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Organic Acids and Exo-Polysaccharide Mediated Zinc Solubilization By Soil Bacteria and Their Plant Growth Promoting Properties.

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ABSTRACT

In soil biota, bacteria possessing the property of solubilizing the insoluble Zn compounds are occasional. Four ZSB (Zn solubilizing bacteria) from pesticide treated, non rhizosphere soil were isolated and evaluated for Zn solubilization potential through plate and broth assay amended with different insoluble Zn sources like ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$, Zn metal powder and ZnS separately. On 16S rRNA gene analysis, the ZSB strains were found closely to *Acinetobacter* sp., *Yokenella* sp., *Pseudomonas putida* and *P. plecoglossicida*. In plate assay, beside other Zn compounds amendment, Zn metal powder and ZnS solubilization was not noticed in 0.1 and 0.2% concentration of Zn, which was later solubilized in the liquid medium. Zn solubilization activity among the ZSB strains varied corresponding to the type of Zn sources used in the broth. Gluconic, formic, lactic and citric acid secreted by ZSB strains owing Zn solubilization was therefore confirmed using High performance liquid chromatography and further quantified. *Acinetobacter* sp. and *Yokenella* sp. showed excellent Zn solubilization in plate and broth assay respectively. This is a pioneer study reported on *Yokenella* sp. for its role in Zn solubilization that is facilitated through exo-polysaccharide, in addition to organic acids secretion.

Keywords: Zinc solubilization, Organic acid, Exo-Polysaccharide, *Pseudomonas*, *Yokenella*, HPLC.

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INTRODUCTION

Zinc (Zn), an essential micronutrient needed for achieving normal growth and reproduction in plants, animals and humans. In particular, Zn plays vital functional role in plants such as maintaining the membrane integrity, pollen formation, resistant to certain pathogens, carbohydrate metabolism during photosynthesis and sugar to starch conversion, auxin and protein metabolism (Alloway 2004). Zn nutrition to plants are limited in soils due to their transformation influenced by different soil type or soil chemical reactions that converts cationic Zn^{2+} to various insoluble forms and thereby inflicts deficiency on plants. Zn deficiency exists world-wide attributing the poor yield and nutrition, especially in cereals crops. Developing countries like India, China, Pakistan and Turkey show Zn deficiency in humans where cereals are consumed as major food source (Alloway 2004). The problem is further exacerbated by zinc sulphate fertilizers application to soils that results in further transformation to insoluble, unavailable forms within seven days of application (Rattan and Shukla 1991). In soil environment, dissociation of insoluble cation like Zn leads to concurrent release of anion like phosphate, thus insoluble Zn compounds solubilization may influence the biogeochemical cycling of that element (Gadd 1993; Morley et al. 1996).

One of the approaches to soil Zn deficiency crisis, is to enhance Zn availability by intervention of bacteria that could solubilize insoluble Zn compounds. This soil microbial Zn solubilization process could subsidise a constant supply of available Zn pool in the soil and augments Zn uptake by the plants system. The term Zn solubilizing bacteria (ZSB) refers to any bacteria that could solubilize the insoluble Zn compounds or mineral forms of Zn and thus could increase the Zn supply to the plant system (Saravanan et al. 2003). Applying ZSB in the fields might reduce or avoid the application of chemical fertilizers. Moreover, an inclined pressure upon plant breeding was brought to produce plant system that is able to tolerate low Zn content in the soil (Neue 1998). The bacterial community colonizing the rhizosphere niche are significant in plant growth promotion (PGP) since they convert various organic and inorganic compounds utilizable for plants (Badalucco and Kuikman 2001). The solubilization of different insoluble compounds by bacteria are due to excretion of various metabolites including organic acids that reduces the pH, thus playing a major role in increasing the availability of certain nutrients in soil (Rodriguez and Fraga 1999; Nautiyal et al. 2000). Beside the release of protons from organic acids, their anion counterpart forms complex with metal cation (Burgstaller and Schinner 1993; Hughes and Poole 1991). Insoluble compounds can also be solubilized by protons that are pumped externally to the medium by membrane associated pumps, creating ionic gradient to acquire nutrients for their growth and survival (Gadd 1993; Hughes and Poole 1991; Jones and Gadd 1990).

Zn solubilization was previously reported in bacteria like *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Gluconacetobacter diazotrophicus* aiding solubilization by producing gluconic, 2 and 5-keto gluconic acid respectively (Di Simine et al. 1998; Fasim et al. 2002; Saravanan et al. 2007a). Mostly, *Acinetobacter* spp. were reported for their role of Zn solubilization only using agar plate assay amended with ZnO at 0.1% (0.12 g L^{-1}) concentration (Indiragandhi et al. 2008; Sachdev et al. 2010; Zamin et al. 2011), therefore quantification of solubilized Zn and acids produced by *Acinetobacter* sp. are need to be focussed. From recent times, Xinxian et al. (2011) reported endophytic bacteria possessing $ZnCO_3$ and $Zn_3(PO_4)_2$ solubilization potential in the broth assay associated with *Sedum alfredii*. Likewise, another study suggests *Bacillus* sp. as promising Zn solubilizers that assimilates significant amount of Zn in Soybean seeds (Sharma et al. 2012). However, it is necessary that yet numbers of bacterial strains are needed to be studied for their role of potential Zn solubilization and to assess their solubilization potential on different insoluble Zn compounds.

Therefore, considering the above conditions, the present study was focused and preceded. Consequently from this, to our knowledge, we first report *Yokenella* sp. possessing Zn solubilizing potential and that employs exo-polysaccharide (EPS) during Zn solubilization; secondly, Zn solubilized by *Acinetobacter* sp. was quantified in the liquid mineral salt medium (MSM) , amended with different Zn compounds and subsequently organic acid secretion aided in Zn solubilization was detected; Finally, the *Pseudomonas putida* and *P. Plecoglossicida* were revealed for their Zn solubilization potential. Later, their roles in direct and indirect PGP properties assessed were measured qualitatively and quantitatively.

