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Measuring the Bioavailability of Iron and Zinc by the DTPA-TEA Method in Moderately Within Halophilic Strains

Masoud Drakhshi[1], Mehrnoush Eskandari Torbaghan[*2]& Masoud Eskandari Torbaghan[3]
[1&2] *Lecturers of Khorasan Razavi Agricultural Education Center, Mashhad, Khorasan Razavi, Iran*
[3] *Academic Member of Agricultural and Natural Resources Research Center of Khorasan Razavi*
Corresponding author's email address: mehrnoosh.eskandary@gmail.com

Abstract
According to the FAO study, about 20 to 50 percent of the world's agricultural soils suffer from different degrees of salinity. Various reasons including low absorption of trace elements in saline and alkaline soils due to high-pH soils accompanied by plenty of lime such as Iran soils, ionic imbalance, low organic matter, carbonate presence in irrigation water, the inexorable and imbalance use of fertilizers and finally the subsequent droughts led to study both the bioavailability of iron and zinc within halophilic bacterial strains collected from terrestrial resources and availability of these nutrients for plants under salt stress by means of DTPA-TEA extraction method. Halophilic bacterial strains were isolated and purified from six saline soils in Khorasan Razavi Province (Iran) using the Ventosa moderately halophilic bacteria culture medium. Afterward, the concentrations of Fe and Zn in these strains with three replicates were measured by the DTPA-TEA method. The results showed that only four strains H2, H1, H11 and H3 of the fourteen isolated had iron; however, the iron concentration of the strains did not differ significantly or to the control (bacteria culture medium). Measurement of zinc showed that all halophilic strains had zinc except for two strains: H9 and H11, but the amount of zinc of strains did not differ significantly between strains or to the control. Strain H2 also showed the highest concentration of extractable iron and zinc. Concentrations of iron and zinc in the strains showed a low correlation coefficient (R^2=0.15). Due to the absence of both iron and zinc in the bacteria medium, the amount of iron and zinc measured in strains may be structural and constitute some part of microbial tissues. Because of measuring these micro nutrients based on the simple method of EDTA-TEA, the chance of bioavailability of the nutrients exists in the rhizosphere of plants under salt stress conditions. In addition, the average electrical conductivity, osmotic pressure and total dissolved solids in halophilic bacterial extracts were 60.85 dS/m, 21.61 atmospheres, 3.89 percent respectively. The high osmotic pressure in bacterial extracts, which is equal to the osmotic pressure of the soil in permanent wilting point (10 to 20 atmospheres), could be another reason for the viability and efficiency of these special bacteria in high salinity. Hence, due to the high bacterial population growth and survival of bacteria under the conditions of high salinity and osmotic pressure of the soil, these bacteria may have the ability to help the stressed plants and biofortification of these important micronutrients in human food.
Key words*:* Moderately halophilic strains, Fe and Zn bioavailability, DTPA-TEA method, Salinity stress

Introduction
Soil salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Recent studies indicate that microorganisms can help crops to cope with saline stress (Venkateswarlu and Shanker 2009). Iran is situated in one of the most arid and semi arid regions of the world. Salinity and drought are among the most important environmental stresses that limit crop production in Iran (FAO report, 2005). Halophiles are salt loving organisms that flourish in saline environments and can be classified as slightly, moderately or extremely halophilic, depending on their requirement for sodium chloride (DasSarma and DasSarma, 2012). Hypersaline environments are found all over the world, in arid, coastal, and deep sea locations, underground salt mines, and artificial salterns. Halophilic microorganisms include a variety of heterotrophic, phototrophic, and methanogenic archaea, photosynthetic, lithotrophic, and heterotrophic bacteria, and photosynthetic and heterotrophic eukaryotes. Halophilic organisms either accumulate internal organic compatible solutes to balance the osmotic stress of the environment or produce acidic proteins to increase solvation and improve function in high salinity (DasSarma and DasSarma, 2012).
Viable plants require the metal nutrients to be in the aqueous phase so that the absorption takes place in the roots and is susceptible to the strong binding or precipitation of metals (Lasat, 2002). Many nutrient deficiencies produce characteristic visual symptoms in cereals. Visual symptoms may be the first clue that the nutrient supply is limiting plant growth. These symptoms in wheat are far more nebulous than in some other species (Snowball and Robson, 1991).
Whereas yeast requires complex nutrients for growth, bacteria are well recognized for their low growth requirements and ability to adapt in new environments (Lasat, 2002). More precisely, halophilic bacteria are the best candidates to be directly inoculated into toxic saline environments along with accumulating metal ions on their surface, cell wall, and intracellular (Massadeh et al., 2005). Pentetic acid or diethylene triamine pentaacetic acid (DTPA) is an aminopolycarboxylic acid consisting of a diethylenetriaminebackbone with five carboxymethyl groups. The conjugate base of DTPA has a high affinity for metal cations. Thus, the penta-anion DTPA5− is potentially an octadentate ligand assuming that each nitrogen centre and each COO−-group counts as a centre for coordination. The formation constants for its complexes are about 100 greater than those for EDTA (Roger Hart, 2005). As a chelating agent, DTPA wraps around a metal ion by forming up to eight bonds. DTPA (diethylenetriaminepentaacetic acid) extraction method has gained significant adoption by agricultural laboratories. DTPA offers a most favorable combination of stability constants for the simultaneous complexing of Fe, Mn, Cu & Zn (Mahashabde and Patel, 2012). The extraction solution should be

