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Molecular characterization of zinc solubilising bacteria isolated from different rhizospheric soil and assessment of their physiological attributes

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Abstract
Zinc is a very crucial micronutrient for crop plants that plays many important functions in their life cycle. Plant growth, development, maturity, vigor, and yield are directly or indirectly affected by zinc. The present study was conducted to isolate and characterize zinc solubilizing bacteria (ZSB) from different rhizospheric soil collected from different crop rhizosphere. ZSB proficiently solubilized both the insoluble zinc compounds although ZnO was effectively solubilized in comparison to $ZnCO_3$ and $ZnPO_4$. The soluble Zn concentration was determined in the culture supernatant using atomic absorption spectrophotometer (AAS). In broth assay, ZSB24 showed high solubilization range in zinc oxide 83.2 mg Zn/l and followed ZSB14 with a value of 68 mg Zn/l. A positive correlation between Zn solubilization and reduction in pH of the culture medium was also noted for most of the ZSB isolates. These 32 Zinc solubilising bacteria were subjected to morphological, physiological and molecular characterization, screening of their plant growth promotion activities, ability of these isolates to survive in abiotic stress conditions *viz.*, salt, pH, drought, antibiotic and temperature stress. The results revealed that all the ZSB isolates were gram negative. The molecular diversity by Amplified Ribosomal DNA Restriction Analysis (ARDRA) was done in thirty two ZSB strains revelled high diversity amongst them. Based on UPGMA clustering analysis, the strains showed significant molecular diversity and the dendogram obtained differentiated 32 strains into 02 major clusters. The Major clusters were further sub divided into four sub clusters. Major cluster A consisted a total 27 strains and sub cluster A1 consisted of 3 strains, A2 consisted of 24 strains and cluster B consisted of 02 strains and 3 strains (ZSB20, ZSB29 and ZSB5) stood apart and were kept as independent minor groups in the dendogram.

Keywords: Zinc solubilising bacteria, rhizospheric soil, physiological attributes

Introduction
The importance of Zn in the living system was first identified by Raulin in 1869 in the bread mold, Aspergillus niger. A proteome analysis of all the three forms of life (bacteria, archaea and eukaryotes) revealed that 4-10% of cellular proteins are basically Zn-binding proteins (Andrieni *et al*. 2006) [3]. Zinc deficiency is widespread among plants grown in calcareous soils of high pH because zinc is made unavailable through adsorption to clay or calcium carbonate (Marschner H, 1995) [24]. In plants, more than 90% of Zn is present in soluble forms. It plays major roles in carbohydrate metabolism, through photosynthesis; in sucrose and starch formation; protein metabolism; membrane integrity; auxin metabolism; and reproduction. Plants absorb available zinc in a reactive form from the soil solution. Available amount of zinc to plants is controlled by soil factors, e.g., total zinc concentration, organic matter, pH, clay, redox conditions, calcium carbonate, microbial activity in the rhizosphere, soil moisture, concentrations of other trace elements, and concentrations of macronutrients, especially climate and phosphorus (Alloway, 2008) [2]. Supply of Zn is mostly affected by the soil pH in soil pools, on account of the fact that this element is easily adsorbed in cation exchange sites at over neutral pH and made available at low pH values (Broadley *et al*. 2007) [4].
Use of microorganisms in sustainable crop production and restoration of fertility is gaining more interest. Zn-solubilizing microbes have been discovered from the soils of many crops and tested as plant growth-promoting factors (Goteti *et al*. 2013; Sunithakumari *et al*. 2016) [14, 36]. Glick (2007) [12] reported the use of PGPR which promotes plant growth through releasing phytohormones, solubilization and assistance in nutrient acquisition, and biocontrol agents to protect plants from different pathogens. Zinc is essential for the synthesis of tryptophan, a precursor of IAA, and is also active in the production of growth hormones such as auxins

(Cakmak, 2000) [6]. Zn-solubilizing microorganisms have great potential as compared to chemical sources of plant nutrients such as fertilizers. Within 7 days of application, applied Zn fertilizers converted into various insoluble forms (Saravanan *et al*. 2004) [34]. Hence, the insoluble form of Zn can be converted into soluble form by treated bacterial cultures with the ability for Zn solubilization. This shortage can be managed by zinc solubilizing microbes, which have great ability to convert many unavailable forms of metals to a readily available form. These microbes can convert unsolubilized zinc such as zinc phosphates, zinc oxide, and zinc carbonates in good amounts, which is not a common feature among the microbes in the top surface soils (Cunninghan and Kuiack, 1992) [8]. ZSBs are capable alternatives that can cater essential zinc to plants through solubilizing complex zinc into soils. Several genera of microbes, such as *Bacillus, Pseudomonas, Acinetobacter, Thiobacillus thiooxidans,* and *Thiobacillus ferrooxidans*, have been found as Zn solubilizers (Saravanan *et al*. 2007) [33]. The solubilized metals are formed by chelated ligands, protons, and the oxido-reductive system available on cell surfaces and in cell membranes. These microbes have many beneficial features to the plants such as producing phytohormones, siderophores, vitamins, antibiotics, and antifungal substances (Goteti *et al*. 2013) [14]. Among the microorganisms, an group of soil bacteria known as plant growth-promoting rhizobacteria (PGPR) have a role in nutrient cycling and, therefore, have attracted special attention for such bio-inoculants in sustainable agriculture (Glick *et al*. 1999) [13]. In this context, application of beneficial rhizosphere microorganisms to convert insoluble zinc into the soluble form for plant assimilation and to achieve objectives of low-cost input is highly essential for sustainable agriculture (He *et al*. 2010).

