Zinc solubilizing bacterial isolates from the agricultural fields of Coimbatore, Tamil Nadu, India

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Zinc plays a pivotal role in physiological and biochemical functions of the plant. Both quantitative and qualitative yield of the plant is strongly dependent on this micronutrient. Supplementation of zinc in the form of synthetic fertilizer is proved to be inappropriate due to its unavailability to plants. This crisis can be prevented by the identification of rhizospheric micro-organisms which has the potential to transform various unavailable forms of the metal to available forms. In the present study about thirty five zinc solubilizing bacteria were isolated from eight different agricultural fields (banana, chilli, field bean, ground nut, maize, sugarcane, sorghum and tomato) in and around Coimbatore district of Tamil Nadu. Five isolates were selected as best strains based on their solubilization efficacy in the qualitative estimation. The selected five isolates were identified using 16S rRNA as *Stenotrophomonas maltophilia* (ZSB-1), *Mycobacterium brisbanense* (ZSB-10), *Enterobacter aerogenes* (ZSB-13), *Pseudomonas aeruginosa* (ZSB-22) and *Xanthomonas retroflexus* (ZSB-23). These strains were subjected to further studies such as quantitative estimation influence of the isolates on the pH of the medium and production of gluconic acid as well as IAA. Of the five bacterial isolates, *Pseudomonas aeruginosa* showed maximum solubilization of zinc in the broth and also maximum decrease in the pH from 7 to 3.3 and recorded highest IAA production. HPLC analysis of gluconic acid production by the selected isolates indicated their potential to solubilize zinc.

Keywords: Agricultural soils, bacteria, zinc solubilization.

DEFICIENCIES in plants due to the imbalanced supply of micronutrients are turning out to be an alarming condition in today's agricultural world. Among the micronutrient deficiencies, zinc (Zn) deficiency appears to be present unanimously. Zinc is a central component of several enzymes that drive and boost the rate of many important metabolic reactions of the plants. Thus Zn deficiency will result in the cessation of physiological and biochemical functions of plants leading to abnormal growth and adverse effect on the yield of crops. Zn deficiency has become a serious problem affecting nearly half of the world's population[1]. This is actually due to low Zn content of the crops grown in Zn-deficient soils. In India, about 50% of the soils are deficient in zinc and this remains the most important nutritional disorder affecting majority of the crop production. The reasons for the zinc deficiency are increased application of chemical fertilizers, intensive agriculture and poor irrigation system that leads to the reduction of zinc content in the Indian soils[2]. Zinc deficiency is expected to increase from 42% to 63% by 2025 due to continuous depletion of soil fertility[3]. Though marked response of crops to zinc application has been noticed, zinc deficiency is a major nutritional constraint for successful crop production in Tamil Nadu[4]. It is estimated that about 53% of the soils in Tamil Nadu are deficient in zinc[5]. Exogenous application of Zn to counter its deficiency in plants in the form of zinc sulphate also gets transformed into different unavailable forms like Zn(OH) and $Zn(OH_2)$ at pH of 7.7 and 9.1 (ref. 6); $ZnCO_3$ in calcium-rich alkali soils, $Zn(PO_3)_4$ in near-neutral to alkali soils of high P application[7] and gets accumulated in the soil. Though there is plenty of zinc in the soil to support crop growth, the crops exhibit deficiency due to the presence of the unavailable fractions. This necessitates a system that releases essential quantity of zinc from the unavailable state in which it is retained in the soil to the plants for good growth. Numerous bacteria, especially those associated with the rhizosphere have the ability to transform unavailable form of a metal into available form through solubilization mechanism[8].

The secretion of organic acids appears to be the functional mechanism involved in metal solubilization[9]. Gluconic acid is considered to be the major organic acid involved in the solubilization of insoluble minerals[10]. Organic acids secreted by micro-flora increase soil Zn availability by sequestering cations and by reducing rhizospheric pH. Therefore, isolation and identification of such bacteria is an eco-friendly approach to eradicate zinc deficiency in plants.

The greatest challenge for researchers is the identification of bacterial taxa from the soil resources. Various phenotypic and genotypic methodologies are being used to identify and characterize bacteria present in the soil

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community. Although phenotypic methods play a significant role in the identification, molecular methods are found to be more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Each bacterial species has at least one copy of the 16S rRNA gene containing highly conserved regions together with hyper variable regions. The use of 16S rRNA gene sequences to identify new strains of bacteria is gaining momentum in recent years[11].

Most of the zinc-deficient plants exhibit low levels of auxin such as indole-3-acetic acid (IAA) because Zn plays an essential role in the biosynthesis of IAA. Many researchers have observed that Zn is required for the synthesis of tryptophan, which in turn is the precursor for the synthesis of IAA. In the absence of IAA, plant growth is stunted[12]. This is because auxin forms a central regulator in many biological functions of plants such as cell division, elongation and differentiation to tropic responses, fruit development and senescence[13]. Many bacteria isolated from the rhizosphere have the capability to synthesize IAA *in vitro* in the presence or absence of physiological precursors, mainly tryptophan[14]. The application of such plant growth promoting rhizobacteria will resolve auxin deficiency in plants.

Thus the present investigation was aimed to isolate and identify soil bacteria that can solubilize insoluble forms of zinc into soluble form. In addition, organic acid secretion and synthesis of IAA by the isolates were also analysed.