selected to solubilize amounts of the micronutrients that are proportional to amounts that will be absorbed by plants during single growing season.

The goals of this study were the possibility of using the simple method "DTPA" to measure of iron and zinc concentrations in the living systems "halophilic bacteria" and investigation of their bioavailability for plants in the salinity stress conditions.

Materials and Methods

Bioavailability of iron and zinc in halophilic bacterial strains collected from terrestrial resources and availability of these nutrients for plants under salt stress were studied by means of DTPA-TEA extraction method. Halophilic bacterial strains were collected from six saline soils in Khorasan Razavi Province (Iran). Composite soil samples were collected from a depth of 0-25 cm from saline different areas (Bardaskan salt lake, Kalate shour Aref Abad (Kashmar), Yonsi and Merendiz (Bajestan), two samples from Astane Ghodes pistachio gardens and kale shour Eshgh Abad (Neyshabour)) in sterile plastic tubes with area recording by GPS and were transferred as soon as possible to lab (not more than 48 h after collection). The samples were kept at 4 °C during this interval and then were stored at 4 °C to separation stage. Halophilic bacterial strains were isolated and purified using the Ventosa moderately halophilic bacteria medium (Ventosa et al., 1982). The medium used for isolation was as follows (g/L): NaC1, 81; $MgC1_2$, 7; $MgSO_4$, 9.6; $CaCI_2$, 0. 36; KCI, 2; NaHCO, 0.06; NaBr, 0.026 supplemented with 5 (g/L) Proteose-peptone, 10 (g/L) yeast extract, 1 (g/L) glucose and 20 (g/L) agar. pH 7.2 adjusted with KOH. Soil samples were diluted with sterile distilled water as 1:1 ratio (1 g soil to 1 ml sterile distilled water).The samples were surface-inoculated on HM medium and incubated at 34 °C for 5 d in sterile Petri dishes. Colonies were spread and successively subcultured on HM to ensure purity. Colonies were controlled daily and observations were recorded at each step of the purification. Finally, colonies were cultivated in HM liquid medium until late log phase and one loop of each colony were suspended with sterile fresh medium containing 10% glycerol. The cells suspensions in plastic presterilized screw-cap vials were placed in a mechanical freezer at -80°C for 1 h and then in a liquid nitrogen (Horikoshi, 1999). Vials were preserved in a freezer at -20°C. To measure the bioavailability and concentration of iron and zinc by the DTPA-TEA method, moderately halophilic strains were recultured. Frozen cultured were recovered by rapid thawing in the sterile HM liquid fresh medium. 20 ml of fresh medium were centrifuged at 6000 × g for 20 min (Gutierrez et al., 2009). 10 ml of culture supernatant after centrifugation were mixed with 20 ml DTPA-TEA extractor. (1.967 g of diethylene tetramine penta acetic acid (DTPA) was dissolved in some distilled water in a 500 ml beaker and then 1.470 g of calcium chloride was weighed and added to the DTPA solution. Next, 14.91 g of tri ethanol amine (TEA) was added and solution volume was increased to 400 ml with distilled water. pH was adjusted to 7.3 with 6 N HCl. Beaker contents were transferred to a 1 liter flask and the final volume was increased to 1 liter with distilled water). Samples were shook for 2 h in the room temperature. Then, samples were filtered by filter paper and stored in plastic tubes. Subsequently, the concentrations of Fe and Zn in these strains with three replicates were measured by atomic absorption spectroscopy (PG9000 model). Electrical conductivity and pH were measured in all strains samples with conductivity meter and pH meter (EW-35414-00). The data were analyzed using the software MSTAT-C. None parametric tests were preformed by SPSS version 11.0. The goodness of fit of the distributions including normal distribution, Uniform distribution, Poisson distribution and exponential distribution were examined by the Kolmogorove-Smirnov Test.