Materials and Method

Collection of rhizospheric soil

Rhizospheric soil from different crop rhizosphere like chickpea, arhar, wheat and barley were collected for isolation of ZSB. For sampling 3-4 locations of fields were identified per selected area. Four-five plants of variable vigour were selected from each field, uprooted with bulk rhizospheric soil, and pooled up to make a representative sample. The samples were placed individually in poly bags, labelled and transported to laboratory shady dried, powdered and stored at 4°C for further research study. Physical and chemical properties of collected soil samples were studied as per the standard protocols (Vance *et al*., 1987, Lindsay and Norvell, 1978) [22, 37].

Isolation of ZSB and Media optimization

ZSB were isolated by serial dilution plate method (Pramer and Schmidt, 1956) [27]. The Tris Mineral salts medium containing (Dextrose-10.0g, $(NH_4)_2$ SO_4-1.0gm, KCl-0.2gm, K_2HPO_4-0.1gm, $MgSO_4$-0.2gm, Zinc Source 0.1%, Agar agar 20 g, pH 7.0, Distilled water 1000 ml) (Saravanan *et al*., 2007) [33] and Bunt & Rovira medium (Glucose 20 g, Peptone 1 g, Yeast Extract 1 g, $(NH_4)_2SO_4$-0.50 g, K_2HPO_4-0.40 g, $MgCl_2$-0.10 g, $FeCl_3$-0.01 g, Zinc source- 0.1%, Agar agar 20 g, pH 7.0, Distilled water 1000 ml) (Bunt and Rovira, 1955) supplemented with different insoluble zinc source [like Zinc phosphate (ZnP), Zinc carbonate (ZnC) and Zinc oxide (ZnO)] was prepared & autoclaved at 121ºC for 30 mins. The sterile agar medium was poured in sterile petri plates under aseptic condition. After solidification 0.1ml from the final dilutions of 10^{-5}-10^{-8} poured in media plates. They were gently rotated clockwise and anti-clockwise with the help of spreader for uniform distribution and incubated at room temperature (28±2ºC) for 24-48 hours. A clear halo zone forming colonies were isolated individually and 79 isolates were purified by streak plate method (Rangaswami, 1993) [28] for further study. There were three biological replicates and the experiment was repeated twice.

Selection of ZSB strains

After the incubation period the grown individual colonies exhibited halozone around the colony from 10^{-4}, 10^{-5}, and 10^{-6} dilution were transferred into nutrient slants. The pure cultures were used for zinc solublization test.

Qualitative estimation of zinc solubilizing potential of the isolates (plate assay)

To assess zinc solublization ability of the strains, the halo zone forming bacterial strains were placed on Bunt and Rovira agar and MSM media plates amended with 0.1% insoluble zinc source. The plates were incubated at 28ºC+2ºC for 48 h. By measuring the diameter of the clear halo zone and colony growth Zn solubilization efficiency was determined.

$$\text{Solubilization efficiency} = \frac{\text{Zone diameter}}{\text{Diameter of colony growth}} \times 100$$

Quantitative estimation of zinc solubilizing potential of the isolates (Broth assay)

To assess the quantum of zinc solubilized by ZSB strains, 32 ZSB were selected and grown in 100 ml conical flasks containing 50 ml of MSM broth medium containing 0.1% Zinc Oxide, Zinc Carbonate and Zinc Phosphate as insoluble zinc source. The broth was inoculated with 100 µl of overnight grown bacterial culture and incubated at 28ºC in an orbital shaker at 120 rpm for 16 days. Appropriate uninoculated controls were also maintained. The cultured broth was filtered through 0.2 µm filter paper then filtrated was fed to atomic absorption spectrometer (AAS). The available zinc concentration was recorded at 4th, 8th and 16th day of incubation (Gandhi *et al*., 2014) [11] and solubilised zinc content was expressed as mg/l.

Determination of physiological attributes

pH tolerance

The ability of the ZSB isolates to grow in acidic or alkaline media was assayed by inoculating them in MSM broth (pH 6.8±0.2) with pH adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 using HCl or NaOH (Jordan, 1982; Graham, 1992) [15, 19]. The cultures were spot inoculated on these media with different pH values, the flasks containing the broth were placed in a BOD incubator at 28ºC. Observations were recorded after 3 days of incubation.

