilva		
Nocardiopsis rhodophaea		
Nocardiopsis chromatogenes		
Nocardiopsis baichengensis		
Haloglycomyces albus		[31]
Isoptericola halotolerans	Saline soil, Qinghai province, North- west China.	[32]
Nocardiopsis litoralis	Sea anemone collected from South China Sea, China	[6]
Nesterenkonia jeotgali	Jeotgal, a traditional Korean fermented seafood	[33]
Nocardiopsis kunsanensis	Saltern in Kunsan, Republic of Korea.	[34]
Nocardiopsis terrae	Saline soil, Qaidam basin, North-west China	[35]
Streptomyces tritolerans	Soil from Gulbarga Karnataka, India	[10]
Saccharomonospora saliphila		[9]
Actinopolyspora species AH1	Marine sediments sample from Alibag coast Maharashtra, India	[5]
Streptomyces clavuligerus	Mithapur, west coast of India	[36]
Nocardiopsis prasina HA-4	Limestone quarry, Ukhrul district, Manipur, India	[4]
Actinopolyspora spp.	Oil field, Sultanate of Oman	[37]
Demequina aestuarii	Tidal flat sediment sample, South Korea	[8]
Nocardiopsis halotolerans	Marsh soil, Kuwait	[38]
Saccharomonospora halophila		[39]
Nesterenkonia aethiopica	Abjata soda lake, Ethiopia	[40]
Streptomyces spp.	Saltpan soil	[41]

Haloactinobacterium: This genus of family *Ruaniaceae* was proposed by Tang et. al. (2010), and has only one member characteized as halophilic actinomycete.

Amycolatopsis: This genus was proposed by Lechevalier et. al. (1986). Out of the forty-one species in it, just one is actinomycete.

Saccharomonospora: It belongs to the family *Pseudonocardiaceae* and was proposed by Nonomura and Ohara (1971). It comprises of eight species, from which three are actinomycetes.

Saccharopolyspora: This genus was proposed by Lacey et al (1975). It comprises of twenty species, from which two are actinomycetes.

Haloactinospora: It is a newly formed genus with only 1 representative member *Haloactinospora alba*.

Nocardiopsis: It belongs to the family *Nocardiopsaceae*, which was proposed by Rainey et al in 1996 [1]. This particular genus was proposed by Meyer (1976) [29]. Currently it comprises of 38 species including 11 actinomycetes.

[#]Box 2. List of media used for isolation of halophilic actinomycetes.

Bennet media [33, 36]
Cellulose casein multi salts media [25]
Chitin agar [36]
Czapek agar [17, 23, 24, 28]
Glucose aspargine agar [27, 28]
Glycerol aspargine agar(ISP5) [22-24, 28, 29]
Inorganic salt starch agar(ISP4) [24, 23, 28]
Maltose yeast extract agar [24, 28]
Marine agar [16, 22, 32] / Marine broth[33]
Modified Horikoshi medium [31]
Medium glycerol glycine MSG media [21]
Nutrient agar [23, 24, 28]
Oatmeal agar(ISP 3) [24, 28]
Potato extract [23,24,28, 29]
Salt starch nitrate agar [38, 39]
Skim milk agar [36]
Starch casein agar [17,23,41]
Starch casein nitrate agar [36]
Trypticase soy broth [32, 33]

[#]Appropriate amount of salts are added in media as per growth requirement of organism(s) in question.

Streptomonospora: This genus of family *Nocardiopsaceae* was proposed by Cui et. al. (2001) [24]. It comprises of 5 representatives of halophilic actinomycetes. Streptomonospora is a group of extremely halophilic filamentous actinomycetes. Members of which are isolated frequently, probably due to the high occurrence of these actinomycetes in the hypersaline soil environment.

ISOLATION AND CULTIVATION

While attempting isolation, sampling is an important aspect demanding considerable attention. After collection, the samples should be examined immediately or aseptically stored under refrigeration. A variety of media are known for the isolation and characterization of different halophilic actinomycetes (Box 2). Growth media may be formulated according to the ecosystem or habitat from which sampling is done, e.g. seawater can be added to isolation agars at different concentrations to match the salt gradient in an estuary. Antifungal agents are usually incorporated into isolation agars to retard the fungal development.