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MATERIALS AND METHODS

Soil samples and physico-chemical properties

Rhizosphere and non rhizosphere soil samples were collected from the different crop growing fields of *Solanum lycopersicum* and *Beta vulgaris* of Vellore, Tamil Nadu, India were collected. Non rhizosphere soil samples includes pesticides treatments such as quinalphos, monocrotophos, lambda cyhalothrin and chlorpyriphos applied at recommended dose on regular basis (45 days interval).

The physicochemical properties of the soils were analysed by standard soil analysis methods by National Agro Foundation, Chennai, India. The soil samples were collected and dried in oven for 70 °C to weigh constant. The organic matter content was estimated by the rapid titration method as described by Walkley and Black (1934), cation exchange capacity (CEC) was measured as per Jackson [31]. The total soil nitrate content was estimated using the alkaline permanganate method (Subbiah and Asija 1956). The pH and the electrical conductivity of the soil were determined by preparing it into soil slurry (soil: water ration, 1:2) Richards (1954).

Isolation of ZSB from soils samples

From each soil sample, 10 g was suspended in 95 mL of sterile distilled water and subjected to shaking for 30 min. Further, 1mL of soil suspension from each sample was serially diluted. Appropriate dilutions were selected and 100 µL suspensions were spread on freshly prepared modified MSM agar (g L^{-1}: D-glucose 10, Tryptone 10 and agar 15) supplemented with ZnO and $ZnCO_3$ at 0.12 % and 0.21 % respectively. The plates were incubated for 48 h at 28 ± 2 ºC, plates showing bacterial colonies with clear halo against an opaque background are considered as ZSB and were streaked further on Zn containing plates to obtain them in pure culture and maintained in glycerol stock at -80 ˚C.

16S rRNA gene analysis of the ZSB strains

The strains were sequenced in DNA sequencing service of Chromous Biotech Pvt. Ltd, Tamil Nadu, India; using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The sequence obtained was subjected to BLAST search and the phylogenetically related neighbour was identified. Using neighbour joining analysis, a phylogenetic tree was constructed using 16S rRNA gene sequence of the ZSB strains with their nearest phylogenetic neighbours. Further, the sequences were submitted in the public GenBank (NCBI) procuring the accession numbers for each strain. The nucleotide accession numbers include JF825890 (*P. plecoglossicida*), JF825891 (*P. putida*), JF825892 (*Yokenella* sp.) and JF825893 (*Acinetobacter* sp.) respectively.

In vitro Zn Solubilization assay

Zn Solubilization in plate assay

Pure cultures of ZSB were retrieved from glycerol stock and they were allowed to grow for 48 h in Zn unamended MSM broth. The plates used for testing Zn solubilization was prepared using Zn compounds that include ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$, Zn metal powder and ZnS were amended separately at 0.1 and 0.2% of final concentration of Zn in the MSM agar medium. From the mass multiplied cultures of ZSB, for each strain, 10 µL (1 x 10^6 CFU mL^{-1}) of aliquot was placed on to the centre of insoluble Zn compounds amended MSM medium. The plates were incubated at 28 ± 2 ºC for subsequent days while the zone around the colonies was recorded till the solubilization halo reaches the maximum diameter. The values are expressed as (solubilization index) in millimetre after subtracting the colony diameter from that of the solubilization halo.

Zn Solubilization in broth assay

Quantification of Zn solubilization in liquid medium were conducted in 250 mL Erlenmeyer flask containing 100 mL MSM broth amended with different Zn sources separately at 0.1% concentration. Test strains were inoculated at 1 % inoculum level to all the flasks and uninoculated flasks were maintained as control. The flasks were incubated in shaking conditions at 100 rpm for 48 h at 28 ± 2 ºC. After 12, 24 and 48 h of incubation, the culture was filtered through 0.22 µm membrane filters, centrifuged at 12,000 rpm for 10

min. and the supernatant was stored at -20 ºC for further analysis. The availability of free Zn^{2+} in the supernatant was quantified directly by injecting the culture filtrate into Varian Atomic absorption spectroscopy (AAS). $ZnCl_2$ was used to prepare standard curve for Zn estimation. The reductions in the pH of broth during the incubation intervals were also noted.

Maximum tolerance level of Zn by ZSB strains

The maximum tolerance level of the ZSB strains were evaluated in minimal salt media (g L^{-1}: glucose 10, $(NH_4)_2SO_4$ 2.0, $Fe_2SO_4.7H_2O$ 0.001, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.01, $Na_2HPO_4.12H_2O$ 1.5, KH_2PO_4 1.5, pH 7) amended with varying Zn concentrations. For testing the tolerance level, ZSB cultures of 1% were inoculated in series of flasks containing increasing concentrations of Zn prepared using $ZnCl_2$. The flasks were maintained in shaker and incubated at 28 ± 2 ºC for 4 days. After incubation, 100 µL aliquot from each concentration containing flask was plated on minimal salts media for detecting the viability of ZSB cultures. Growth of ZSB in maximum concentration containing flask was denoted as maximum Zn tolerance level of that strain.

HPLC analysis of organic acids in the culture inoculated broth

Using HPLC, the production of organic acids by different strains in the MSM broth was quantified. The broth was centrifuged after 48 h at 10,000 rpm for 10 min. The supernatant was filtered through 0.22 µm millipore filter and the filtrate was injected to Waters 1525 binary High Performance Liquid Chromatography (HPLC) pump equipped with C18 column (150 mm X 4.5 µm) with waters 2487 dual λ absorbance detector. The chromatograms were developed using a mobile phase consisting of 50 mM KH_2PO_4 moving at a constant flow rate of 0.7 mL $min.^{-1}$ in isocratic mode. Retention time of each signal was recorded at a wavelength of 210 nm. Thus, the organic acids were identified and quantitatively determined by comparing the retention time and peak area of chromatograms with those of standards.

