Short communication

# Screening for Zn solubilisation potential of soil bacteria from Zn deficient soils of Kerala

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**Abstract**

Zinc plays an important role among the micronutrients that influence plant growth and productivity. Various microorganisms like bacteria and fungi have been reported to solubilise insoluble sources of zinc present in soil. In the present study, microorganisms capable of solubilising insoluble forms of zinc like zinc oxide and zinc phosphate were isolated from zinc deficient soils of Kerala. Ten isolates of bacteria which were able to solubilise insoluble form of zinc (zinc oxide) were obtained. The size of the solubilisation zone ranged from 8.67 mm to 13.33 mm for zinc oxide and 1.00 mm to 5.33 mm for zinc phosphate incorporated selective medium. In broth culture, maximum zinc solubilisation in both sources were observed on 30$^{th}$ day in the range of 35.91 ppm to 104.08 ppm for zinc oxide supplemented medium and 1.38 ppm to 4.15 ppm for zinc phosphate supplemented medium. The isolate ZSB-4 showed highest solubilisation of zinc in plate assay and broth assay. Morphological and molecular characterization studies revealed the identity of the bacteria as *Bacillus cereus*. The isolate ZSB-4 significantly increased the available zinc content in soil from 0.55 ppm to 9.47 ppm under soil incubation studies.

**Keywords:** *Bacillus,* Screening of bacteria, Zinc solubilization

## Introduction

Zinc plays a vital role in metabolism, mitosis, seed development and mitochondrial activities (Hughes and Poole, 1989). It is a constituent of about 59 enzymes and has an important role in auxin production from tryptophan and is present in relatively small amounts (5 - 100 mg kg$^{-1}$) in plant tissues. Soil zinc deficiency is a worldwide problem in crop production which affects the crop growth in over 50 per cent of agricultural lands. The All India Coordinated Research Project (AICRP) on Micro and Secondary Nutrients and Polluted Elements in Soils and Plants estimated about 49 per cent of soil samples to be deficient in zinc (Singh, 2001). The available zinc content in soils of India is low whereas the total zinc content, which exists in fixed forms such as smithsonite, sphalerite, zincite, franklinite, wellemite and hopeite, is high.

In Kerala, zinc defiency is most prevalent in Wayanad district and localised deficiencies are reported in the districts of Ernakulam, Idukki, Thiruvananthapuram, Kollam, Alappuzha, Thrissur and Kozhikode and need based application is recommended (Kerala State Planning Board, 2013). To correct the zinc deficiency, exogenous application of water soluble $ZnSO_4$ is advocated. In Kerala, zinc sulphate has been recommended @ 20 kg ha$^{-1}$ for rice and sesamum, 30 kg ha$^{-1}$ for ginger, turmeric and black pepper, 25 kg ha$^{-1}$ for cardamom and 10 kg ha$^{-1}$ for tomato and cassava in Package of Practices recommendation of KAU (KAU, 2011).

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It has been reported that 96 to 98 per cent of the applied soluble zinc is converted in soil, to the unavailable form. The water soluble zinc applied as zinc sulphate gets transformed into zinc hydroxide in soils with relatively high pH, zinc carbonate in alkali soils rich in calcium, zinc phosphate in near neutral to alkali soils with high phosphorus content and zinc sulphide under reduced conditions.

The zinc solubilising potential of microbial genera such as *Bacillus*, *Pseudomonas* and *Aspergillus* was explored by researchers recently (Saravanan et al., 2003). Microbial mobilisation of unavailable forms of native and applied zinc is of much importance to avoid pollution by repeated application of zinc sulphate. The present study has been designed to screen Zn deficient soils of Kerala for isolation and characterization of zinc solubilising bacterial isolates.