Salinity tolerance

The ability of the isolates to grow at different concentrations of salt (NaCl) were tested by streaking isolates on MSM media plates containing 1.0, 2.0, 3.0, 4.0, 5.0 up to 10.0 percent (w/v) NaCl as described by Hashem *et al*. (1998) [16]. The plates were incubated at 28°C in BOD incubator for 7

days. Growth of the ZSB isolates was observed after one week.

Temperature tolerance

ZSB growth was determined at different temperatures on MSM media plates. The streaks from the isolates were incubated at different temperatures, *viz.,* 20, 25, 30, 35, 40 and 45ºC. Observations on growth were recorded after 48 hr. of incubation at these temperatures. (Graham, 1992) [15].

Screening for drought tolerance

Growth of all ZSB strains were evaluated on Luria & Burtani broth supplemented with different concentration of polyethylene glycol (PEG 6000) (Abolhasani *et al*., 2010) [1].

Antibiotic resistance

Antibiotic resistance test was done on LB agar by using antibiotic disc like Penicillin, Kanamycin, Ampicillin, Rifampicin and Cefixime (Li and Ramakrishna, 2011) [21].

DNA isolation of potent ZSB strains

DNA was isolated from ZSB culture (32 strains) following the protocol of Sagervanshi *et al*. (2012) [32]. 16S rRNA gene for each bacterial isolate was amplified using DNA template (50 ng) 2µl, Taq buffer (1X) 2 µl, dNTPs (100 µM) 2µl, Primer (0.25 µM) each 2 µl, Sterile distilled water - 11.4 µl Taq polymerase (1 Unit) 0.6 µl. The polymerase chain reaction was run by using two primers (one forward 5'AGAGTTTGATCCTGGCTCAG 3') and the other reverse 5'ACGGCTACCTTGTTACGACTT 3') for amplifying 16S rDNA gene. The thermal cycling program for amplification consisted of 1 cycle of 94°C for 4 min (Denaturation), 30 cycles of 94°C for 1 min, 52°C for 1 min (Annealing) 72°C for 2 min and a Final elongation cycle of 72°C for 8 min. The DNA fingerprinting profiles were then used to score the data, presence of amplified product was scored as 1 and its absence as 0 for all ZSB strains. The data matrices obtained were then entered into NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System Program) as developed by Rolf (1997). The data were analyzed using SIMUQUAL Jaccard's similarity coefficients. The similarity coefficients were then used to construct a dendrogram by employing the un-weighted pair group method with arithmetic average (UPGMA) method and a common dendrogram was finally constructed. Based on the dendogram, the genetic diversity amongst ZSB strains was assessed.

Result and Discussion

In the present study Rovira & Bunt media and Mineral Salt Media (MSM) was used for the growth of ZSB strains. Both media were supplemented with different Zinc sources *viz*., ZnO, $ZnPO_4$ and $ZnCO_3$. A prominent growth was observed in MSM media as compare to R&B media. Solubilization efficiency was calculated for both medium are as below (Figure No. 1)**.** A total 79 ZSB strains were isolated from 8 soil samples. All strains were selected on the basis of their zone forming ability in zinc supplemented media plates. For further experiments 32 ZSB strains were selected those found to be effective in solubilization of multiple forms of insoluble zinc *viz.,* ZnO, $ZnPO_4$ and $ZnCO_3$ in plate assay. Out of 32, two strains ZSB14 and ZSB24 produced high zinc solubilization. ZSB14 exhibited the highest solubilization index with zinc oxide with the diameter (71.4 mm), zinc carbonate with the diameter (47.2 mm) and zinc phosphate with the diameter (73.7 mm) followed by the strain ZSB24 with 55.0 mm in ZnO, 47.4 mm in $ZnCO_3$ and 40.9 mm in $ZnPO_4$ as compared with other strains In broth assay, ZSB24 showed high solubilization range in zinc oxide 83.2 mg Zn/l and followed ZSB14 with a value of 68 mg Zn/l (Figure NO. 1, Table No. 2). There was a decline in pH of the medium were observed in most of the ZSB strains in inoculated broth over uninoculated control. Results of physiological stress related to ZSB growth are represented in graphical format (Figure No. 2, Table No. 4).

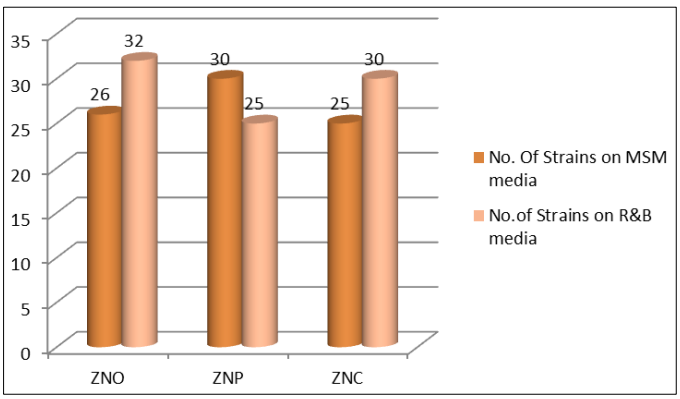
Table 1: Solubilization index of zinc oxide, zinc carbonate and zinc phosphate by ZSB isolates on Rovira & Bunt media.