Materials and methods

Collection of soil samples

Soil samples were collected from eight different agricultural fields in and around Coimbatore district, Tamil Nadu, India during the months of May and June 2012. The field areas that were deficient in zinc and were cultivated with plants that require zinc as vital nutrients for growth were taken as the criteria for soil sample collection. Samples were randomly collected ten times from the rhizosphere of young plants at a depth of 6–15 cm using soil augur. The soil samples that were collected from each location were air-dried, crushed and passed through 2 mm sieve before being mixed into a single composite sample. Then the soil samples were stored at 5°C for further study.

Enumeration and isolation of zinc solubilizing bacteria

Enumeration of zinc solubilizing bacteria (ZSB) present in the soil samples was done by adopting plate count method. Addition of different insoluble sources of zinc (ZnO, $ZnCO_3$ and Zn $(PO_3)_4$) at 0.1% in the modified Bunt and Rovira agar medium helped in the enumeration of bacteria. The plates were incubated for three days at 30°C in an incubator. The colonies that exhibited the clearing zone were considered as zinc solubilizers. The clear-zone forming organisms were counted, isolated and purified. The 35 most predominant and morphologically distinct bacterial colonies were selected for qualitative assay and were designated ZSB-1, ZSB-2 … ZSB-35.

Qualitative estimation of zinc solubilizing potential of the isolates

In the qualitative study, all the 35 bacterial isolates were tested for solubilization efficiency by means of plate assay using modified Bunt and Rovira agar medium containing 0.1% of ZnO, $ZnCO_3$ and Zn $(PO_3)_4$ as insoluble source. The plates were incubated at 30°C for 48 h. By measuring the diameter of the clear zone and colony growth, Zn solubilization efficiency was tested[15]

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

Based on the results of the plate assay, five isolates (ZSB-1, ZSB-10, ZSB-13, ZSB-22 and ZSB-23) which showed the best solubilization of zinc were identified using molecular marker 16S rRNA. They were subjected to further experimental studies such as quantitative estimation (broth assay), influence of the isolates on pH of the medium and production of gluconic acid as well as IAA.

Identification of Zn solubilizing microorganisms using molecular markers

Bacterial genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit according to the manufacturer's instruction. Using 16S rRNA universal primers, gene fragment was amplified using a thermal cycler (MJ Research PTC-225 Peltier Thermal Cycler). The PCR product was purified using a kit (Montage PCR clean-up kit (Millipore)). Sequencing reactions were performed using terminator cycle sequencing kits (BigDyeTM) ABI PRISM with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). A comparison of the 16S rRNA gene sequences was done with those available in GenBank (National Centre for Biotechnology Information; NCBI) using Basic Local Alignment Search tool (BLAST)[16,17] to obtain the best homologous sequences. Multiple sequence analysis was carried out using ClustalX (ref. 18). To obtain the confidence values, the original dataset was resampled 1000 times using bootstrap. The bootstrap dataset was used directly for constructing the phylogenetic tree using neighbour joining method of Saitou and Nei[19]. All the analyses were performed using MEGA 6.0.6 (ref. 20).

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

The five bacterial isolates were screened to find out the amount of zinc solubilized in the broth by growing them in 100 ml Erlenmeyer flasks containing 50 ml of Bunt and Rovira broth supplemented with 0.1% ZnO, $ZnCO_3$ and $Zn(PO_3)_4$. Appropriate uninoculated controls were maintained. All the treatments were replicated. The bacterial cultures were withdrawn after the sixth, eighth and tenth day of incubation at 30°C for the estimation of soluble Zn. The bacterial cultures were centrifuged at 15,000 rpm for 20 min and the supernatant was passed through 0.2 µm membrane filter so as to obtain the culture filtrate containing only the soluble forms of metal[21].

Then the sample was fed to an atomic absorption spectrometer (Shimadzu 7000AA) to find the concentration of available zinc present in the sample. The minimum detection limit of zinc in the instrument is 0.2 mg/kg and the linearity over the concentration range is 0.9952 (fit factor).

Influence of zinc solubilizing organisms on pH of the growth medium

The selected strains were inoculated in the flasks containing 50 ml of sterilized Bunt and Rovira agar medium containing 0.1% of ZnO, $ZnCO_3$ and $Zn(PO_3)_4$ as insoluble sources. An uninoculated sample was also maintained. The samples were drawn on the sixth, eighth and tenth day after incubation. The bacterial cultures were centrifuged at 15,000 rpm for 10 min and filtered using Whatman No. 42 filter paper. pH of the ZSB culture filtrates was measured using a pH meter (Elico).

Quantitative estimation of IAA by zinc solubilizing bacterial isolates

The selected bacterial isolates were tested for their ability to produce IAA by inoculating in the flasks containing 50 ml of Bunt and Rovira medium supplemented with 0.1% ZnO. Another set devoid of Zn material was also inoculated. All the treatments were amended with 0.1% tryptophan and incubated for seven days. The quantity of IAA produced by the organisms was estimated by the method of Brick *et al.*[22].