Results and Discussion

The results of the experiment showed that the concentration of Fe was higher in four strains than to control (Fig 1). H2, H1, H11 and H3 strains had 18.5, 6.4, 1.0 and 0.2 percent increase than to control. But, they did not show any significant difference to each other or to the control. Iron concentration was observed lower in the other strains than to the control. Minimum of Fe concentration was observed in H12, H6, H10, H7,H4 and H9 with -50, -35.8, -30.6,-29.8, 28.6 and -25.8 percent decrease compared to control respectively (Fig. 1). They also showed significant differences with the control. But, no significant difference was observed among these strains. The remained strains showed lower Fe concentration than to the control (Fig. 1). Although, they had no significant difference to each other or to the control (Fig. 1). Researchers expressed that *Halobacterium cutirubrum*, *H. salinarium*, and *H. halobium* required at least 10 p.p.m. ferrous ions for maximal growth in aerated cultures. The addition of iron up to 50 p.p.m. increased the rate of growth *H. cutirubrum* but amounts greater than 10 p.p.m. had little effect on the final population (Sehgal and Gibbons, 1960). A few preliminary experiments with Fe59 indicated a considerable amount of iron is taken up by the cells, most of which seems to be in the cytoplasm (Sehgal and Gibbons, 1960). It seems that results of this experiment showed the same phenomenon occurred in some strains (Fig 1).

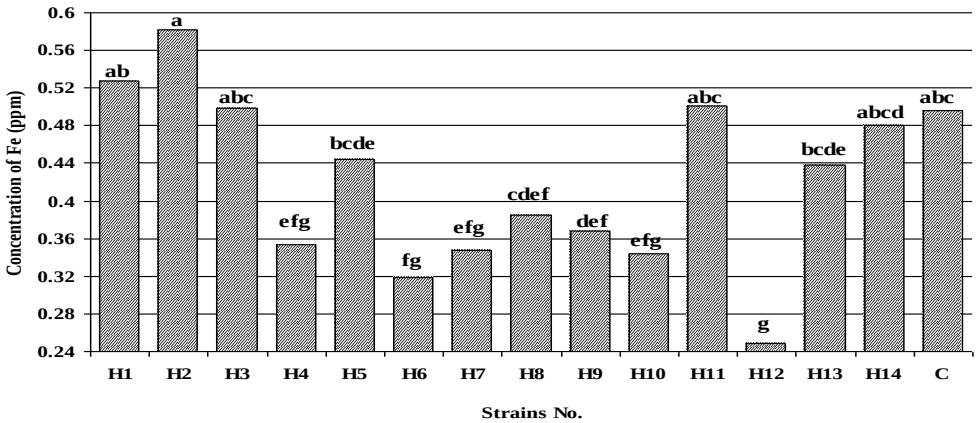

Fig 1. Concentration of Fe in halophilic strains media (p.p.m)

None of the strains showed significant difference in zinc concentration to each other or to the control (Fig. 2). All of them had higher zinc concentration than to control except of H11 and H9. These two strains had -10.8% and -2.9% reductions compared to control. Maximum increase in Zn concentration was observed in two strains (H2 and H13) with +42.2 and +39.3 increase respectively (Fig. 2). The growth of *H. cutirubrum* was stimulated by 0.05 p.p.m. manganese and 1 p.p.m. zinc ions and 2 p.p.m. calcium ions but more addition of divalent zinc or calcium had no effect on the growth of this organism (Sehgal and Gibbons, 1960). The accumulation of the absorbed metals was found to be maximum in the protoplast of all cultures (Al-Momani et al., 2007). Uptake of zinc and copper by halophilic bacteria isolated from the Dead Sea shore in Jordan (Al-Momani et al., 2007) showed that the accumulation of the uptake of Zn or Cu of three selected isolates was detected in three cellular parts (cell wall, plasma membrane, and cyto-plasm). The analysis found that the highest Zn accumulation in the cell wall was achieved by culture 6, with 0.072%, and that accumulated in each bacterial cell wall was 0.015×10^{-4}% per viable cell; the absorption between the cell wall and the plasma membrane was achieved by culture 8, with 0.1%, and accumulation in cytoplasmic fluid was 6.86%, achieved by culture 2 (Al-Momani et al., 2007). All the moderately and extremely halophilic bacteria tested were sensitive to zinc concentrations and zinc concentration is needed for growth in halobacteria, halococci and moderately halophilic eubacteria 2.5 mM at least (Rodriguez-Valera, 1991).