Assessment of EPS production

After 48 h of incubation, the culture broth was centrifuged (5433g for 30 min.) for the quantification of EPS. After centrifugation, the supernatant was mixed with chilled acetone at 1:3 ratios content to precipitate the EPS in the medium. Later, the EPS precipitated was washed thrice using water and acetone alternatively. The precipitate was dried on the filter paper and weighed (Mody et al. 1989).

Assessment of PGP activities

Production of indole-3-acetic acid (IAA)

The quantification of IAA was performed following the method of Brick et al. (1991). Briefly, nutrient broth was amended with100 µg tryptophan mL^{-1} and inoculated with ZSB strains and incubated at 28 ± 2 °C under dark conditions for 24 h in an incubator shaker at 120 rpm. Later, to their culture supernatant, 2 mL of Salkowsky reagent was added and incubated at 28 °C in darkness for 1 h. Thus the color intensity was measured at 530 nm in UV-Spectrophotometer. Standard curve was constructed using pure IAA for quantification.

Siderophore production

Siderophore secretion by each strain was detected employing the universal method of Schwyn and Neilands (1987) using blue coloured agar plates containing the dye Chrome azurol S. Briefly, 10 µL culture was spot inoculated on to the centre of the plate and colony with orange halos against bluish background were indicative of siderophore secretion.

NH_3 production

Bacterial strains were tested for the production of ammonia using peptone water. Freshly grown cultures were inoculated in 10 mL peptone water and incubated for 48-72 h at 28 ± 2 ºC. Ammonia production was confirmed by addition of 0.5 mL of Nessler's reagent that lead to appearance of brown to yellow colour indicating positive result (Cappuccino and Sherman 1992).

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HCN production

Strains were screened for the production of hydrogen cyanide using the method of Lorck (1948). Briefly, nutrient agar was amended with 4.4 g glycine L^{-1} and the bacteria were streaked on to the modified agar plate. A Whatman filter paper No. 1 soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed to the top of the plate. The plates were sealed with parafilm and incubated at 28 ± 2 ^{o}C for 4 days. Development of orange to red colour indicates HCN production.

Aminocyclopropane-1-carboxylate (ACC) deaminase activity

The bacterial supernatant was quantified for ACC deaminase activity by estimating the amount of α-ketobutyrate produced through the enzymatic hydrolysis of ACC (Belimov et al. 2005). According to the method of Honma and Shimomura (1978) the culture sample absorbance at 540 nm was compared against the α-ketobutyrate standard curve ranging from 0.1 to 1.0 mmol according to the method of.

Phosphorus solubilization

The test strains were quantified for their P solubilization in NBRIP broth (g L^{-1}: Glucose 10; $Ca_3(PO_4)_2$ 5; $MgSO_4.7H_2O$ 0.25; $MgCl_2.6H_2O$ 0.5; KCl 0.2; $(NH_4)_2SO_4$ 0.1; pH 7.0). The available P in the broth was estimated using vanado-molybdate method as described by Gulati et al. (2008) using the calibration standard curve constructed using KH_2PO_4. The concurrent change in pH following tri-calcium phosphate (TCP) solubilization was also recorded.

Statistical analysis

All the experiments were conducted in triplicates. The statistical analysis was carried out using Graphpad Prism, Version 5.03, the significant difference were calculated at $P \leq 0.05$ using two way ANOVA method.

RESULTS

Screening of ZSB from rhizosphere and non rhizosphere soils

ZSB were able to isolate only from the pesticide treated-non rhizosphere soils collected from *Solanum lycopersicum* and *Beta vulgaris* field (Fig. 1). The ZSBs were absent in the rhizosphere soils of *Sorghum bicolor*, *Capsicum annum* and *Solanum melongena*. The maximum number of ZSB was recorded from the soils collected from *Solanum lycopersicum* field. Based on the potential solubilization and different colony morphology, six bacterial isolates were selected and subculture several times to obtain them in pure cultures and maintained at -20 ºC.

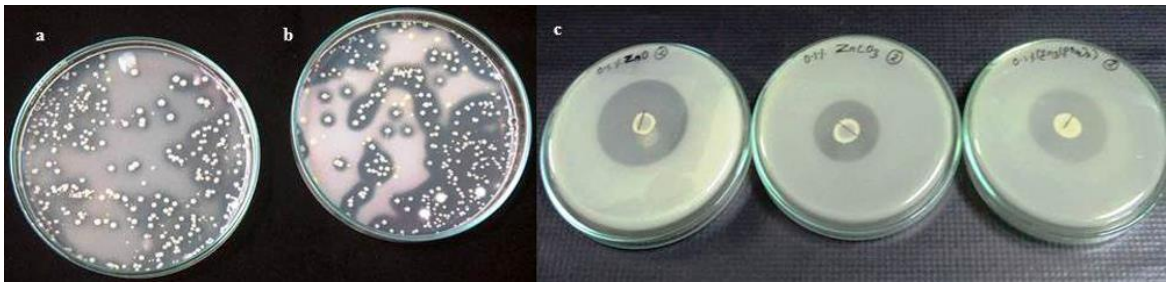

Fig 1: Zn solubilization by ZSB strains on MSM agar plates. Fig. (a) and (b) shows $ZnCO_3$ and ZnOsolubilization on plates, during the isolation of ZSB from pesticide treated non rhizosphere soil at 10^{-2} dilution factor. Fig. (c) depicts zone of solubilization by *Acinetobacter* sp. on ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ amended plates at 0.1% Zn concentration respectively

ISSN: 0975-8585

16S rRNA gene analysis of the ZSB strains

Based on the assessment for Zn solubilization on plate assay, four strains were phylogenetically categorized. The 16S rRNA gene analysis showed the ZSB strains to be *Acinetobacter* sp., *Yokenella* sp., *Pseudomonas putida* and *P. plecoglossicida*. The 16S rRNA sequences of those strains were subjected to BLAST search and were deposited in NCBI in the following accession numbers JF825893 (*Acinetobacter* sp.), JF825892 (*Yokenella* sp.), JF825891 (*P. putida*) and JF825890 (*P. plecoglossicida*) respectively (Fig. 2).