S. N.	Strain	SI ON R & B ZNO	Strain	SI ON R & B ZNC	Strain	SI ON R & B ZNP
1	ZSB-1	5.43±0.05	ZSB-1	3.78±0.02	ZSB-1	4.1±0.02
2	ZSB-2	5.57±0.03	ZSB-2	3.14±0.16	ZSB-2	3.26±0.03
3	ZSB-3	2.47±0.16	ZSB-3	2.78±0.12	ZSB-3	3.28±0.01
4	ZSB-4	5.54±0.04	ZSB-4	5.39±0.15	ZSB-4	5.11±0.03
5	ZSB-5	4.49±0.02	ZSB-5	7.02±0.06	ZSB-5	5.13±0.15
6	ZSB-6	4.58±0.01	ZSB-7	2.88±0.02	ZSB-6	4.84±0.03
7	ZSB-7	4.78±0.09	ZSB-8	2.38±0.02	ZSB-7	2.85±0.03
8	ZSB-8	2.37±0.02	ZSB-9	3.18±0.08	ZSB-8	4.14±0.06
9	ZSB-9	2.78±0.02	ZSB-10	3.09±0.04	ZSB-9	3.08±0.08
10	ZSB-10	4.92±0.05	ZSB-11	2.62±0.02	ZSB-10	2.7±0.02
11	ZSB-11	2.88±0.02	ZSB-14	4.72±0.02	ZSB-11	3.13±0.15
12	ZSB-12	7.12±0.05	ZSB-15	5.14±0.06	ZSB-13	2.31±0.02
13	ZSB-13	4.92±0.03	ZSB-16	5.81±0.01	ZSB-14	7.37±0.03
14	ZSB-14	3.64±0.02	ZSB-17	3.09±0.08	ZSB-15	4.16±0.03
15	ZSB-15	3.79±0.02	ZSB-21	4.12±0.06	ZSB-16	6.28±0.02
16	ZSB-16	2.63±0.03	ZSB-22	3.58±0.01	ZSB-17	6.56±0.01
17	ZSB-17	2.85±0.04	ZSB-24	4.74±0.01	ZSB-18	2.67±0.01
18	ZSB-18	2.19±0.04	ZSB-25	4.71±0.01	ZSB-19	3.41±0.02
19	ZSB-19	4.68±0.02	ZSB-26	4.91±0.03	ZSB-20	2.44±0.02
20	ZSB-20	4.59±0.09	ZSB-27	5.36±0.02	ZSB-21	3.52±0.01
21	ZSB-21	5.5±0.02	ZSB-28	4.87±0.02	ZSB-22	3.86±0.01
22	ZSB-22	4.89±0.27	ZSB-29	4.85±0.02	ZSB-24	4.09±0.08
23	ZSB-23	4.89±0.03	ZSB-30	5.56±0.05	ZSB-25	3.18±0.03
24	ZSB-24	4.71±0.02	ZSB-31	4.1±0.04	ZSB-26	3.32±0.02
25	ZSB-25	3.47±0.04	ZSB-32	3.67±0.03	ZSB-27	3.58±0.01

26	ZSB-26	3.83±0.02			ZSB-28	4.76±0.04
27	ZSB-27	5.43±0.05			ZSB-29	3.59±0.01
28	ZSB-28	5.57±0.03			ZSB-30	4.07±0.06
29	ZSB-29	2.47±0.16			ZSB-31	4.21±0.01
30	ZSB-30	5.54±0.04			ZSB-32	4.07±0.06
31	ZSB-31	4.49±0.02				
32	ZSB-32	4.58±0.01				
	Se	0.2487		0.2277		0.0802
	CD5%	0.7028		0.6433		0.2266
	CD1%	0.9339		0.8548		0.3011
	CV	8.63		7.65		3.28

Table 2: Zinc solubilizing potentiality of ZSB isolates in broth and pH fall.