Analysis of gluconic acid produced by zinc solubilizing bacteria using HPLC

The selected bacterial cultures were tested for the production of gluconic acid by growing them in 50 ml of Bunt and Rovira medium supplemented with 0.1% ZnO. After incubation of the culture for 10 days at 30°C, 20 µl culture filtrate was injected in to a HPLC system (Shimadzu HPLC LC-20AD) using a separon SGX C18 column. Elution was performed with an isocratic flow consisting of acetonitrile : water (30 : 70 v/v) with a flow rate of 1.0 ml/min at 210 nm using UV/Vis detector[23]. The detection limit of the instrument is up to 0.5 ppm and the standard gluconic acid is detected in the range 50 ppm. Thus the gluconic acid present in the culture filtrate is determined by comparing the retention time and peak area of the sample with the standard of gluconic acid.

Results and discussion

Bacteria present in the rhizosphere play a vital role in the environmental cycling process such as solubilization of insoluble forms of metal into soluble forms for plant consumption, which will be significant for their optimum growth. Zinc is an essential micronutrient which plays a macro role in the metabolic and physiological activities of plants due to its central role as an enzyme cofactor.

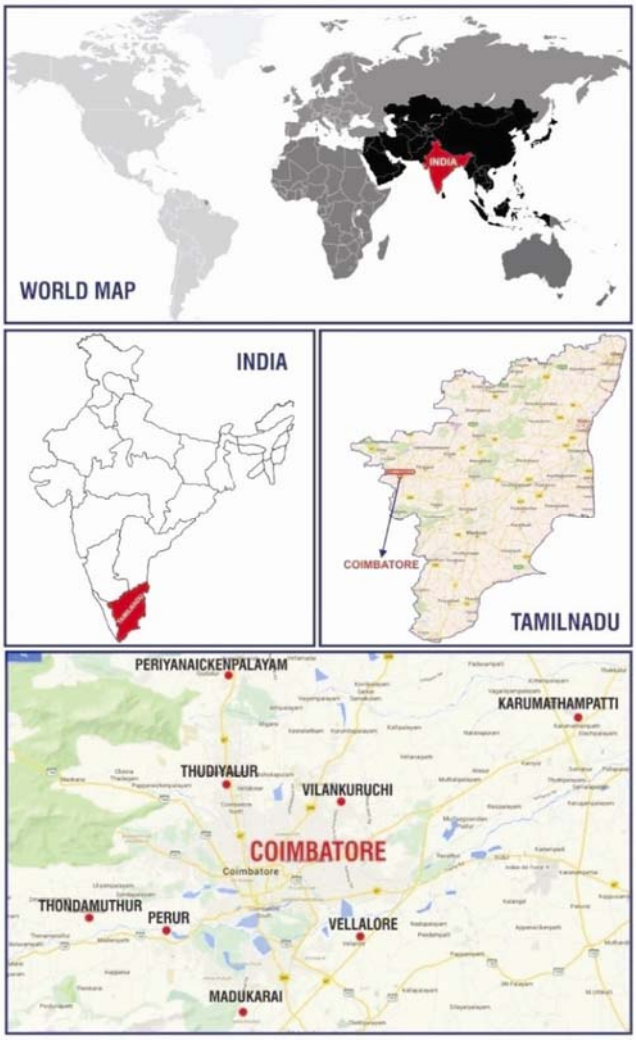

Figure 1. Map depicting the soil sampling locations used for the isolation of zinc solubilizing bacteria (ZSB).

Table 1. Physico-chemical characteristics of rhizospheric soil samples

Area	Field	Geographic location	Soil type	pH	EC (dS m^{-1})	N (%)	P (%)	K (%)	Zn (mg/kg)
Madukkarai	Banana field	10.9°N, 76.97°E	Sandy	7.89	0.24	79	112	219	3.97
Thudiyalore	Chilli field	11.4°N, 76.96°E	Sandy loam	6.9	0.22	116	7	210	1.206
Periyanaiken palayam	Field bean	11.15°N, 76.93°E	Sandy loam	7.29	0.24	74	114	117	1.198
Thondamuthur	Groundnut field	11.00°N, 76.49°E	Sandy	6.8	0.79	112	90	117	1.973
Vellalore	Maize field	10.58°N, 77.01°E	Sandy loam	6.7	0.37	117	8.4	290	1.09
Vilankurichi	Sorghum field	11.04°N, 77.00°E	Sandy loam	7.3	0.55	117	121	210	5.594
Perur	Sugarcane field	10.97°N, 76.9°E	Sandy loam	7.8	0.17	90	7.4	112	1.249
Karumathampatti	Tomato field	11.67°N, 77.33°E	Sandy loam	7.69	0.29	114	14.5	210	0.851