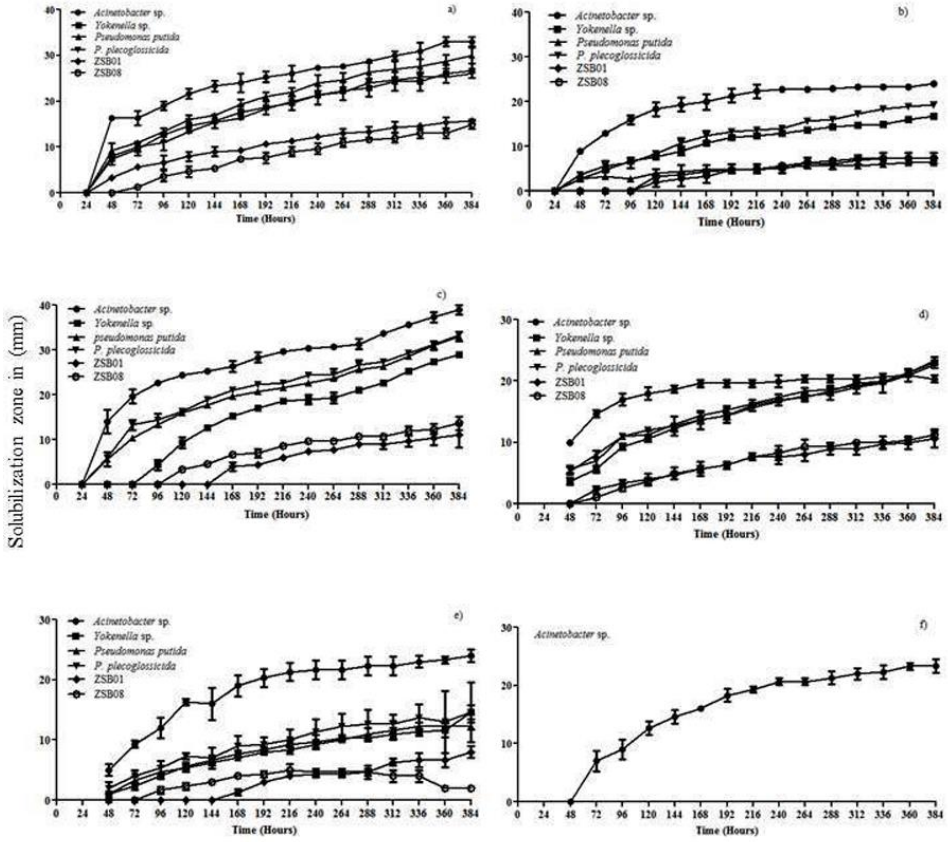

Fig 2: Solubilization of different Zn Compounds by ZSB strains on agar plate assay. ZSBs showing progressive increase in zone size on Tryptone agar plate amended with different Zn source at 0.1% and 0.2% concentration separately and incubated for 384 h. a) ZSBs on 0.1% ZnO b) ZSBs on 0.1% $ZnCO_3$ c) ZSBs on 0.1% $Zn_3(PO_4)_2$ d) ZSBs on 0.2% ZnO e) ZSBs on 0.2% $ZnCO_3$ f) Solubilization of 0.2% concentration of $Zn_3(PO_4)_2$ by *Acinetobacter* sp.

***In vitro* assay of Zn Solubilization by ZSB**

Zn Solubilization assay on agar plates

On plate assay, the selected six ZSBs were evaluated for Zn solubilization by adding ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$, Zn metal powder and ZnS separately at 0.1% and 0.2% concentrations. In 0.1% Zn concentration, the ZSB strains produced significant solubilization halos on ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ added plates. In 0.2% dosage of Zn, all strains produced halo on ZnO and $ZnCO_3$ added plate but not on $Zn_3(PO_4)_2$ amended plate whereas *Acinetobacter* sp. is the only strain that produced halo on $Zn_3(PO_4)_2$. None of the strains produced zone on Zn metal and ZnS amended plates on either dosages. The maximum diameter of solubilization on overall Zn compounds was observed in *Acinetobacter* sp. and the least was noted in ZSB01 and ZSB08 in 0.1% and 0.2% Zn concentrations. The solubilization of different Zn compounds and the progressive increase in haloes on plate assay is illustrated in fig. 3.