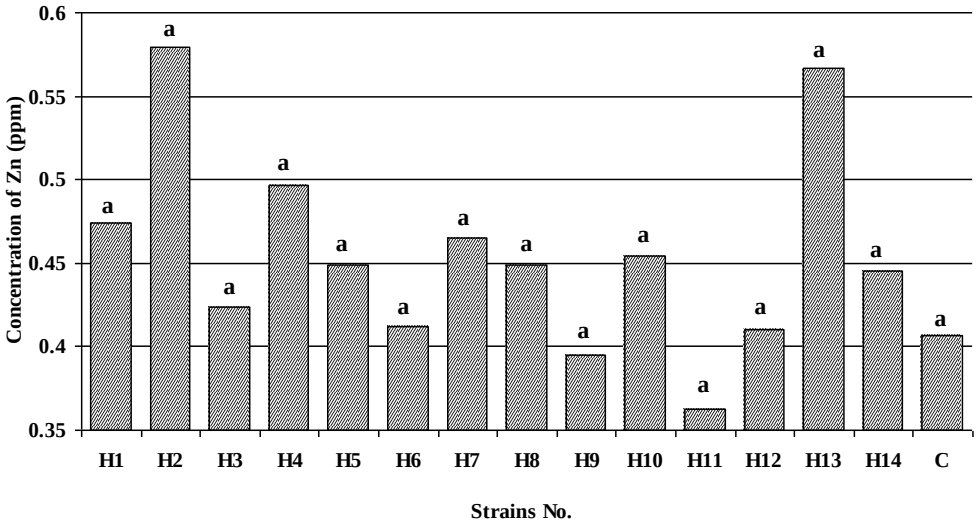

Fig 2. Concentration of Zn in halophilic strains media (p.p.m)

None parametric tests performance for iron concentration showed that data followed by the two tests (normal and uniform distribution) as the Kolmogrov-Smirnov values were 0.711 and 0.381 ($P_v > 0.05$) for these two tests respectively. However, this value was very higher in normal distribution. So, the most appropriate distribution for iron concentration data was normal distribution (Tables 1 and 2).

One-Sample Kolmogorov-Smirnov Test

		FE
N		45
Normal Parameters[a,b]	Mean	.4221
	Std. Deviation	.10715
Most Extreme Differences	Absolute	.104
	Positive	.081
	Negative	-.104
Kolmogorov-Smirnov Z		.700
Asymp. Sig. (2-tailed)		.711

a. Test distribution is Normal.

b. Calculated from data.

Table 1. The normal distribution test for Fe concentrations

One-Sample Kolmogorov-Smirnov Test

		FE
N		45
Uniform Parameters[a,b]	Minimum	.23
	Maximum	.65
Most Extreme Differences	Absolute	.135
	Positive	.135
	Negative	-.051
Kolmogorov-Smirnov Z		.909
Asymp. Sig. (2-tailed)		.381

a. Test distribution is Uniform.

b. Calculated from data.

Table 2. The uniform distribution test for Fe concentrations

The data did not follow by the Poisson and exponential distribution for iron concentration (Tables 3 and 4).

One-Sample Kolmogorov-Smirnov Test

		FE
N		45[c]
Poisson Parameter[a,b]	Mean	.4221

a. Test distribution is Poisson.

b. Calculated from data.

c. Poisson variables are non-negative integers. The value .23 occurs in the data. One-Sample Kolmogorov-Smirnov Test cannot be performed.