APPLIED ASPECTS

Bioremediation

Heavy metal tolerance

Heavy metals are being introduced into the water bodies by various industries. Heavy metals such as lead, cadmium, copper, zinc, mercury, arsenic, and chromium in effluents from tanneries and fertilizer industries are released into nearby streams and rivers. Many heavy metals are released as by-products of different industries like leather industries, sugar mills, textiles, and fertilizer industries. Heavy metal contamination of the water bodies affects the flora of water bodies, thus disturbing the ecosystem. It can also cause health problems to animals and human beings [42]. Sponges filter large volumes of seawater and potentially accumulate heavy metals and other contaminants from the environment into their tissues. One study concerning the heavy metal resistance of sponge associated bacteria was reported to develop suitable biological indicator [43].

There are few reports on interaction between moderate halophiles and heavy metals [44, 45]. Bacteria tolerating heavy metals have been studied for the mechanism of tolerance. Some are responsible for environmental transformation and thus can be used as bioassay indicator in saline/non-saline, polluted and non-polluted environments [46]. Salinity of the medium may have an impact on metal tolerance of the organism [47]. Hypersaline soil and water often get polluted with heavy metals and other toxic material. During the manufacture of chemicals such as herbicides, pharmaceuticals, pesticides and during oil and gas recovery processes hypersaline waste water is generated. Normal remediation treatment may not work at such high salt concentration, so here halophilic organisms that work well at high salt concentrations can prove a useful alternative [48].

Some halophilic actinomycetes (*Streptomyces sp*.) were evaluated for their efficiency to bioremediate heavy metals. Two strains VITDDK1 and VITDDK2 isolated from Ennore saltpan showed significant heavy metal resistance. Both isolates grew well in the presence of zinc sulphate, cadmium, lead acetate, and cadmium sulphate [49]. Acidophilic and chemolithotrophic microorganisms are widely used for bioleaching purposes. Several alkaliphilic microorganisms also have bioleaching capacity. One such organism which prefers light, alkaline conditions (pH 8.5) for growth is *Nocardiopsis metallicus*. It is a halophilic actinomycete isolated from Germany. Due to its ability to solubilise metals, it may prove useful in mobilizing metals from soils, solid waste materials, and ores [50].

Crude oil degradation

Oil spillage is one of the main causes of environmental pollution. In a study involving multiple cultures of alkaliphilic, halophilic, and thermophilic actinomycetes, a thermophilic actinomycete *Thermoactinomyces dichotomicus* 84TH, isolated from soil in Georgia showed a high efficiency of oil hydrocarbon detoxification [51]. *Rhodococcus sp*. NCIM 5126, a marine actinomycete isolated from Bombay harbour could degrade aliphatic and aromatic fraction of crude oil, so it can be useful for bioremediation of soil and aquatic systems contaminated by hydrocarbons [52].

Enzymes

Biocatalysts offer advantages over the use of conventional chemical catalysts for numerous reasons, for example they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts and are economically viable.

Alkaline proteases are produced by different bacteria and fungi, but less study is being done on production of alkaline proteases by alkaliphilic actinomycetes. Proteases are very important industrial enzymes which constitute 60% of global enzymes sales. They constitute two thirds of the total enzymes used in various industries and it account for at least a quarter of the total global enzyme production [53-54]. Few reports describe extracellular alkaline proteases produced by certain halophilic and alkaliphilic bacteria [55]. *Nocardiopsis prasina* HA-4, isolated from Manipur (India) produced protease, and could tolerate a wide range of temperature (20-42^{0}C) and pH (7-10). The optimum temperature for the enzyme was 55°C and it had two pH optima at 7 and 10. Effect of certain metal ions (Ca^{2+}, Fe^{2+}, Mg^{2+}, Hg^{2+}) was also studied on enzyme production. Fe^{2+} (as $FeSO_4$) had stimulatory effect on HA4 enzyme activity, Ca^{2+} and Mg^{2+} had negative effect, whereas Hg^{2+} completely inhibited HA4 protease. Enhancement of protease activity by Fe^{2+} has been rarely reported [4]. Another salt tolerant alkaliphilic actinomycete *Streptomyces clavuligerus* strain Mit-1 isolated from Mithapur, western coast of India has also been reported to produce alkaline protease [36], and so as the alkaliphilic actinomycetes isolated from marine sediments of the Izmir Gulf, Turkey, strain MA1-1 [2].