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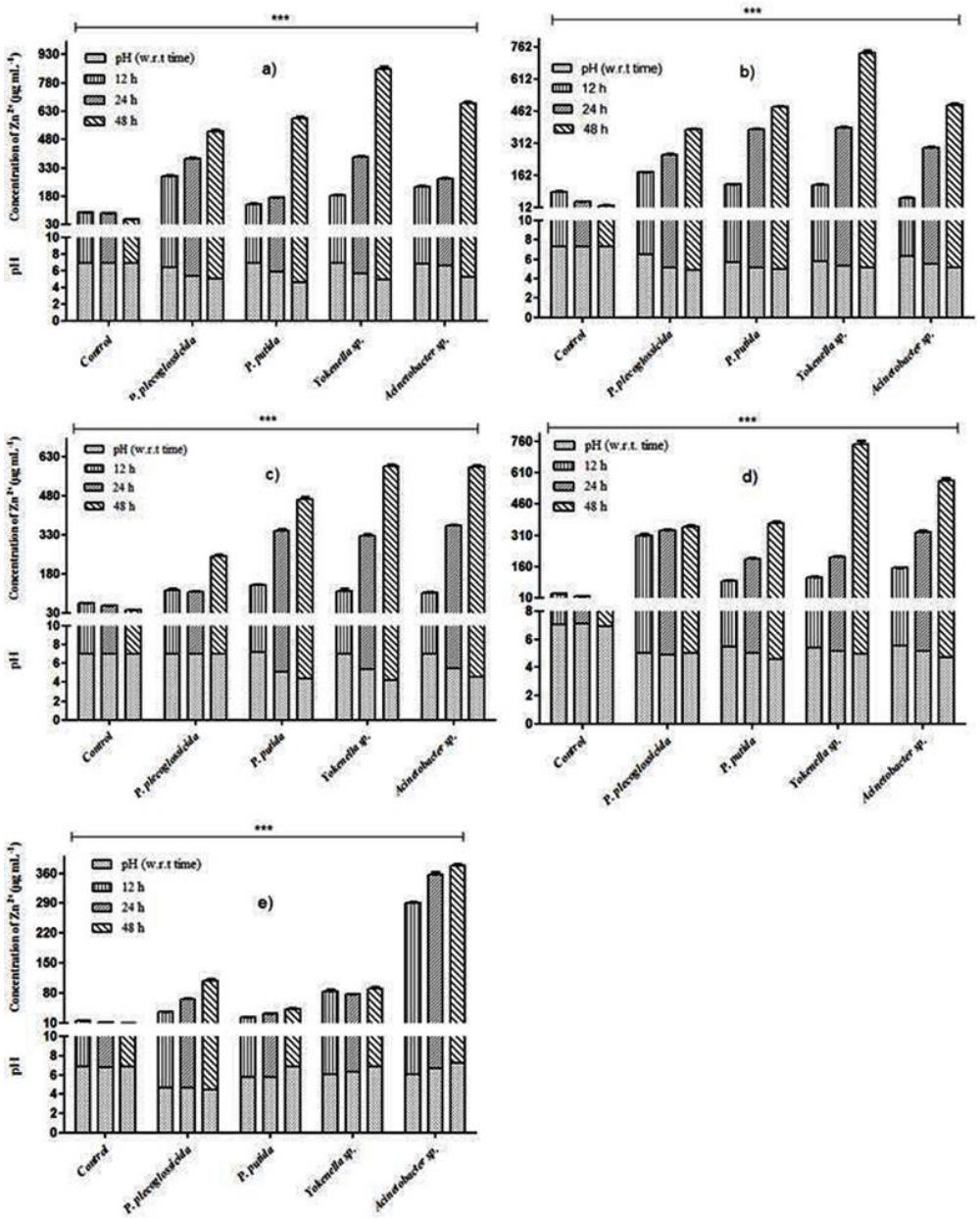

Fig 3: Solubilization and quantification of different Zn compounds in MSM broth inoculated with Zn solubilizing strains. Each value represents the mean ± SD of three replicates per treatment. Significant differences at P ≤ 0.05 levels are indicated by asterisk according to two-way ANOVA by Bonferroni's test comparing each concentration over control. Strains showing Zn solubilization in a) ZnO b) $ZnCO_3$ c) $Zn_3(PO_4)_2$ d) Zn metal powder and e) ZnS amended MSM liquid medium and corresponding pH of the medium with respect to (w.r.t) time are mentioned below the concentration of available Zn

Quantification of Zn solubilization in liquid medium was performed using 0.1% concentration of Zn compounds that were used in the plate assay. All strains exhibited high solubilization, and Zn availability was higher in ZnO amended broth. The maximum availability of Zn^{2+} was noted after 48 h in the broth inoculated with *Yokenella* sp. exhibiting the concentration of 849 µg Zn^{2+} mL^{-1} and the least was recorded in *P. plecoglossicida* inoculated broth showing a concentration of 519 µg Zn^{2+} mL^{-1}. The least availability of Zn was noticed in all ZnS amended flasks except *Acinetobacter* sp. that solubilized fair amount of Zn. However, with Zn metal amendment, significant Zn availability was observed in in *Acinetobacter* sp., *Yokenella* sp., *P. putida* and *P. plecoglossicida* inoculated broth that interestingly did not show solubilization zone on Zn metal power or ZnS amended agar plates. The role of Zn solubilization by *Yokenella* sp. was observed in all Zn sources and was noticed to be in an increased manner to than that of the *Acinetobacter* or *Pseudomonas* spp (Fig. 4). The

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solubilization and increase in the available Zn was associated with the acidification of the medium, may be through the excretion of organic acids by the ZSB strains in the broth.