Table 3. The Poisson distribution test for Fe concentrations

One-Sample Kolmogorov-Smirnov Test

		FE
N		45
Exponential parameter.[a,b]	Mean	.4221
Most Extreme Differences	Absolute	.427
	Positive	.216
	Negative	-.427
Kolmogorov-Smirnov Z		2.863
Asymp. Sig. (2-tailed)		.000

a. Test Distribution is Exponential.

b. Calculated from data.

Table 4. The exponential distribution test for Fe concentrations

Results for zinc concentration also showed that data only followed by the normal distribution (Table 5). P_{value} was very low ($P_v < 0.05$) in other distributions for Zn concentration (Tables 6, 7 and 8).

One-Sample Kolmogorov-Smirnov Test

		ZN
N		45
Normal Parameters[a,b]	Mean	.4527
	Std. Deviation	.10243
Most Extreme Differences	Absolute	.077
	Positive	.077
	Negative	-.052
Kolmogorov-Smirnov Z		.516
Asymp. Sig. (2-tailed)		.953

a. Test distribution is Normal.

b. Calculated from data.

Table 5. The normal distribution test for Zn concentrations

One-Sample Kolmogorov-Smirnov Test

		ZN
N		45
Uniform Parameters[a,b]	Minimum	.23
	Maximum	.74
Most Extreme Differences	Absolute	.270
	Positive	.270
	Negative	-.118
Kolmogorov-Smirnov Z		1.812
Asymp. Sig. (2-tailed)		.003

a. Test distribution is Uniform.

b. Calculated from data.

Table 6. The uniform distribution test for Zn concentrations

One-Sample Kolmogorov-Smirnov Test

		ZN
N		45[c]
Poisson Parameter[a,b]	Mean	.4527

a. Test distribution is Poisson.

b. Calculated from data.

c. Poisson variables are non-negative integers. The value .23 occurs in the data. One-Sample Kolmogorov-Smirnov Test cannot be performed.

Table 7. The Poisson distribution test for Zn concentrations

One-Sample Kolmogorov-Smirnov Test

		ZN
N		45
Exponential parameter.[a,b]	Mean	.4527
Most Extreme Differences	Absolute	.445
	Positive	.228
	Negative	-.445
Kolmogorov-Smirnov Z		2.985
Asymp. Sig. (2-tailed)		.000

a. Test Distribution is Exponential.

b. Calculated from data.

Table 8. The exponential distribution test for Zn concentrations

Measurement of electrical conductivity in media's strains also showed that minimum of EC was observed in H2, H1, H4, H3, H9 and H5 with -35.6, -33.9, -18.6, -16.9, -8.4 and -6.7 percent reduction compare to control (Fig. 3). H10 showed -1.7% decrease and none significant difference compare to control. Media in these remained strains showed higher EC than the control. But, these increases were not significant (Fig 3).

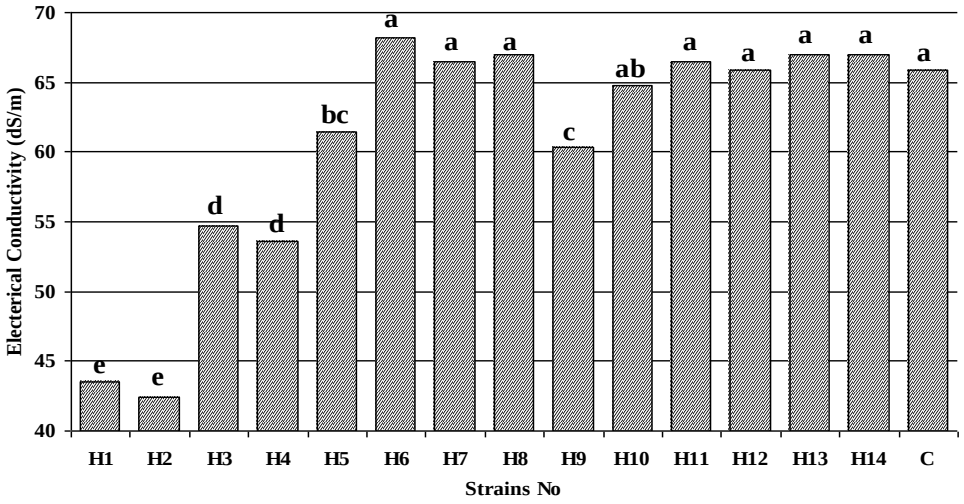

Fig 3. Electrical conductivity in halophile strains (dS/m)

Data analysis for osmotic pressure, total dissolved solids and sum of cations or anions showed the same trend such as electrical conductivity for halophilic strains were isolated (Table 9).