Nocardiopsis halotolerans isolated from salt marsh in Kuwait was found to be slightly keratinolytic. It grew at 28–35^{o}C in salt concentrations of 0–15% [38]. *Saccharomonospora halophila*, another isolate from Kuwait, utilized keratin as sole carbon and nitrogen source [39].

One lignin oxidizing enzyme, lignin peroxidase (ligninase) is produced by *Streptomyces psammoticus*. Lignin is the most complex biopolymer and it plays a major role in carbon cycle. Lignin degrading enzymes have industrial applications such as textile dye decolourization, delignification of pulp and effluent treatment [56].

Biosurfactants

Chemical surfactants are surface active agents that reduce the surface tension, and are mostly petroleum derivatives. They are highly toxic and non-degradable in nature. Bio-surfactants are surface active agents produced by certain microorganisms. They are amphiphilic, non toxic, eco-friendly, with high foaming ability, and biodegradable in nature [57, 58]. They are stable at extreme conditions like high temperature, high salt concentration, etc. Bio-surfactants can thus be used as an alternative for chemical surfactants [49]. Marine actinomycetes are good candidates for bio- surfactant production [59]. *Streptomyces orientalis* and *S. aureomonopodiales* exhibited good biosurfactant activity at high temperature, pH and salt concentration [49].

Solvent tolerance

Solvent tolerance is a strain-specific property, and the toxicity of a given solvent correlates with the logarithm of its partition coeficient in n-octanol and water (log P_{ow}). Organic solvents with a log P_{ow} between 1.5 and 4 are extremely toxic for microorganisms and other living cells because they partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions [36], changing structural and functional integrity followed by cell lysis [60]. In presence of organic solvents, normally enzymes are easily denatured and their catalytic activities deminish [61]. Thus enzymes which naturally remain stable in the presence of solvents could be very helpful for biotechnological applications in which these types of solvents are used [62]. Halophilic extremozymes can keep their catalytic properties in organic solvents, even at low salt concentrations. The use of these extremozymes in organic media could open new fields of application [63].

The halophilic α-amylase isolated from *Nesterenkonia sp.* strain F from Aran-Bidgol lake (Iran) could tolerate various organic solvents such as chloroform, cyclohexane, benzene, and toluene. Such properties of halophilic α-amylases could be exploited in starch-processing industries, baking, brewing, textile, and distilling industries [64]. A salt-tolerant alkaliphilic actinomycete, *Streptomyces clavuligerus* isolated from Mithapur, coastal region of Gujarat, India could grow up to 15% salt and pH 11, the optimum being 5% salt and pH 9. It was able to tolerate, and secrete alkaline protease in the presence of xylene, ethanol, acetone, butanol, benzene, and chloroform (each solvent upto 2%v/v). The solvents were tested with crude, partially purified and purified enzyme. The enzyme secretion was increased by 50-fold in presence of butanol. It could also utilize these solvents as the sole source of carbon with significant enzyme production [36].

FINAL COMMENTS

Several halophilic actinomycetes have been studied for solvent tolerance, and metal tolerance, and how presence of solvents or metals affect extracellular enzyme production in them. It will be interestingly useful to decipher the strategy by which they tolerate such multiple stresses (high salt, metal, solvent) and still maintain catalytic efficiency of their enzymes. Whether and how these enzymes differ from those produced by other organisms needs to be explored, which may lead to significant novel biotechnological applications. Additionally such hardy organisms which are simultaneously withstanding high salt, multiple metal ions or organic solvents, can serve as good model for investigating stress response in prokaryotes.