S. N.	Treatment	Zink solubalization 4th day (mg/l)	Zink solubalization 8th day (mg/l)	Zink solubalization 16th Day (mg/l)	pH
1	Contol	2.03±0.05	5.87±0.05	8.75±0.04	7.0
2	ZSB-1	5.18±0.04	14.57±0.04	17.30±0.06	3.2
3	ZSB-2	4.78±0.03	12.58±0.05	15.25±0.08	4.5
4	ZSB-3	3.40±0.03	7.87±0.06	12.37±0.05	4.4
5	ZSB-4	4.63±0.04	6.50±0.03	12.21±0.04	4.5
6	ZSB-5	9.03±0.14	13.36±0.08	18.22±0.08	5.8
7	ZSB-6	3.92±0.03	8.74±0.07	14.35±0.10	4.6
8	ZSB-7	5.41±0.03	11.32±0.05	13.54±0.09	5.2
9	ZSB-8	4.67±0.04	9.87±0.05	13.51±0.07	4.2
10	ZSB-9	3.01±0.0	7.46±0.04	13.26±0.32	4.3
11	ZSB-10	4.86±0.05	10.40±0.05	11.61±0.38	5.2
12	ZSB-11	2.20±0.05	8.17±0.06	12.65±0.65	4.2
13	ZSB-12	3.87±0.02	4.22±0.05	13.41±0.24	4.1
14	ZSB-13	4.16±0.04	4.56±0.06	10.67±0.55	6.9
15	ZSB-14	9.83±0.02	9.67±0.06	18.55±0.39	6.8
16	ZSB-15	4.59±0.04	8.13±0.07	15.32±0.40	7.4
17	ZSB-16	9.38±0.03	15.69±0.04	18.48±0.38	6.2
18	ZSB-17	6.11±0.02	14.25±0.06	14.65±0.62	6.1
19	ZSB-18	2.95±0.03	6.45±0.04	11.11±0.59	7.3
20	ZSB-19	4.20±0.62	6.18±0.08	12.94±0.22	7.0
21	ZSB-20	2.19±0.06	8.17±0.06	10.22±0.34	7.2
22	ZSB-21	3.10±0.08	6.52±0.04	13.76±0.60	7.3
23	ZSB-22	5.05±0.09	7.73±0.10	12.04±0.10	7.3
24	ZSB-23	4.56±0.04	9.12±0.07	12.16±0.12	7.4
25	ZSB-24	4.17±0.04	15.73±0.04	17.06±0.60	6.9
26	ZSB-25	5.73±0.03	6.45±0.06	13.20±0.08	7.1
27	ZSB-26	8.32±0.02	10.49±0.04	13.57±0.07	7.1
28	ZSB-27	6.22±0.04	11.37±0.03	15.09±0.09	6.9
29	ZSB-28	2.840.04	5.50±0.03	12.44±0.07	6.9
30	ZSB-29	5.34±0.01	8.93±0.04	13.89±0.08	6.5
31	ZSB-30	3.89±0.04	8.02±0.09	13.67±0.08	6.4
32	ZSB-31	4.03±0.54	6.23±0.06	12.25±0.08	6.8
33	ZSB-32	4.42±0.02	6.02±0.09	12.24±0.08	7.4
	Se	0.08	0.03	0.60	
	CD5%	0.25	0.10	1.70	
	CD1%	0.33	0.13	2.26	
	CV	3.20	0.70	7.66	



Graph 1: Graphical representation of ZSB growth on different media supplemented with different Zinc source.

Metallic Zn solubilisation was reported by the *Thiobacillus thioxidans*, *Thiobacillus ferroxidans* and facultative thermophilic iron oxidizers (Hutchins *et al*., 1986) [18], *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* genera (Fasim *et al.*, 2002; Sachdev *et al*., 2010) [10, 31] which involved the production of gluconic acid and its derivatives in the culture broth (Saravanan *et al*., 2007) [33]. The above result of salt stress and temperature stress agreed with the findings of Kumar *et al*. (2014) [20], pH stresses (Chennappa *et al.* 2016) [7] and antibiotic resistance (Sheng *et al*. 2008) [35]. Reports are available on PGP bacteria tolerance to drought stress (20-40%) induced by PEG by Marulanda *et al.* (2009) [25].

Molecular characterization of ZSB

Amplified rDNA (Ribosomal DNA) Restriction Analysis (ARDRA) is the extension of the technique of RFLP (restriction fragment length polymorphism) to the gene encoding the small (16S) ribosomal subunit of bacteria. The technique involves an enzymatic amplification using primers directed at the conserved regions at the ends of the 16S gene, followed by digestion using restriction enzymes. The pattern obtained is said to be representative of the species analyzed and important for their molecular characterization. In the present study ARDRA produced a fingerprint based on length polymorphism for molecular characterization of ZSB isolates.

Isolation and purification of bacterial DNA

ZSB isolates were grown in LB broth for 14-16 hours at 28^0C in an incubator shaker at 200 rpm. Total genomic DNA was isolated from ZSB isolates and checked on agarose gel electrophoresis.

Quantification of DNA

Reliable measurements of DNA concentration are important for any application in molecular biology including complete digestion of DNA by restriction enzymes and amplification of DNA by PCR. The DNA quantification was carried out by nanospectrophometer measurements. The concentration of DNA obtained for all the 32 ZSB isolates in given (Table No.3). The maximum DNA concentration (312ng/µl) was recorded in ZSB3 whereas; the minimum DNA concentration (117ng/µl) was recorded in ZSB12.