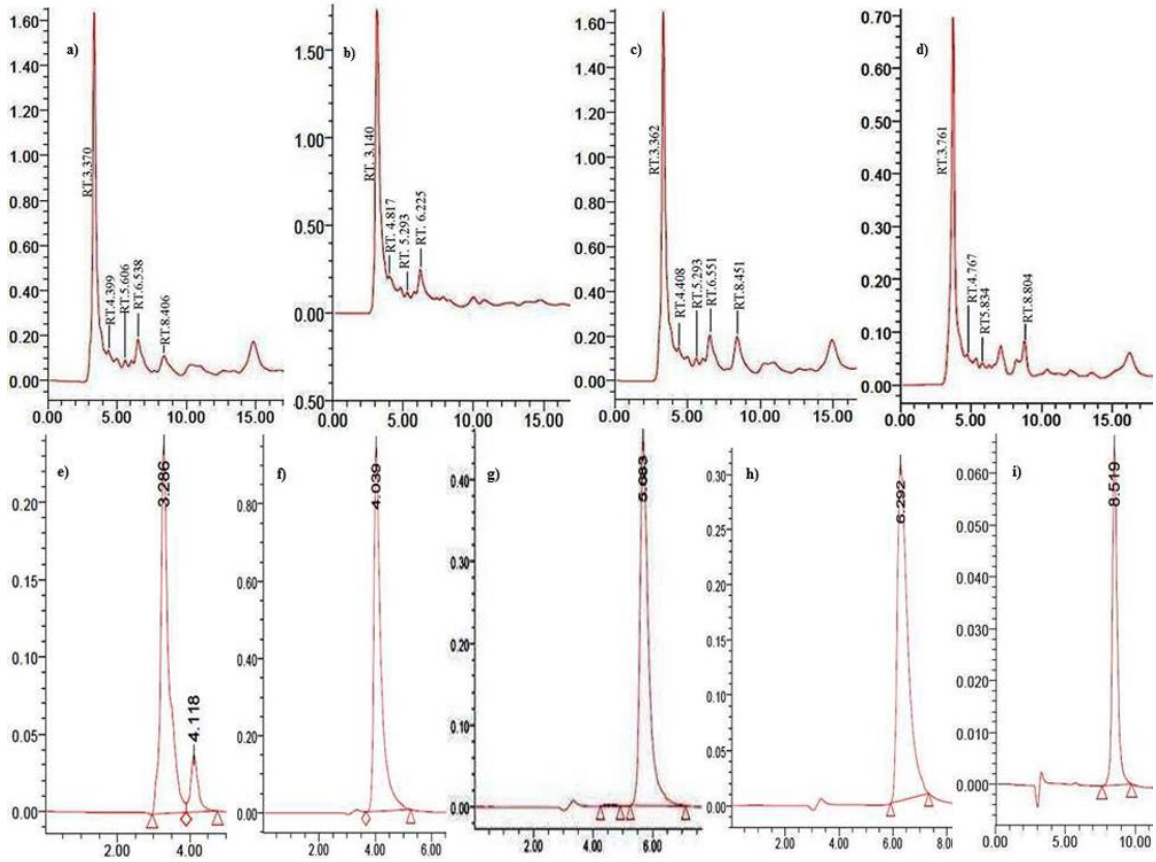

Fig 4: HPLC chromatogram of culture supernatant.HPLC chromatogram peaks obtained after 48 h of incubation from MSM broth inoculated with ZSB strains separately showing various kind of organic acids production. a) culture supernatant analysis of *P. plecoglossicida* showing the presence of different organic acids produced during ZnOsolubilization b) Culture supernatant of *Acinetobacter* sp. c) Culture supernatant of *P. putida* d) Culture supernatant of *Yokenella* sp. respectively. Standard chromatogram peaks of organic acids e) gluconic acid f) formic acid g) lactic acid h) acetic acid i) citric acid

HPLC analysis of Organic acids identification

The organic acids attributed for the Zn solubilization in the broth assay were identified and quantified using HPLC after 48 h of incubation. On quantification assay, the production of organic acids differed among the ZSB strains tested. Gluconic, formic and lactic acids were found commonly in all ZSB inoculated broth. Among the strains tested, *Acinetobacter* sp. produced the highest concentration of gluconic acid of 48 µg mL^{-1} and the least production was observed in *Yokenella* sp. inoculated broth, it recorded a concentration of 17 µg mL^{-1}. The synthesis of formic acid and lactic acid were least produced by ZSBs compared to other organic acids excreted in the broth. Presence of acetic acid in the culture filtrate of *P. puitida*, *P. Plecoglossicida* and *Acinetobacter* sp. was noticed nonetheless absent in *Yokenella* sp. In addition, noticeable amount of citric acid was produced by *P. putida*, *P. plecoglossicida* and *Yokenella* sp. ranging 1463, 889 and 190 µg mL^{-1} respectively (Fig. 5).

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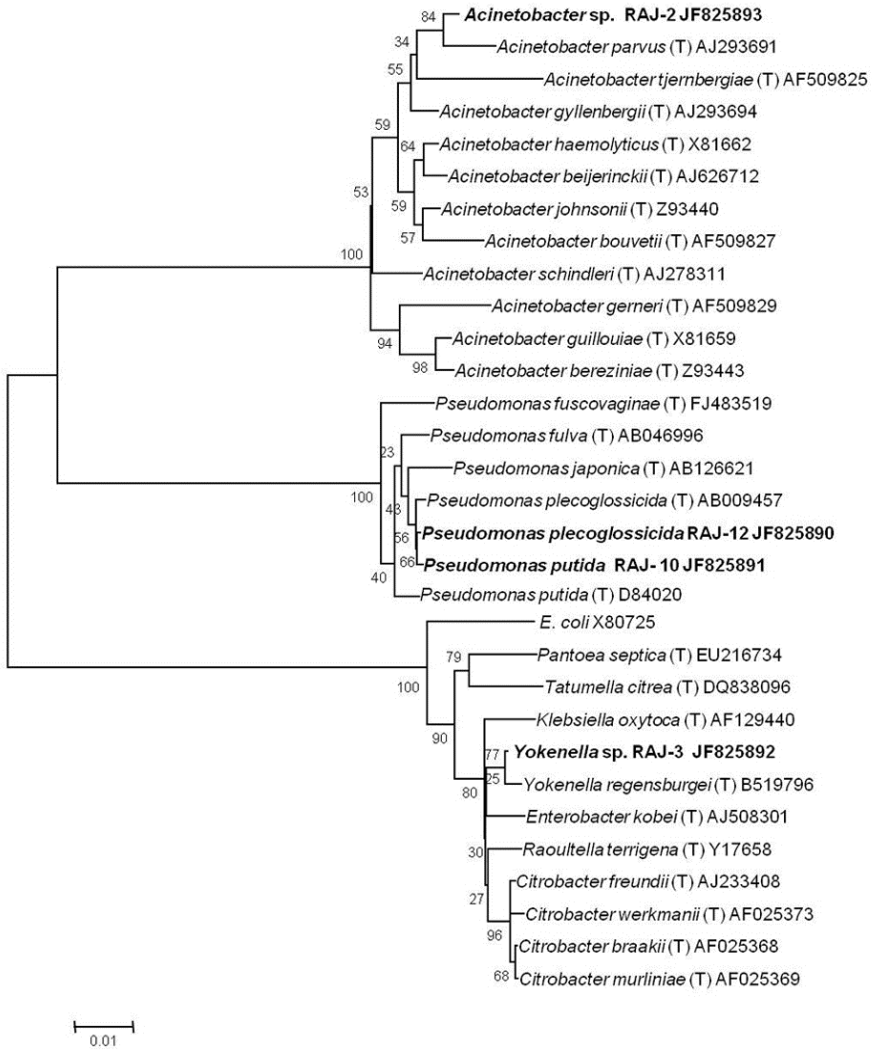