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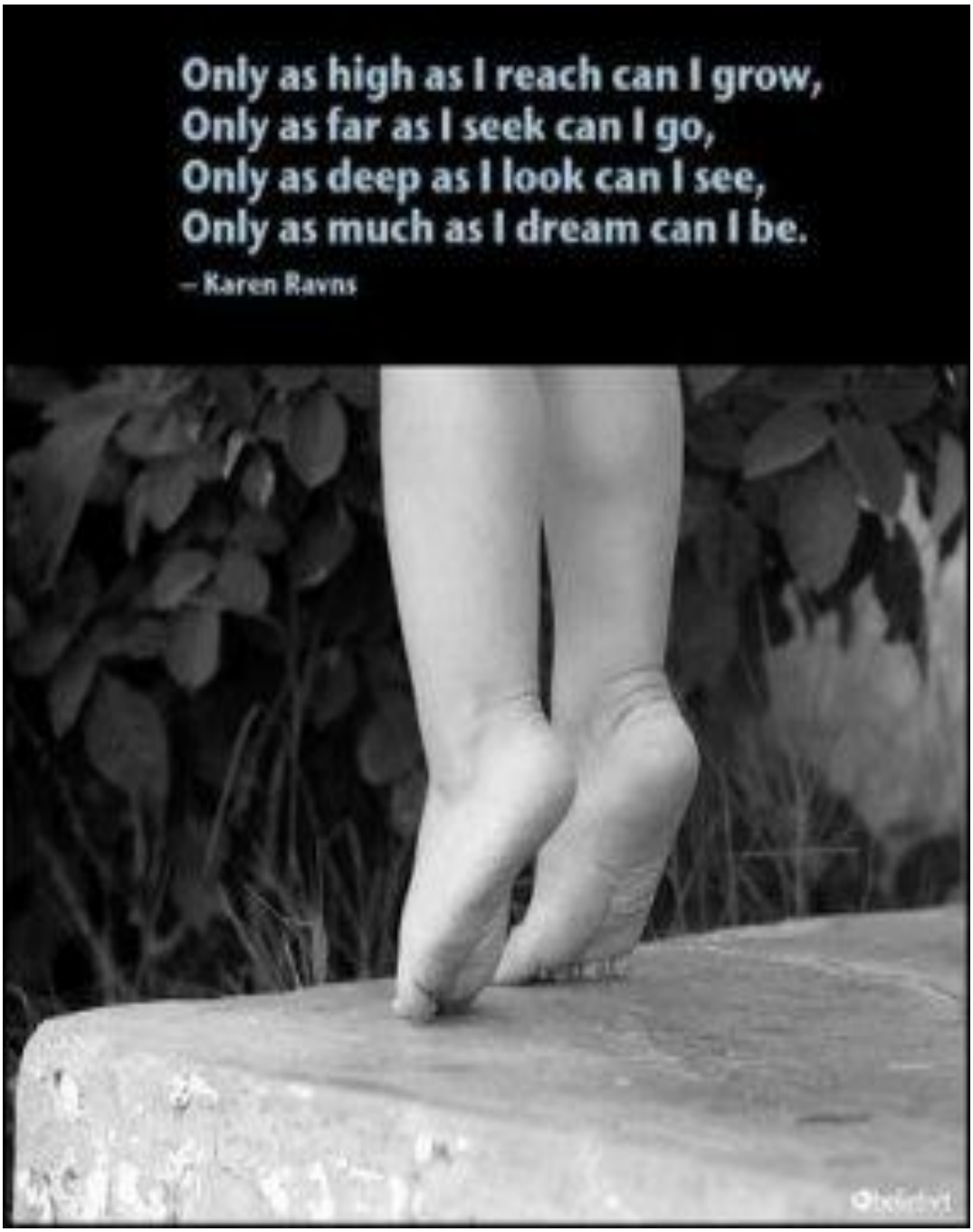
Only as high as I reach can I grow,
Only as far as I seek can I go,
Only as deep as I look can I see,
Only as much as I dream can I be.
– Karen Ravns