Table 9.The effect of strains' type on O.P (bar), T.D.S (ppm) &(%),C(meq/l)

Strain No.	O.P (bar)	T.D.S (ppm)	T.D.S (%)	C (meq/l)
1	15.69 e	27885.3 e	2.78 e	435.7 e
2	15.28 e	77170.3 e	2.71 e	424.5 e
3	19.71 d	35035.3 d	3.50 d	547.4 d
4	19.31 d	34320.3 d	3.43 d	536.3 d
5	22.12 bc	39325.4 bc	3.93 bc	614.5 bc
6	24.53 a	43615.4 a	4.36 a	681.5 a
7	23.93 a	42542.9 a	4.25 a	664.7 a
8	24.13 a	42900.4 a	4.29 a	670.3 a
9	21.72 c	38610.4 c	3.86 c	603.3 c
10	23.33 ab	41470.4 ab	4.14 ab	648.0 ab
11	23.93 a	42542.9 a	4.25 a	664.7 a
12	23.73 a	42185.4 a	4.21 a	659.1 a
13	24.13 a	42900.4 a	4.29 a	670.3 a
14	24.13 a	42900.4 a	4.29 a	670.3 a
C	23.73 a	42185.4 a	4.21 a	659.1 a

Means within a column followed by the same letter are not significantly different at p = o.o5

Maximum of pH was observed in H6, H7 and H5 with 9.3, 8.0 and 6.6 percent increase and significant difference to control (Fig. 4). Strains of H3 and H8 had a 1.3% increases without significant difference compared to control. But, strains of H12 and H4 had a -1.3 and -2.6 percent decrease and none significant difference than to control. All remained strains showed lower pH compare to control (Fig. 4). H10, H14, H1, H2 and H13 showed -34.6, -34.6, -33.3, -33.3, -33.3 and -32 percent decrease and significant difference compare to control (Fig. 4).

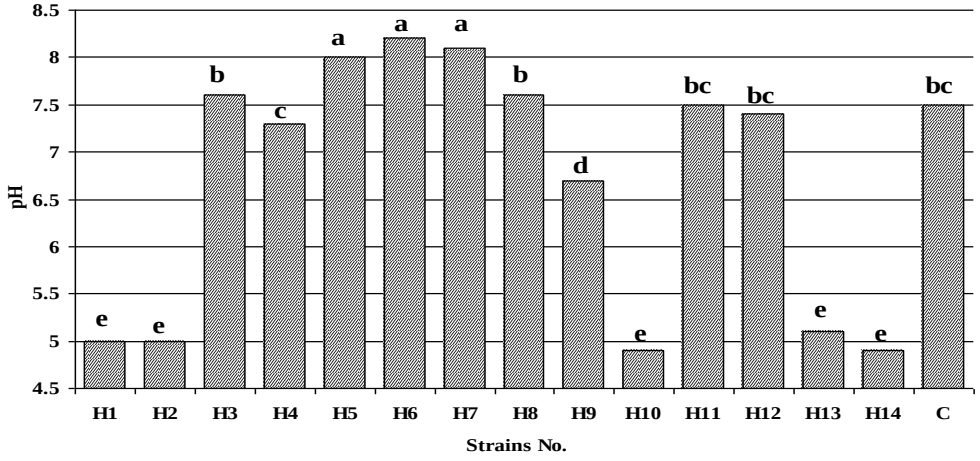

Fig 4. pH concentration in halophile strains (dS/m)

The pH content was adjusted at 7.2 in media but in some strains pH decreased up to 4.9. Only a very few extreme halophiles are able to grow optimally under alkaline conditions. Microorganisms with pH values for optimal growth above 8.5 carry with them the unique energetic problems of alkaliphiles, e.g., an inverted pH gradient and thus a suboptimal proton motive force (Bowers and Wiegel, 2011). In the case of extremely halophilic alkaliphiles these problems are exacerbated by the need to keep the intracellular sodium concentration below toxic levels, which is frequently as low as a few mM (Bowers and Wiegel, 2011). This complication may explain the more prevalent occurrence of microorganisms growing optimally in environments that are pH neutral or near neutral. However, as discussed by Bowers et al. (2009a), this could be due to the fact that researchers may simply have not tried to isolate microorganisms able to thrive under these combined stressors. Gochnauer and Kushner (1969) showed that H. *halobium* produced considerable acid only when grown in the presence of 2% glycerol.