Table 2. Total zinc solubilizing bacteria (ZSB) in the soil samples

Sample	Zinc source in the medium	Total bacteria (CFU × 10^{-7}/g)	ZSB (CFU × 10^{-7}/g)	Percentage of ZSB
Banana field	ZnO	54.33 ± 2.52	6.67 ± 2.52	12.15 ± 4.11
	$ZnCO_3$	40.67 ± 2.52	4.33 ± 2.31	10.50 ± 5.02
	$Zn(PO_3)_4$	35.00 ± 3.00	3.67 ± 0.58	10.45 ± 1.03
Chilli field	ZnO	43.00 ± 3.61	5.00 ± 3.61	9.22 ± 1.59
	$ZnCO_3$	47.33 ± 2.08	5.33 ± 1.53	11.20 ± 2.78
	$Zn(PO_3)_4$	61.67 ± 1.53	7.33 ± 2.08	11.85 ± 3.18
Field bean	ZnO	48.67 ± 4.04	6.33 ± 2.31	12.84 ± 3.60
	$ZnCO_3$	73.33 ± 4.16	11.00 ± 2.65	14.90 ± 2.69
	$Zn(PO_3)_4$	42.00 ± 2.65	5.67 ± 2.52	13.27 ± 5.28
Groundnut field*	ZnO	45.00 ± 1.73	9.67 ± 1.15	21.53 ± 3.07
	$ZnCO_3$	50.67 ± 2.31	7.67 ± 2.52	15.01 ± 4.42
	$Zn(PO_3)_4$	54.00 ± 4.58	8.00 ± 3.00	14.58 ± 4.34
Maize field	ZnO	36.33 ± 5.03	5.67 ± 4.04	14.78 ± 9.04
	$ZnCO_3$	47.67 ± 5.51	7.67 ± 3.06	15.74 ± 4.54
	$Zn(PO_3)_4$	46.67 ± 4.51	7.00 ± 2.00	14.81 ± 2.88
Sorghum field	ZnO	55.67 ± 2.31	4.33 ± 0.58	7.78 ± 0.90
	$ZnCO_3$	48.67 ± 6.03	5.33 ± 3.21	10.55 ± 5.07
	$Zn(PO_3)_4$	44.33 ± 3.06	7.00 ± 1.73	16.02 ± 5.15
Sugarcane field	ZnO	50.67 ± 3.21	7.33 ± 2.31	14.39 ± 3.93
	$ZnCO_3$	45.33 ± 6.81	6.33 ± 3.21	13.50 ± 4.72
	$Zn(PO_3)_4$	45.67 ± 2.08	5.67 ± 1.53	12.33 ± 2.88
Tomato field	ZnO	51.67 ± 2.31	2.33 ± 1.15	4.46 ± 2.08
	$ZnCO_3$	34.33 ± 3.06	3.00 ± 1.00	8.61 ± 2.18
	$Zn(PO_3)_4$	34.33 ± 1.15	2.67 ± 0.58	7.73 ± 1.45
SEd		–	–	3.24983
CD ($P < 0.05$)				6.53457

Values are mean ± SD of three samples in each group.

Most of the soils present in Tamil Nadu exhibit Zn deficiency due to factors such as tropical climate, low total Zn content, neutral or alkaline pH, high salt concentration, high calcium carbonate content and calcareous soil[24]. Hence in the present investigation an attempt was made to isolate ZSB from the rhizospheric soil samples of eight different agricultural fields (banana, chilli, field bean, groundnut, maize, sugarcane, sorghum and tomato) in and around Coimbatore district (Figure 1). The physico-chemical characteristics of the samples show that most of the collected soils are sandy loam, rich in pH with low zinc content, which indicates Zn deficiency (Table 1).

Assessment of total bacterial counts along with solubilizers of ZnO, $ZnCO_3$ and $Zn(PO_3)_4$ and total bacterial population in the soils was made. The result of the study clearly shows the natural occurrence of ZSB in all the collected samples. But the population is limited in all the soils, which accounts for 7–21% of the total bacterial count depending on the soil type and zinc source. Maximum ZSB were found in soil samples of the groundnut field when all the three insoluble sources of Zn [ZnO (21.53%), $ZnCO_3$ (15.01%) and $Zn(PO_3)_4$ (14.58%)] were used as substrate, whereas tomato soil showed limited populations of ZSB compared to other field soils (Table 2).

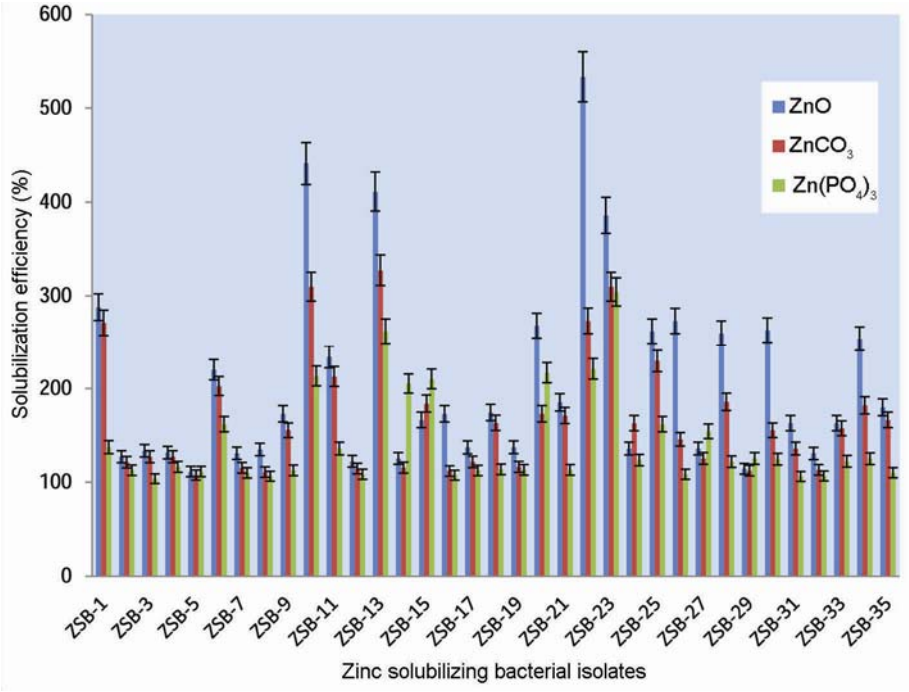

Figure 2. *In vitro* zinc solubilizing potential of the bacterial isolates (plate assay).