Table 3: Quantification of DNA

S. N.	Isolates	Concentrations of DNA (ng/µl)	S. N.	Isolates	Concentrations of DNA (ng/µl)
1.	ZSB 1	267	17.	ZSB17	283
2.	ZSB2	192	18.	ZSB18	302
3.	ZSB3	312	19.	ZSB19	264
4.	ZSB4	307	20.	ZSB20	311
5.	ZSB5	259	21.	ZSB21	247
6.	ZSB6	261	22.	ZSB22	218
7.	ZSB7	306	23.	ZSB23	128
8.	ZSB8	271	24.	ZSB24	132
9.	ZSB9	254	25.	ZSB25	141
10.	ZSB10	237	26.	ZSB26	298
11.	ZSB11	282	27.	ZSB27	291
12.	ZSB12	117	28.	ZSB28	121
13.	ZSB13	198	29.	ZSB29	283
14.	ZSB14	213	30.	ZSB30	307
15.	ZSB15	144	31.	ZSB31	253
16.	ZSB16	256	32.	ZSB32	206

Table 4: Effect of Physiological attributes on Growth of ZSB strains.

Salt concentration	No. of ZSB strains	pH	No. of ZSB strains	Temperature	No. of ZSB strains	Drought	No. of ZSB strains	Antibiotic	No. of resistant ZSB strains	No. of sensitive ZSB strains
1%	14	pH 3	0	20˚C	7	10%	13	Kanamycin	2	30
2%	12	pH 4	0	25˚C	30	20%	9	Cefixime	6	26
3%	7	pH 5	11	30˚C	32	30%	6	Penicillin	21	11
4%	4	pH 6	32	35˚C	27	40%	2	Ampicillin	16	16
5%	0	pH 7	32	40˚C	25			Rifampicin	1	31
		pH 8	8	45˚C	22					
		pH 9	2							

16S rDNA amplification and ARDRA analysis

The genetic diversity amongst the thirty two ZSB isolates was assessed by the amplification of 16S rDNA and restriction analysis of the 16S amplicon. The 16S rDNA region was amplified by using PCR and all the ZSB isolates had amplified the specific size ~ 1.4 kb amplicon of 16S rDNA region. Three restriction endonucleases *viz.*, *Hinf*I, *Hae*III and *Taq*I were used for restriction fragment analysis of amplified 16S rDNA. The banding pattern of the representative ZSB isolates are shown in (Figure No.), with standard molecular weight marker. Total, 27 bands of varying sizes were observed in all the thirty two strains when digested with three restriction enzymes. Upon digestion of 16S rDNA amplicon with *Hinf* I, 9 different DNA fragments were produced whereas with *Taq*I digestion 7 different DNA fragments and with *Hae*III restriction enzyme 11 different DNA fragments were observed on agarose gel. All were polymorphic.

Genetic similarity estimates the variations based on ARDRA banding patterns, which were calculated using method of Jaccard's coefficient analysis. The similarity coefficient matrix generated was subjected to algorithm "Unweighted Pair Group Method for Arithmetic Average (UPGMA)" to

generate clusters using NTYSYS 2.02 pc program (Rohlf, 1997) [30]. The pair wise comparison of ARDRA patterns based on both shared and unique amplification products was made to generate a similarity matrix. Similarity indices established on the basis 27 bands of three restriction enzymes ranged from 0.2 to 1.

The dendrogram (Figure No. 3) is a close representation of the values obtained in the similarity matrix. From the data obtained in the dendrogram the three restriction enzymes discriminated all ZSB isolates. The relationship among the strains clearly divided into two major cluster at a similarity coefficient 0.5. Genetic relationships among thirty two ZSB strains were also visualized by performing PCoA based on ARDRA. The results of PCoA were comparable to the cluster analysis (Figure No.). ZSB strains were grouped within the same cluster in the dendrogram was also occupying the same position in two dimensional and three dimensional scaling. Majeed *et al*., (2015) [23] reported that nine wheat rhizo/endophytic bacteria yielded four IGS patterns. Among these four patterns, one comprising of *Bacillus* sp. was not sequenced but from other three types, one strain each was sequenced based on 16S rRNA gene. The PGPR isolate AJK-3 was identified as *Stenotrophomonas rhizophila* strain having 99% similarity with the reported gene sequence. AJK-7 showed 98% similarity with *Acetobactor pasteurianus.* However, isolate AJK-9 95% similarity with *Stenotrophomonas* spp. Yadav *et al*., (2015) [38] molecularly identified *Bacilli* isolates using ARDRA and amplification of 16s rRNA was done using universal primers and digested 16S rDNA with *Alu*I, *Hae*III and *Msp*I restriction enzymes.