Conclusion

At an elementary level, the nutritional requirements of a bacterium are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo. These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells (Todar, 2012). Iron is an essential nutrient forming 0.2% of dry

weight of bacteria. The iron results from iron salts and functions a component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions. Trace elements are metal ions required by certain cells in such small amounts that it is difficult to detect (measure) them, and it is not necessary to add them to culture media as nutrients. Trace elements are required in such small amounts that they are present as "contaminants" of the water or other media components. As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell. One organism's trace element may be another's required element and vice-versa, but the usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo (Todar, 2012).

Data showed that most strains consumed the iron in media and only a few strains had greater iron concentration control (Fig. 1). As figure 5 shows, maybe H2 and H1 are iron bacteria that derive the energy they need to live and multiply by oxidizing dissolved ferrous iron. The resulting ferric oxide is insoluble, and appears as brown gelatinous slime that will stain plumbing fixtures, and clothing or utensils washed with the water carrying it (Simon et al., 2013). They are known to grow and proliferate in waters containing as low as 0.1 mg/l of iron. However, at least 0.3 ppm of dissolved oxygen is needed to carry out oxidation. Iron is one of the essential elements for a proper plant development. Providing plants with an accessible form of iron is crucial when it is scant or unavailable in soils. Chemical chelates are the only current alternative and are highly stable in soils, therefore, posing a threat to drinking water (Radzki et al., 2013). Bacteria depend upon iron as a vital cofactor that enables a wide range of key metabolic activities. Bacteria must therefore ensure a balanced supply of this essential metal. To do so, they invest considerable resources into its acquisition and employ elaborate control mechanisms to elevate both iron-induced toxicity as well as iron deficiency. In addition, various combinations of nano-chelats iron, zinc and manganese use to strengthen microorganism environment and also provides good conditions for plant growth.

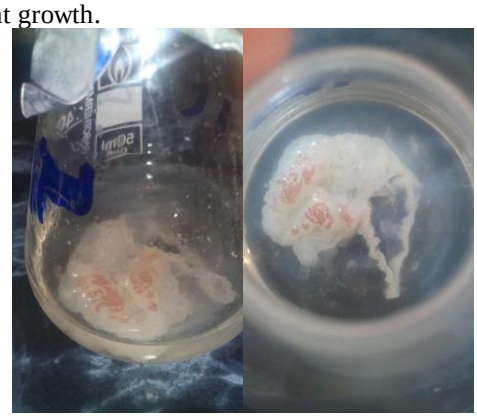

Fig 5. The images of H2 strain

Only H11 and H9 had lower zinc concentration compared to control. However, data did not show any significant difference between all strains. The effect of one of the widely used heavy metal, zinc (Zn) and zinc oxide nanoparticles (ZnO NPs) on extremely halophilic archaea (Salgaonkar et al.,2015) was showed that all the haloarchaeal genera (*Halococcus*, *Haloferax*, *Halorubrum* and *Haloarcula*) investigated were resistant to both $ZnCl_2$ and ZnO NPs at varying concentrations. *Halococcus* strain BK6 and *Haloferax* strain BBK2 showed the highest resistance in complex/minimal medium of up to 2.0/1.0 mM $ZnCl_2$ and 2.0/1.0–0.5 mM ZnO NP. Accumulation of $ZnCl_2$/ZnO NPs was seen as *Haloferax* strain BBK2 (287.2/549.6 mg g^{-1}) *Halococcus* strain BK6 (165.9/388.5 mg g^{-1}) *Haloarcula* strain BS2 (93.2/28.5 mg g^{-1}) *Halorubrum* strain BS17 (29.9/16.2 mg g^{-1}). Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM–EDX) analysis revealed that bulk $ZnCl_2$ was sorbed at a higher concentration (21.77 %) on the cell surface of *Haloferax* strain BBK2 as compared to the ZnO NPs (14.89 %) (Salgaonkar et al., 2015). While investigating the antagonism of toxic zinc ions by magnesium ions, MacLeod and Snell (1950) found that zinc was antagonized by a number of divalent cations. They suggest the possibility of inactivation of certain proteins (apoenzymes) by zinc during the formation of metal loenzymes. It was further suggested by them that the antagonizing ions form active complexes with these proteins.

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