Thirty-five bacterial cultures were isolated and designated as ZSB-1, ZSB-2,…, ZSB-35.

In the qualitative study, the isolates were tested using plate assay. The 35 bacterial isolates were inoculated in the Bunt and Rovira agar medium containing different insoluble sources (ZnO, $ZnCO_3$ and $Zn(PO_3)_4$) of Zn at 0.1%. The solubilization efficiency of the isolates was calculated by measuring the diameter of the colony growth and the solubilization zone. Zinc solubilizing potential varies with each isolate and solubilization efficiency ranged between 103% and 533% depending on the zinc sources used (Figure 2). Among the isolates; ZSB-22, ZSB-13 and ZSB-23 showed highest dissolution zone and solubilizing efficiency of ZnO [533.33%], $ZnCO_3$ [327.30%] and $Zn(PO_3)_4$ [304.17%] respectively. The formation of halo zone by the microorganisms is due to the movement of acidity corresponded with the solubilization of the metal compound[25]. Saravanan *et al.*[26] also conducted a similar study and found that *Bacillus* sp. exhibited maximum clearing zone in zinc sulphide-containing medium, whereas *Pseudomonas* sp. produced higher halo zone in a medium consisting of ZnO and $ZnCO_3$ as substrates.

Based on the results of the plate assay, five isolates (ZSB-1, ZSB-10, ZSB-13, ZSB-22 and ZSB-23) that showed best solubilization of zinc (Figure 3) were identified using molecular marker 16S rRNA. They were subjected to further experimental studies such as quantitative estimation (broth assay), influence of the isolates on pH of the medium and production of gluconic acid as well as IAA.

The five bacterial strains which showed highest Zn solubilization efficiency were identified using molecular marker 16S rRNA. The data obtained from the partial 16S rRNA sequencing of the 1400 bp long PCR-amplified product were subjected to BLAST. The query sequence revealed that the isolates were *Stenotrophomonas maltophilia* (ZSB-1), *Mycobacterium brisbanense* (ZSB-10), *Enterobacter aerogenes* (ZSB-13), *Pseudomonas aeruginosa* (ZSB-22) and *Xanthomonas retroflexus* (ZSB-23) (Figure 4). The obtained sequences were also deposited in Genbank with the accession numbers KT037116, KT148588, KT148589, KT148590 and KT825693 respectively.

In the quantitative assay, the five bacterial isolates were tested by growing them in Bunt and Rovira liquid medium supplemented with 0.1% of ZnO, $ZnCO_3$ and $Zn(PO_3)_4$. The bacterial cultures were withdrawn after the sixth, eighth and tenth day of incubation at 30°C for estimation of soluble Zn in the broth using AAS. The amount of Zn solubilized by the five isolates varied, which may due to the differences in the location from which they were isolated. Among the five isolates, *P. aeruginosa* (ZSB-22) recorded maximum solubilization of Zn in all the three insoluble sources, i.e. ZnO [51.33 mg/l], $ZnCO_3$ [19.87 mg/l] and $Zn(PO_3)_4$ [31.30 mg/l] at the tenth day of incubation (Table 3). The solubilization efficiency of the isolates increases as the incubation period increases. Though ZSB-22 shows lesser dissolution in the solid medium, its efficiency is more in the broth. The results are in agreement with the findings of Louw and Webley[27], who reported which isolates did