In the present study, soil samples were collected from zinc deficient locations such as Agroecological units 20, 21 (Wayanad) and 8 (Thiruvananthapuram) (Kerala State Planning Board, 2013). The locations of zinc deficiency were selected by analyzing the soil samples for the available zinc status by extraction method using 0.1N HCl followed by Atomic Absorption Spectrophotometer reading (Sims and Johnson, 1991). Soil samples were collected from non-cropped, undisturbed sites covered by natural vegetation and 50 g of soil samples were taken from the upper 30 cm of the soil profile from different sites and pooled to get a representative sample.

Microorganisms capable of solubilising zinc were isolated from zinc deficient areas by serial dilution and plating on medium (Bunt and Rovira, 1955) supplemented with 0.1% insoluble zinc oxide. After 3-days of incubation at room temperature (28±2ºC), zinc solubilising microorganisms developed clear halo zones around colonies. The colonies with clearance zones were further purified by sub culturing on Bunt and Rovira agar medium (glucose 20.0 g, peptone 1.0 g, yeast extract 1.0 g, $(NH_4)_2SO_4$ 0.5 g, $K_2HPO_4$ 0.4 g, $MgCl_2$ 0.1 g, $FeCl_3$ 0.01 g, agar 20.0 g, distilled water 1000 ml, pH 6.6-7.0) supplemented with 0.1 per cent zinc oxide. Ten morphologically different zinc solubilising bacterial isolates were selected for further assay.

Sterilized Bunt and Rovira agar medium supplemented with 0.1 per cent insoluble zinc compounds, either zinc oxide or zinc phosphate was poured into sterilized Petri dishes. The bacterial isolates were spot inoculated on solidified agar plates aseptically and incubated at $28^0$C for 72 hours. The halo zones around the colonies were observed and measured in millimetres. Three replications for each isolate and a control without zinc solubilising microorganisms were maintained.

The bacterial isolates were further inoculated separately in basal medium with 0.1 per cent insoluble zinc compounds, either zinc oxide or zinc phosphate. The flask containing 100 ml of sterile broth was inoculated with 1 ml of 24 hour old, actively growing test bacterial cultures and incubated at room temperature with shaking at 140 rpm. Three replications were maintained for each isolate along with an un-inoculated control. The available zinc content in the broth culture was analyzed 0, 7, 14, 20 and 30 days after inoculation. Ten millilitre of each sample were transferred aseptically to centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes to remove cell debris. The supernatant was collected and filtered through Whatman filter paper No. 42. The clear solution was collected in 30 ml vials and the quantity of available zinc released into the medium was assessed using Atomic Absorption Spectrophotometer (AAS).

The isolate with maximum zinc solubilisation potential was selected for characterization studies. The bacterial culture was characterized phenotypically to the genus-level based on its colony morphology (size, margin, elevation), microscopic observation (Gram reaction, shape and

arrangement of cells) and biochemical tests. Species level identification was carried out at Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, by 16S rRNA gene sequencing.

Talc, lignite, vermiculite, perlite and vermicompost were selected as carrier materials for development of a formulation of the selected zinc solubilising bacterial isolate. One kilogram of each carrier material was transferred into polythene bags and autoclaved for 3 consecutive days. One hundred millilitres of the overnight culture in the nutrient broth was mixed aseptically with 1000 g of each sterile carrier, packed in polyethylene bags, sealed and incubated under room temperature. The total count of zinc solubilising microorganism was monitored at monthly intervals for a period of three months by dilution plating technique on zinc solubilizing medium supplemented with 0.1 per cent insoluble zinc oxide.