Fig 5: Phylogenetic tree of ZSB strains. The Neighbour Joining phylogenetic tree was constructed from the 16S rRNA gene sequences of ZSB. The sequences were labelled with respective genus and species names along with the nucleotide Acc. No. The ZSB cultures used in the present study were shown in bold. The numbers at nodes indicate the levels of bootstrap support, based Neighbour Joining analysis of 1000 re-sampled data sets. Bar 0.005 substitutions per site

Assessment of Plant growth promoting activities and Maximum tolerance level of Zn metal by ZSB strains

The selected ZSB strains were assessed for different direct and indirect plant growth promoting traits. In IAA production, the highest synthesis was represented by *P. plecoglossicida* at the range of 43 µg mL^{-1} and the least was detected in *P. putida* of 33 µg mL^{-1}. The IAA activity was not shown by the *Acinetobacter* sp. and *Yokenella* sp. Behind Zn solubilization, all strains solubilized significant quantity of TCP in the liquid medium. The maximum solubilization of $Ca_3(HPO_4)_2$ in NBRIP broth was noted in *P. plecoglossicida* solubilizing 319 µg P mL^{-1} and *Acinetobacter* sp. solubilized 197 µg P mL^{-1} at the lower range. Additionally, all strains secreted siderophore and produced NH_3. All the strains were checked for HCN production and ACC deaminase activity and the consolidated activity of PGP traits are mentioned in the Table 1. The maximum tolerance to Zn in the medium allows the strain to continue in their role of Zn solubilization by tolerating the Zn stress. The increased tolerance to Zn was exhibited by *Yokenella* sp. at 1700 ppm, while the least tolerance was noted in 1500 ppm by *Acinetobacter* sp.

ISSN: 0975-8585

Table 1: Plant growth promotion activity of ZSB strains

ZSB strains	IAA[a] (µg mL^{-1})	Siderophore[b] (mm)	Phosphate solubilization[c] (µg mL^{-1})	pH	MIC[d] (ppm)	EPS[e] (µg mL^{-1})	ACC[f] Deaminase (µm α-KB mg^{-1} h^{-1})	NH_3[g]	HCN[h]
*Acinetobacter*sp.	_	14 ± 0.5	197 ± 2.8	4.6	1500	11 ± 1.0	28 ± 2.0	+	_
*Yokenella*sp.	_	7 ±0.5	319 ± 2.0	4.7	1700	21 ± 1.0	26 ± 3.3	+	_
P. putida	33 ± 1.0	12 ±0.5	302 ± 2.5	4.2	1600	15 ± 1.1	22 ± 2.8	+	+
P. plecoglossicida	43 ± 0.5	9 ±0.0	233 ± 2.8	4.1	1600	16 ± 1.1	30 ± 3.2	+	_

The values represent mean ± S.D. of three replicates per treatment. a – indole-3- acetic acid (µg mL-1), b – chrome azurol S agar was used, c– Phosphate solubilization and pH of NBRIP broth, d- minimum inhibitory concentration of Zn, e- exo-polysaccharide, f-1-aminocyclopropane-1-carboxylate, g– Ammonia production,h – Hydrogen cyanide, '+'Presence of activity; '-'Absence of activity

DISCUSSION

Soil dwelling bacteria possessing Zn solubilizing property is considered as rare feature among the bacterial community and they were been previously discussed and isolated from rhizosphere soil (Di Simine et al. 1998), air environment (Fasim et al. 2002), as endophytes (saravanan et al. 2007a). In our study, the ZSB strains were isolated from pesticide treated non rhizosphere soils of crop fields and were studied. The pesticide treated soil screened in the study showed less native bacterial population even in the minimum dilution factor, yet the number of ZSB was even lesser. Pesticide added soil contains less bacterial population is due to the consequent stressful effect of pesticides that is applied to the soil, thereby that render only the resistant bacteria to survive. However, soil collected from *Solanum lycopersicum* field contained modest population of ZSB than the other samples, which may have utilized the pesticide residues as nutrients that is applied to the field. Previously, increases in the population of the phosphate solubilizing bacteria in soil was documented which was based on the utilization of pesticides like BHC and Phorate as their energy and nutrients (Das and Mukherjee 1994).