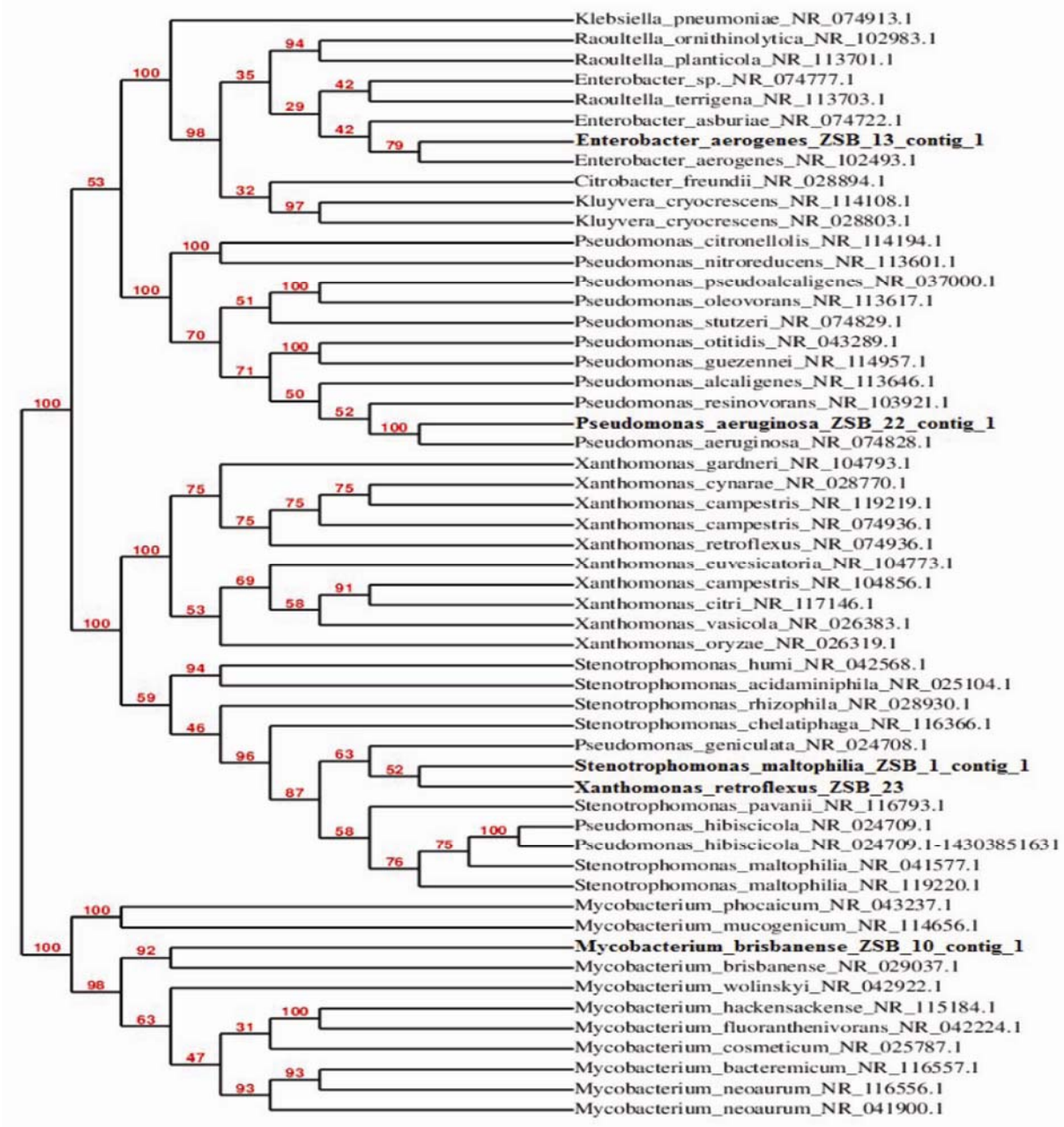

Figure 3. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of ZSB.

not produce a large halo zone on agar plates mobilized significant amount of metal in liquid medium. All the isolates show efficient solubilization of zinc when ZnO is used as substrate compared to $ZnCO_3$ and $Zn(PO_3)_4$, even after ten days of incubation. The results are in accordance with those of Franz *et al.*[28], who studied the efficacy of *Pencillium simplicissimum* to solubilize ZnO and concluded that this compound stimulated the efflux of organic acid because of its buffering capacity. Production of H^+ and organic acids was found to be the most important mechanism for heterotrophic metal solubilization. Dissolution of the ore and other materials may be due to production of organic acids like gluconic acids[29]. Zinc phosphate solubilization by *Pseudomonas fluorescens* was studied by Simine *et al.*[30], who concluded that gluconic acid and 2-keto gluconic acid produced in the culture broth helped in the solubilization of the zinc salts.

The influence of growth of ZSB isolates on pH of the medium was assessed at different intervals (sixth, eighth and tenth day after incubation) using a pH meter (Elico). All the culture filtrates showed a drop in pH when the incubation period was increased. Among the five isolates,