Soil incubation study was conducted using soils with low available zinc collected from Agro ecological unit – 8 (Neyyatinkara taluk) in the Department of Agricultural Microbiology at College of Agriculture, Vellayani. Sterilized soil of one kilogram each was filled in plastic containers of dimension 24 cm x 13 cm x 10 cm and maintained at field capacity with sterile distilled water. Each box was covered with polythene sheet and incubated at room temperature (28±2ºC). The isolate with maximum zinc solubilisation potential (ZSB - 4) was selected for the study. The experiment was laid out in completely randomized design and the treatments were designed as $T_1$ and $T_2$ with Zn at two levels (1 kg $ha^{-1}$ and 2 kg $ha^{-1}$ respectively) as ZnO, $T_3$ with addition of talc based product of Zn solubilising culture alone @ 2 kg $ha^{-1}$, $T_4$ and $T_5$ with Zn solubilising bioformulation @ 2 kg $ha^{-1}$ supplemented with Zn at two levels (1 kg $ha^{-1}$ and 2 kg $ha^{-1}$ respectively) as ZnO and T6, the absolute control with no insoluble zinc supplementation and without the inoculation of the bacterial formulation. The soil samples were analysed for soil chemical parameters and population dynamics of the bacterial isolate for a period of three months.

Ten isolates of bacteria solubilising insoluble forms of zinc (zinc oxide) were obtained from zinc deficient areas by serial dilution technique on zinc solubilizing medium. These isolates were allotted code numbers from ZSB-1 to ZSB-10. The Bunt and Rovira medium was used to isolate zinc solubilising microorganisms since it is a simple way to detect zinc solubilisation through the formation of halo zone around the potential microbial colonies on agar plates. Zinc solubilising capability has already been reported in several bacterial genera belonging to *Thiobacillus thiooxidans, Thiobacillus ferroxidans*, facultative thermophilic iron oxidizers (Hutchins et al., 1986), *Gluconacetobacter diaztorophicus* (Saravanan et al., 2007) and *Pseudomonas fluorescens* (Di Simine et al., 1998; Saravanan et al., 2003).

All the selected isolates could effectively solubilise the insoluble Zn compounds used, viz., zinc oxide and zinc phosphate, under the assay conditions. The zone of solubilisation was comparatively large in zinc oxide amended medium than in zinc phosphate medium (Table 1). After three days of incubation on test plates, all the ten isolates solubilised zinc and produced clearing zone around the colonies on

*Table 1.* Zinc solubilising potential of bacterial (ZSB) isolates with different insoluble zinc sources on plate assay

| | Clearing zone (mm) 3 days after inoculation* | |
|---|---|---|
| Isolates | Zinc oxide | Zinc phosphate |
| ZSB - 1 | 9.00 | 1.33 |
| ZSB - 2 | 10.00 | 2.00 |
| ZSB - 3 | 9.33 | 1.66 |
| ZSB - 4 | 13.33 | 5.33 |
| ZSB - 5 | 10.67 | 1.00 |
| ZSB - 6 | 9.67 | 2.33 |
| ZSB -7 | 10.33 | 1.33 |
| ZSB - 8 | 10.00 | 1.00 |
| ZSB -9 | 8.67 | 2.33 |
| ZSB - 10 | 8.33 | 2.00 |
| CD (0.05) | 2.06 | 0.94 |

*Mean of three replications

solid media. The size of the solubilisation zone ranged from 8.67 mm to 13.33 mm in zinc oxide and from 1.00 mm to 5.33 mm in zinc phosphate incorporated medium. Similar results was reported by Desai et al. (2012) for organisms like *Pseudomonas, Azospirillum* and *Bacillus* with a solubilisation zone ranging from 5 mm to 7.6 mm. The results are also in agreement with the findings of Saravanan et al. (2003) who stated that *Bacillus* sp and *Pseudomonas* sp registered a clearance zone of 1.8 cm and 3.3 cm respectively when grown in Bunt and Rovira media containing 0.1 per cent insoluble zinc oxide. Previous studies conducted in plate assay with *Gluconacetobacter diazotrophicus* strains for zinc solubilisation also revealed strain level variability (Saravanan et al., 2007). Similar results were also obtained in studies conducted by Ramesh et al. (2014) with *Bacillus aryabhattai* strains recording a solubilisation zone upto 13 mm on Tris-minimal medium supplemented with zinc phosphate at 0.1 per cent zinc concentration. Since plate assay was more of qualitative nature, broth culture experiment was carried out to assess the efficiency of the isolates to solubilize insoluble Zn.