Pesticide treated, non rhizosphere soil (5 different soil samples) screening recovered 12 ZSB strains on total. Based on Zn solubilization and different colony morphology, six bacterial strains were preferred for the further study. On plate assay, the ZSB strains were evaluated for Zn solubilizing potential showed different degree of haloes. This might possibly be due to the production of various organic acids by the ZSB strains that diffuses through the agar surface by utilising the readily available glucose in the medium. Through direct oxidation pathway, certain bacteria convert significant amount of glucose to gluconic acid that could be expressed stoichiometrically at the concentration of 1 mol L^{-1} or more (Goldstein et al. 1993). Gluconic acid and their derivatives possess multiple conformation that chelate metal cations beside solubilization (Goldstein 1995). In our study, the proficient Zn solubilization on MSM agar plates amended with different Zn compounds was observed in *Acinetobacter* sp. throughout the solid medium assay. In Particular, on agar plate with 0.2 % dose of $Zn_3(PO_4)_2$, only *Acinetobacter* sp. exhibited solubilization halo that may have probably facilitated through the high amount of gluconic acid produced than the other ZSB strains. Conversely, *Acinetobacter* sp. was previously studied for their phosphate solubilization activity mainly through the production of gluconic acid in the medium (Ogut et al. 2010). In response to plate assay, none of the ZSB strain produced zone on Zn metal powder or ZnS amended plates on either doses. Our plate assay results are in contrast with Saravanan et al. (2007b) while, *G. diazotropicus* showed halo growth on Zn metal amended LGI agar medium. The insoluble compounds like metal ion containing Phosphorus could possibly converted to soluble form through the production of organic acids, therefore the metal ions and phosphates are separated (Kepert et al. 1979). Hence, like such conditions, the dissolution of $Zn_3(PO_4)_2$ to Zn and P can serve dual nutrition to the plants, as it is mentioned that the co-existence of Zn and P paucity occurs frequently, specifically in calcareous soil system (Hoffland et al. 2006). Thus, the solubilization of $Zn_3(PO_4)_2$ in plate and broth assay by ZSBs in our study may aid both the nutrition when applied in the soil ecosystem.

Under broth conditions, the solubilization of insoluble Zn compounds and increase in the available Zn was associated with the acidification of the medium, mainly through the excretion of organic acids by the ZSB strains. As an indication, in Zn amended and the culture inoculated flasks, decrease in the pH was observed. The organic acids may have played role in dissociation of Zn^{2+} ions from their respective insoluble compound and their presence in the broth was detected using HPLC. The production of proton (H^+) or organic acid in the medium plays a predominant role in metal solubilization; however, based on the type of organism and their growth conditions, excretion of other metabolites or siderophore like factors also contributes to the solubilization (Morley et al. 1996; Nautiyal et al. 2000).

Likewise, solubilization of Zn metal power and ZnS in broth also resulted in the acidification of medium that led to release a substantial amount of cationic Zn^{2+} over control. While the same Zn sources were used in agar plates, Zn solubilizing activity by ZSB strains were not observed by halo formation. Similar effect was observed by Nautiyal (1999) in phosphate solubilization assay by *Pseudomonas* spp. that released significant amount of P in liquid medium even so showing no solubilizing zone on Phosphate containing plates. Among the Zn sources used, Zn metal powder being the more toxic and constrained to solubilize, the ZSB strains in our study showed substantial amount of solubilization in the liquid medium. Saravanan et al. (2007b) studied the effect of Zn metal on bacterial cells that showed pleomorphism in *G. diazotrophicus* when inoculated in the broth amended with metallic Zn. In addition to gluconic acid in Zn solubilization, citric acid was produced by *P. putida*, *P. plecoglossicida* and *Yokenella* sp. Moreover, the excretion of citric acid in the surrounding medium can also contribute or act as metal complexing agent or H^+ donors (Müller et al. 1995). The quantification of Zn solubilized by the ZSB strains measured excellent in the broth assay, mainly the total available Zn solubilized by *Yokenella* sp. from most of the insoluble Zn sources measured distinctly high compared to the previous reports of Di simini et al (1997), Fasim et al. (2002), Saravanan et al. (2007a), Sharma et al. (2012) and Xinxian et al. (2012).

The maximum Zn solubilization measured in *Yokenella* sp. showed less organic acids production in the broth compared to *Pseudomonas* spp. Conversely, this effective Zn solubilization may be due to the co-existence of EPS with organic acids in the medium that aided in the better solubilization. Moreover, the EPS production among the ZSB strains were found positive and *Yokenella* sp. evidenced to measure increased EPS production. The oligosaccharide of *Yokenella regensburgei* possesses undecasaccharide and does not contain phosphate but contains negative charge in their core (Niedziela et al. 2010); hence this negative part may complex with the metal cation. In addition to organic acids secretion, synthesis of EPS in the medium showed increased phosphate solubilization in *Enterobacter* sp., *Arthrobacter* sp. and *Azotobacter* sp. (Yi et al. 2008). Similarly, it is reported that certain bacteria were known to enhance the silicate (Vandevivere et al. 1994) and plagioclase (Welch et al. 1999) dissimilation by the production of different EPS, fastening the process. Hence from our experiments, we conclude that *Yokenella* sp. mediate the mechanism of producing EPS for Zn solubilization and that may have also attributed overcoming the increased concentration of Zn solubilized that probably causes toxicity. The results of EPS production in our study also showed maximum production by *Yokenella* sp. that supports the high Zn tolerance. The maximum tolerance to Zn in the medium allows the strain to continue in their role of Zn solubilization. High concentration of Zn^{2+} liberated in the medium may serve for Zn toxicity for the culture, however, the production of protein rich Zn binding moiety serve *P. fluorescence* to evade Zn toxicity (Appanna and Whitmore 1995). Furthermore, high Zn solubilization at *in vitro* conditions may not be accomplished in the rhizosphere regions that are merely dependent on the availability of carbon sources (Grayston et al. 1997). Hence, the Zn toxicity process does not take place in the rhizosphere soil that may affect the plant system.

The roles of ZSB used in this study are found effective under *in vitro* Zn solubilization. Furthermore, these strains possess multiple PGP traits and tolerate high Zn concentration in addition to Zn solubilization. Therefore, these strains could be exploited for supplying the cationic Zn nutrition in cereal cultivations or in the bioremediation process where soil contaminated with Zn metal. The compatibility among these strains and efficacy in pot culture need to be studied further for preparing them in consortium for the supply to field conditions.

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