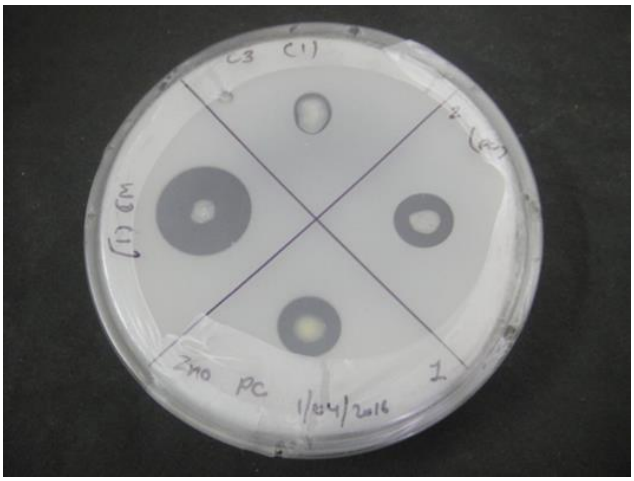
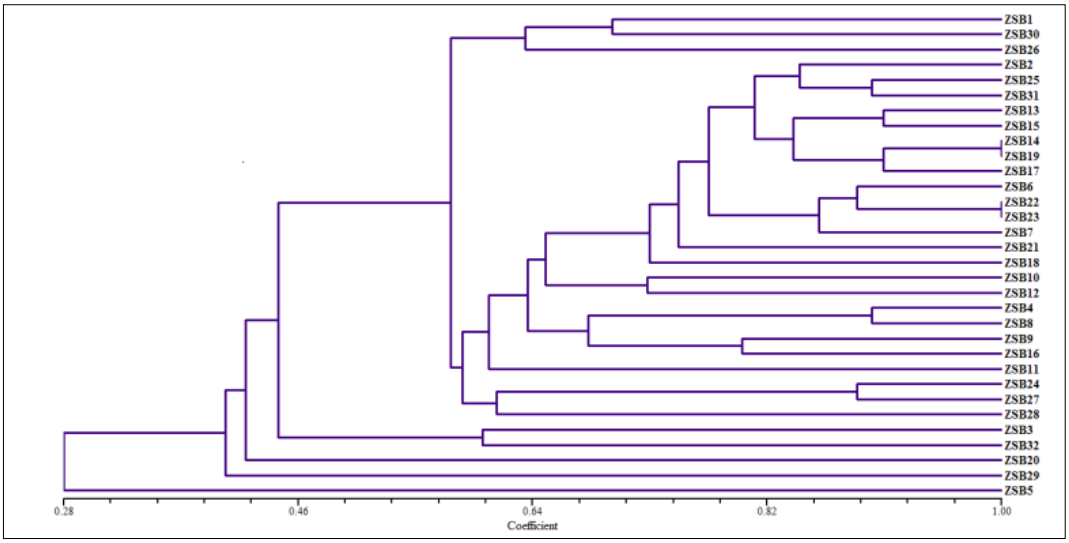
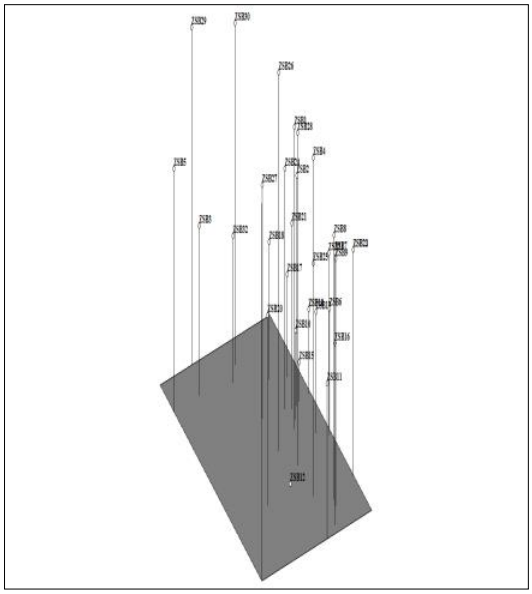