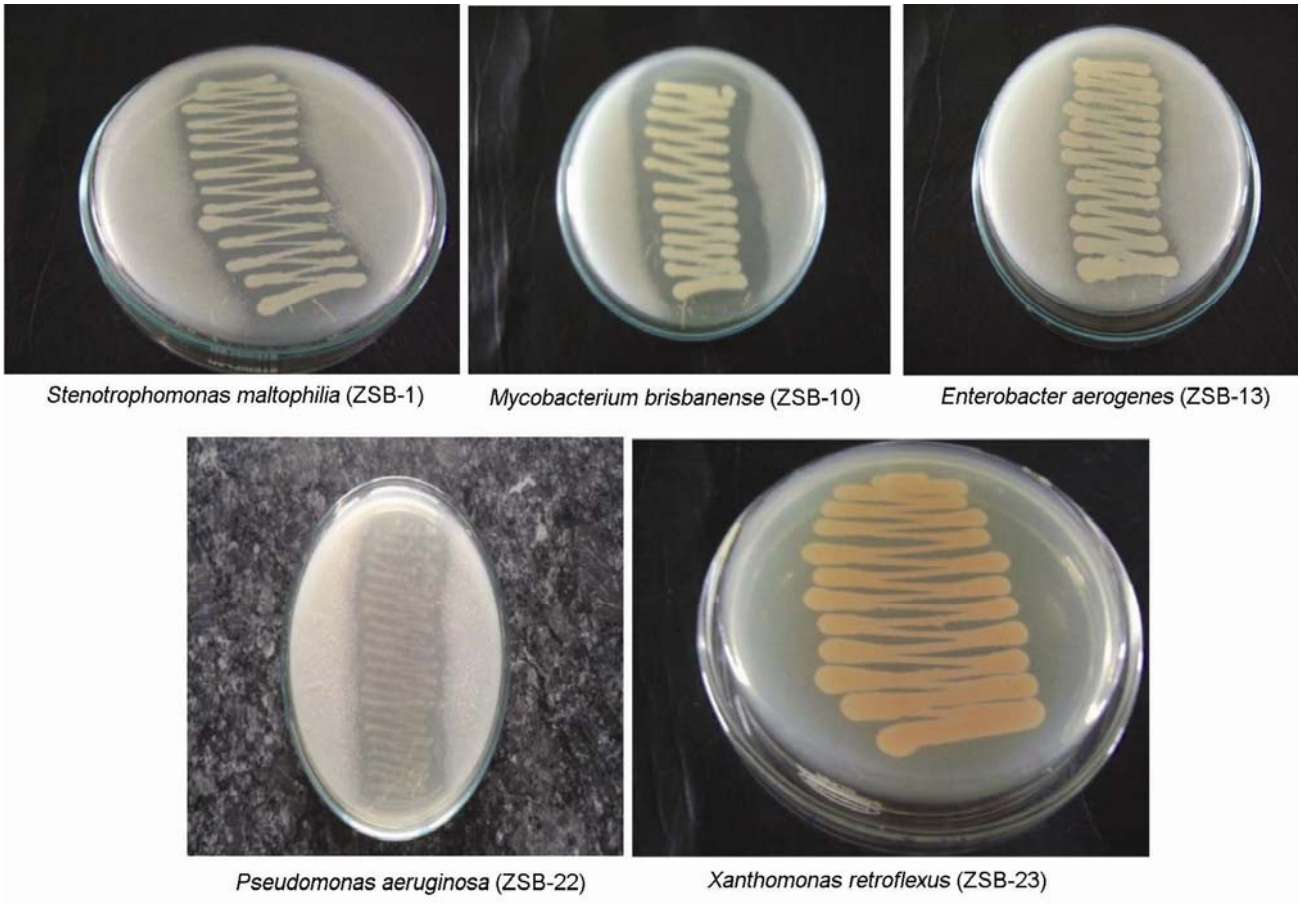

Figure 4. Best ZSB isolates.

Table 3. Zinc solubilizing potential of the bacterial isolates in broth culture

Isolate	Zinc source in the medium	Amount of zinc present in the cultural filtrates (mg/l)		
		6 DAI	8 DAI	10 DAI
ZSB-1	ZnO	15.02 ± 0.29	14.80 ± 0.47	15.71 ± 0.77
	$ZnCO_3$	9.84 ± 0.15	10.11 ± 0.38	9.78 ± 0.31
	$Zn(PO_3)_4$	15.11 ± 0.24	13.70 ± 0.30	13.87 ± 0.34
ZSB-10	ZnO	12.71 ± 0.58	11.54 ± 0.29	11.58 ± 0.31
	$ZnCO_3$	9.49 ± 0.24	9.05 ± 0.08	9.70 ± 0.12
	$Zn(PO_3)_4$	12.10 ± 0.08	12.35 ± 0.56	12.17 ± 0.65
ZSB-13	ZnO	33.20 ± 1.58	27.02 ± 4.67	30.60 ± 2.28
	$ZnCO_3$	15.79 ± 0.50	17.38 ± 0.77	18.07 ± 0.82
	$Zn(PO_3)_4$	22.62 ± 5.57	28.74 ± 2.41	32.33 ± 4.83
ZSB-22	ZnO	47.25 ± 1.34	34.27 ± 2.98	51.33 ± 6.88
	$ZnCO_3$	20.80 ± 0.40	22.89 ± 0.19	19.87 ± 0.51
	$Zn(PO_3)_4$	30.26 ± 2.70	30.11 ± 1.62	31.30 ± 5.47
ZSB-23	ZnO	10.56 ± 0.15	10.39 ± 0.57	46.39 ± 6.57
	$ZnCO_3$	19.73 ± 1.15	16.91 ± 0.73	17.85 ± 1.36
	$Zn(PO_3)_4$	25.15 ± 1.72	28.20 ± 0.53	30.34 ± 6.56
SEd			6.0453	
CD ($P < 0.05$)			10.4728	

*DAI, Days after incubation. Values are mean ± SD of three samples in each group.