In broth culture, maximum solubilisation of zinc in both sources was observed on 30$^{th}$ day in the range of 35.91 ppm to 104.08 ppm in zinc oxide supplemented medium and 1.38 ppm to 4.15 ppm in zinc phosphate supplemented medium (Table 2). Among the isolates, ZSB – 4 showed highest solubilisation of zinc with both sources ie., upto 104.08 ppm in zinc oxide supplemented medium and 4.514 ppm in zinc phosphate supplemented medium and was significantly superior to all other isolates during all the days. Similar results was reported by Desai et al. (2012) for organisms like *Pseudomonas, Azospirillum* and *Bacillus* with available zinc ranging from 9.46 ppm to 13.12 ppm on 9$^{th}$ day of incubation in broth assay.

The best isolate among the ten bacteria tested ie., ZSB–4 was subjected to morphological and molecular characterization. The isolate was Gram positive, rod shaped, with flat to raised colony, having creamy white colour, with smooth texture and capable of endospore formation. The molecular characterisation by 16S rRNA gene sequencing and BLAST analysis data comparison identified the isolate as *Bacillus cereus.* It is well known that zinc mobilizing capabilities appear to be widespread within bacterial taxa (He et al., 2010). The genus *Bacillus* is one of the most studied soil bacterium as it is found to be ubiquitous in nature and

*Table 2.* Efficiency of native bacterial isolates to solubilize zinc

| | Percentage Zn solubilised* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Zinc oxide** | | | | | Zinc phosphate** | | | | |
| | Days after inoculation | | | | | | | | | |
| Isolates | 0 | 7 | 14 | 20 | 30 | 0 | 7 | 14 | 20 | 30 |
| ZSB 1 | 0.01 | 0.11 | 0.33 | 3.43 | 7.71 | 0.00 | 0.09 | 0.23 | 0.25 | 0.34 |
| ZSB 2 | 0.01 | 0.10 | 0.43 | 3.42 | 9.21 | 0.00 | 0.05 | 0.14 | 0.29 | 0.36 |
| ZSB 3 | 0.03 | 0.11 | 0.35 | 1.68 | 6.14 | 0.00 | 0.10 | 0.24 | 0.32 | 0.53 |
| ZSB 4 | 0.04 | 0.29 | 0.98 | 6.57 | 12.49 | 0.00 | 0.24 | 0.53 | 0.95 | 1.17 |
| ZSB 5 | 0.03 | 0.10 | 0.39 | 3.90 | 6.50 | 0.00 | 0.15 | 0.17 | 0.49 | 0.70 |
| ZSB 6 | 0.03 | 0.11 | 0.47 | 3.88 | 7.15 | 0.00 | 0.08 | 0.28 | 0.52 | 0.80 |
| ZSB 7 | 0.02 | 0.11 | 0.48 | 4.15 | 7.77 | 0.00 | 0.08 | 0.27 | 0.47 | 0.87 |
| ZSB 8 | 0.03 | 0.09 | 0.36 | 3.60 | 8.37 | 0.00 | 0.14 | 0.23 | 0.51 | 0.80 |
| ZSB 9 | 0.02 | 0.07 | 0.31 | 3.23 | 4.30 | 0.00 | 0.07 | 0.22 | 0.28 | 0.64 |
| ZSB 10 | 0.02 | 0.04 | 0.26 | 3.09 | 5.90 | 0.00 | 0.06 | 0.12 | 0.22 | 0.36 |
| Control | 0.01 | 0.03 | 0.03 | 0.03 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CD (0.05) | NS | 0.28 | 0.58 | 10.85 | 5.09 | NS | 0.23 | 0.29 | 0.55 | 0.57 |

*Expressed as the amount of Zn ions released into the medium proportional to the total Zn in the combined form added into the medium **Mean of three replications