Fig 1: ZSB isoation





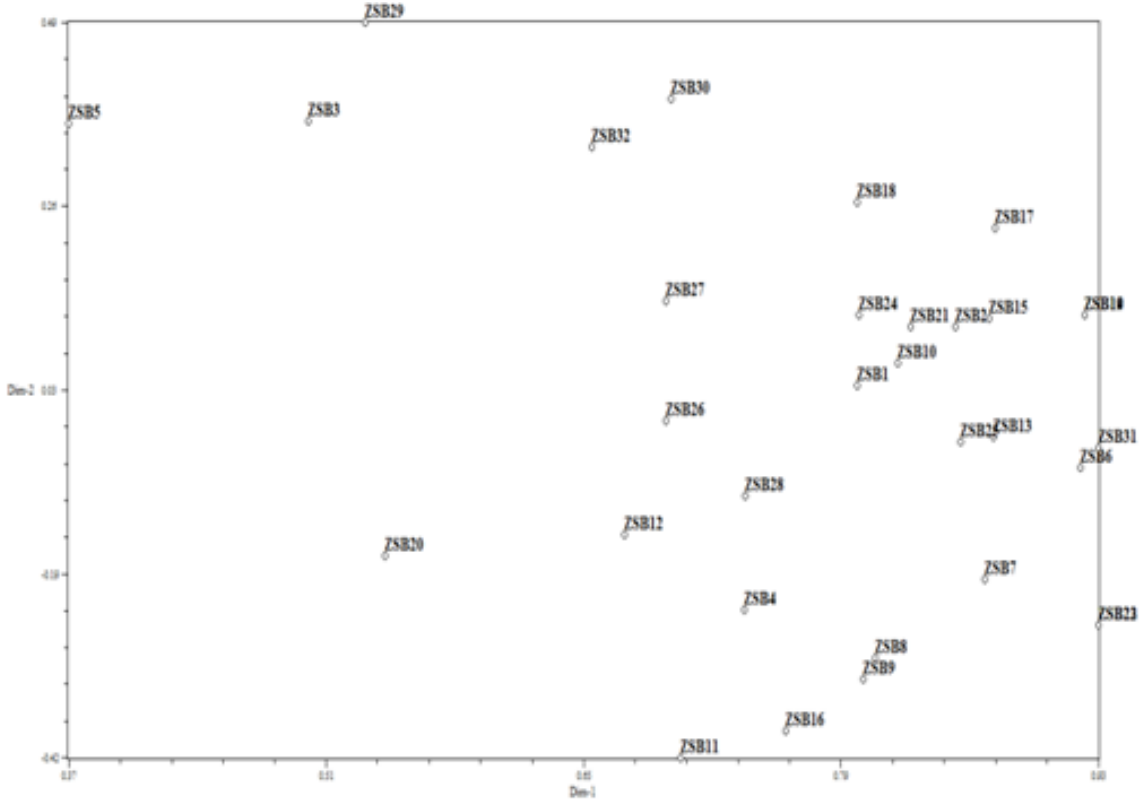


Fig 2: Dendogram based on the shared polymorphic amplification products resultant from thirty two ZSB isolates: (a) Two- dimensional PCA scaling of 32 ZSB isolates (b) Three- dimensional PCA scaling of 32 ZSB isolates

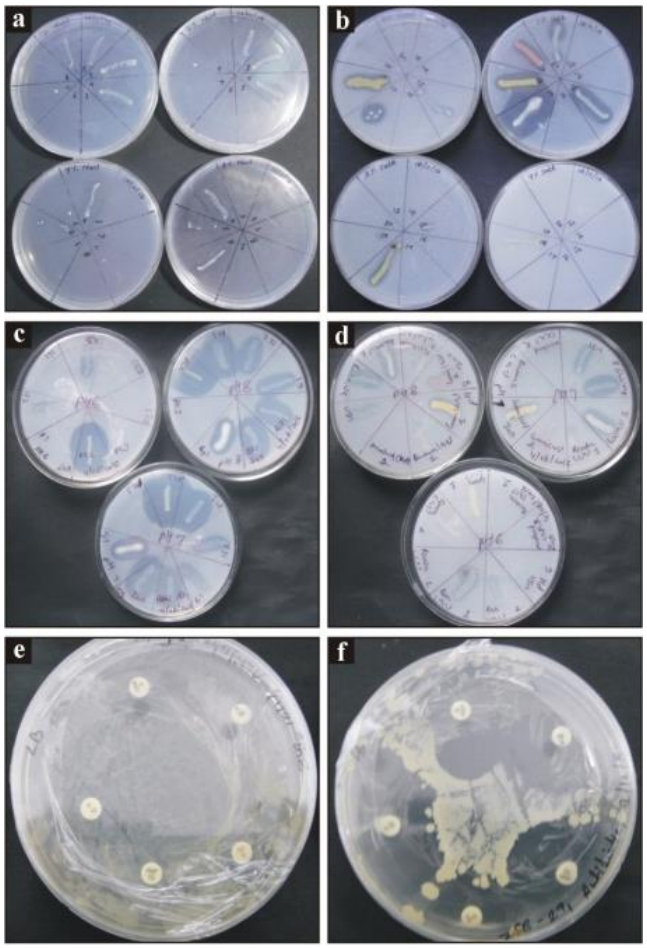


Fig 3: Physiological attributes of ZSB isolates from soil of Rajasthan (a) Growth on different salt concentration (b) Growth on different pH (C) Antibiotic resistance test

Summary

The application of zinc as chemical fertilizer in crop production increases cost of cultivation and may cause harm to the environment. The availability of Zn in soil is differ and may be unavailable to plants due to soil environment, but it can be made available to them by inoculating zinc solubilizing bacterial species as an inoculant. From the present study, based on growth efficiency and zinc solubilizing potentiality, among the 79 zinc solubilizing bacterial isolates ZSB-1 and ZCSB-17 were further selected for making availability zinc to crop plant from unavailable zinc sources such as zinc oxide, zinc phosphate and zinc carbonate. Even though the eight bacterial strains showed better results in laboratory level, further it should be tested by conducting field experiments on maize to confirm their performance and also for recommendation to farmers. Selection and inoculation of zinc solubilizing bacteria either alone in soil inherently rich in native zinc or along with cheaper insoluble zinc compounds, like zinc oxide and zinc carbonate, will lead to lot of saving in crop cultivation. In this present situation, isolation and identification of effective PGPR isolates can be used as bio-inoculants that may be cost effective and eco-friendly in nature compared to chemical fertilizer.

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