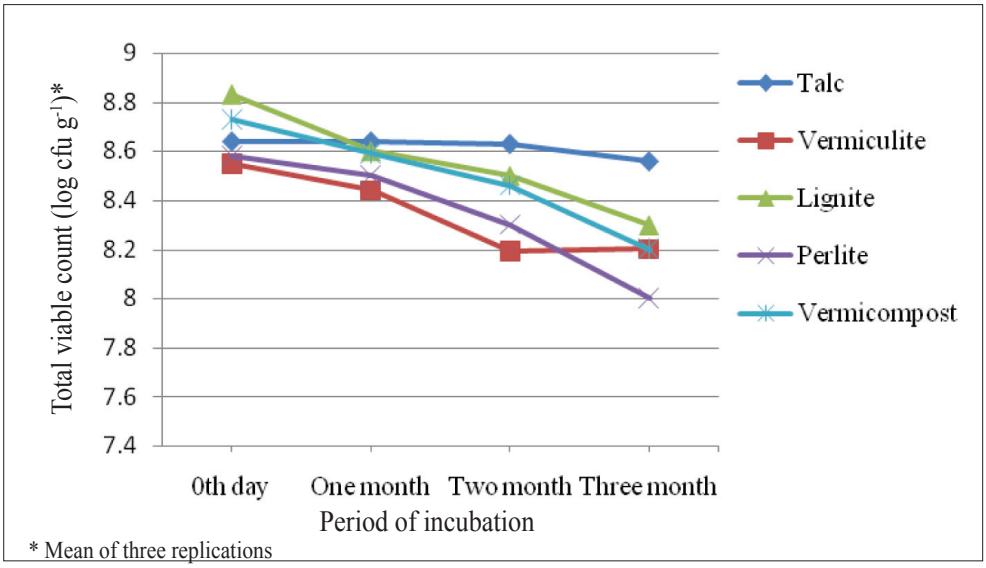


*Figure 1*. Population of zinc solubilising microorganisms in different carrier materials

possesses multiple plant growth promoting traits (Ramirez and Kloepper, 2010; Zhao et al., 2011).

During the course of development of a carrier based inoculum with the best isolate, on the first day of inoculation, highest population of 6.8 x $10^8$ cfu $g^{-1}$ was recorded in lignite followed by talc (6.6 x $10^8$ cfu $g^{-1}$), vermicompost (6.0 x $10^8$ cfu $g^{-1}$), perlite (3.9 x $10^8$ cfu $g^{-1}$) and vermiculite (3.6 x $10^8$ cfu $g^{-1}$) (Fig. 1). Among the different carriers tested, the talc powder supported the maximum population of 3.9 x $10^8$ cfu $g^{-1}$ during the 3rd month. Studies conducted by Gade et al. (2014) were also in agreement with the above results and talc based formulation of *P. fluorescens* sustained the maximum population of 18.0×$10^8$cfu $g^{-1}$ at 180 days of storage. Rajalaxmi et al. (2012) reported that talc based formulations of *P. fluorescens* retained mean population of 11×$10^7$cfu $g^{-1}$ after 300 days of storage.

The isolate ZSB–4 significantly increased the available zinc content in soil from 0.55 ppm to 9.47 ppm in treatment $T_4$ (ZSB–4 @ 2 kg $ha^{-1}$ + zinc oxide @ 1 kg $ha^{-1}$) during the incubation period (Table 3). Similar results were also obtained by Vaid et al. (2013) and Sirohi et al. (2015) who reported an

*Table 3*. Soil chemical parameters during soil incubation

| Treatments | At the start of experiment | | | | | | 3 months after inoculation | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Available zinc (ppm) | Total zinc (%) | Available boron (ppm) | Available phosphorus (kg $ha^{-1}$) | Soil pH | ZSB Populat-ion (log cfu $g^{-1}$) | Available zinc (ppm) | Total zinc (%) | Available boron (ppm) | Available phosphorus (kg $ha^{-1}$) | Soil pH | ZSB Population (log cfu $g^{-1}$) |
| $T_1$ | 0.42 | 0.07 | 0.35 | 9.17 | 4.9 | 0.00 | 0.67 | 0.07 | 0.16 | 9.26 | 4.9 | 0.00 |
| $T_2$ | 0.37 | 0.05 | 0.39 | 9.10 | 4.9 | 0.00 | 0.62 | 0.08 | 0.17 | 9.40 | 4.9 | 0.00 |
| $T_3$ | 0.52 | 0.06 | 0.34 | 8.60 | 5.0 | 2.23 | 2.37 | 0.07 | 0.30 | 12.04 | 4.9 | 3.91 |
| $T_4$ | 0.55 | 0.07 | 0.33 | 9.37 | 4.9 | 2.11 | 9.47 | 0.07 | 0.33 | 12.21 | 4.9 | 3.94 |
| $T_5$ | 0.47 | 0.07 | 0.36 | 8.58 | 4.9 | 2.16 | 6.95 | 0.07 | 0.34 | 12.16 | 4.9 | 3.91 |
| $T_6$ | 0.42 | 0.07 | 0.27 | 9.22 | 4.9 | 0.00 | 0.55 | 0.07 | 0.16 | 8.88 | 4.9 | 0.00 |
| CD (0.05) | NS | NS | NS | NS | NS | 0.12 | 0.64 | NS | 0.09 | 1.15 | NS | 0.03 |

*Mean of four replications
ZSB: Zinc Solubilising Microorganisms

increase in DTPA extractable Zn in soils inoculated with bacterial strains. Sharma et al. (2012) stated that inoculation with *Bacillus firmus*, *Bacillus amyloliquefaciens*, *Bacillus* sp, and *Bacillus cereus* significantly increased zinc assimilation in soybean seeds as compared with the uninoculated control under microcosm conditions. Inoculation with the P-solubilizing bacteria *Bacillus* M-13 also significantly enhanced Mn, Zn and Cu contents in soil (Canbolat et al., 2006). The treatment $T_4$ also registered the highest mean value for available phosphorus content of 12.26 kg $ha^{-1}$ on the 3rd month. This was in agreement with the findings of Canbolat et al. (2006) who reported that *Bacillus* M-13 and *Bacillus* RC01 inoculations increased available P by 16.9 percent and 8.8 percent respectively.

There was no significant difference observed in soil pH among treatments inoculated with zinc solubilising microorganisms ($T_3$, $T_4$, $T_5$) and for uninoculated treatments ($T_1$, $T_2$, $T_6$). On 3rd month, all the treatments were showing a pH of 4.9. Iqbal et al. (2010) also reported that there was no influence of bacterial inoculation in soil pH. Similar results were also reported by Canbolat et al. (2006).

The total zinc content of the soil ranged from 0.05% to 0.08% during the entire three months of soil incubation. During the 3rd month, highest total zinc content was recorded by $T_2$ with 0.08% while lowest mean value was registered by $T_5$ with 0.07%. This may due to more zinc available due to solubilisation of insoluble zinc compounds by microorganisms in sterile soil. The maximum colony count of 9.3 x $10^3$ cfu $g^{-1}$ of soil was recorded in the treatment $T_4$ during the 3rd month which was on par with $T_5$ (9.1 x $10^3$ cfu $g^{-1}$) and $T_3$ (8.8 x $10^3$ cfu $g^{-1}$). Similar results were reported by Sirohi et al. (2015), that the strain of *P. fluorescens* was able to maintain the cell density of approximately $10^8$ cfu $ml^{-1}$ for 4 weeks in soil.

Based on zinc solubilising potentiality, *Bacillus cereus* ZSB-4 was selected as elite zinc solubiliser among the ten zinc solubilising isolates obtained in the present study which was capable of making zinc available from unavailable zinc sources such as zinc oxide and zinc phosphate. However, there exist serious limitations for the use of the strain as a biofertilizer in the field conditions, as several of the *Bacillus cereus* isolates have been reported to be pathogenic to human beings and animals. Our study shows the potential of isolating native Zn solubilising microorganisms from Zn deficient soils of Kerala.

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