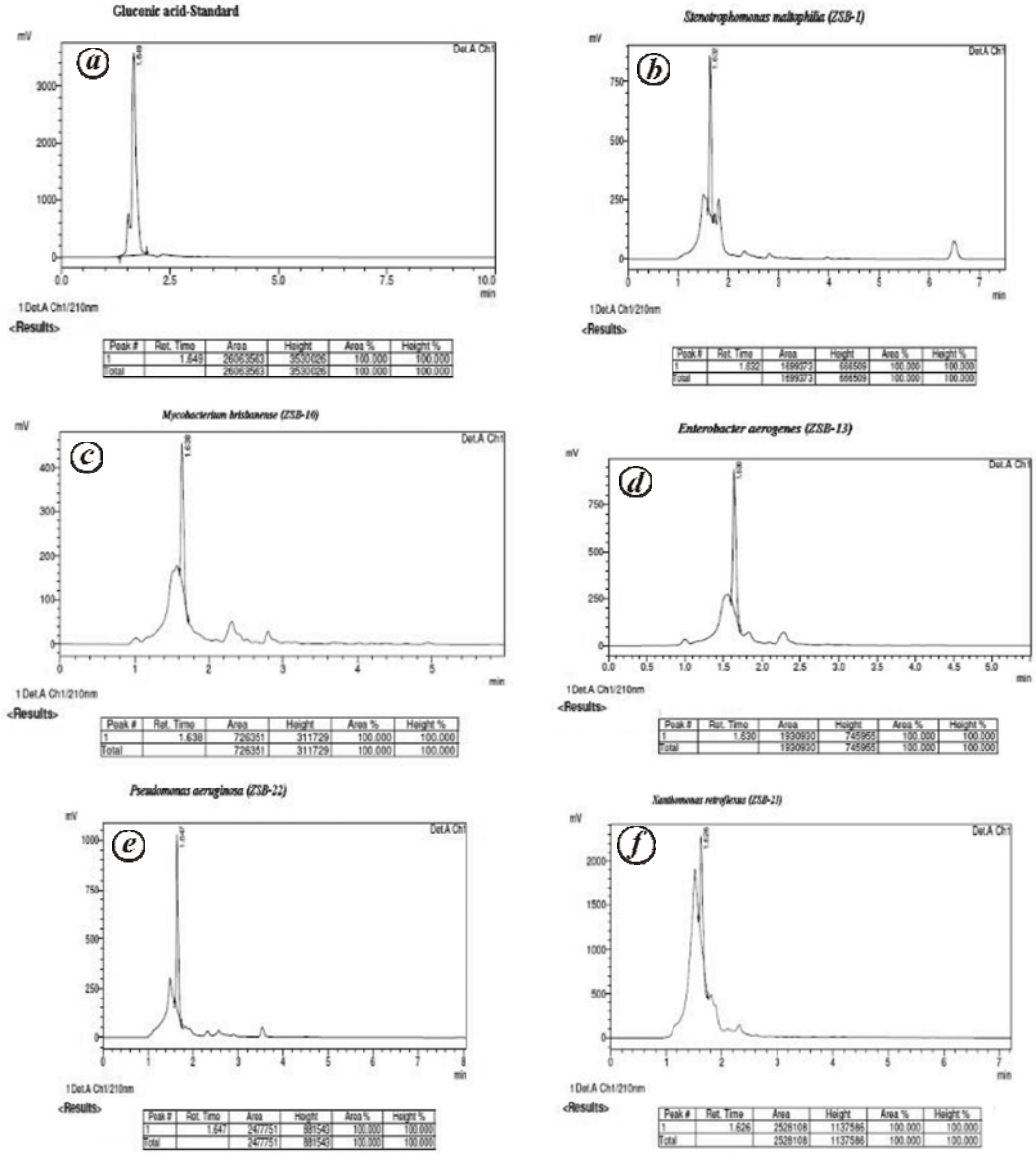

Figure 5. HPLC chromatographs depicting the peaks of gluconic acid secreted by ZSB. ***a***, Chromatograph of gluconic acid standard for a comparative study of its retention time (1.649) and peak area (26063563) with the selected samples. ***b*–*f***, Chromatographs of the samples ZSB-1, ZSB-10, ZSB-13, ZSB-22 and ZSB-23 of 10-day culture supernatant. Peak identified as gluconic acid is indicated.

P. aeruginosa (ZSB-22) caused maximum decline in pH (Table 4). It showed a shift in pH from 7 to 3.3 after ten days of incubation. A drop in the pH of the broth amended with insoluble Zn compounds has been argued by various authors to result from organic acid production and subsequent acidification of the medium[25,30]. The FT-IR spectral analysis by Fasim *et al.*[25] concluded that gluconic acid was attributed to the release of Zn ions formed from the Zn metal due to decrease in the pH caused by bacterial growth. Recently, it has been unequivocally proved that during the metabolism of glucose by *Gluconacetobacter diazotrophicus*, gluconic acid and its derivative acids are the dominant and central products of the metabolic pathway[31,32]. This acid is also commonly encountered during the process of microbial phosphorus solubilization[33].

The synthesis of gluconic acid produced by ZSB in the presence of ZnO was determined using HPLC. The retention time for standard gluconic acid was found to be 1.65. The presence of gluconic acid in the culture filtrate was detected on the basis of retention time obtained in the chromatograph of the gluconic acid standard. All the isolates produced gluconic acid in the medium incorporated with ZnO as insoluble source (Figure 5). Peak height and area of gluconic acid in the chromatograph of *P. aeruginosa* (ZSB-22) were found to be more compared to the

Table 4. Influence of zinc solubilizing organisms on pH of the growth medium

Isolate	Zinc source in the medium	pH of the growth medium		
		6 DAI	8 DAI	10 DAI
ZSB-1	ZnO	6.50 ± 0.60	6.27 ± 0.40	4.77 ± 0.40
	$ZnCO_3$	5.43 ± 0.35	5.10 ± 0.20	4.17 ± 0.21
	$Zn(PO_3)_4$	5.27 ± 0.40	4.97 ± 0.21	4.50 ± 0.36
ZSB-10	ZnO	6.70 ± 0.50	6.23 ± 0.38	6.40 ± 0.46
	$ZnCO_3$	6.70 ± 0.36	6.43 ± 0.35	5.00 ± 0.20
	$Zn(PO_3)_4$	5.10 ± 0.46	4.73 ± 0.55	4.50 ± 0.50
ZSB-13	ZnO	5.53 ± 0.50	4.83 ± 0.50	3.73 ± 0.50
	$ZnCO_3$	3.70 ± 0.40	3.70 ± 0.46	3.60 ± 0.56
	$Zn(PO_3)_4$	3.83 ± 0.45	3.7 ± 0.60	3.73 ± 0.65
ZSB-22*	ZnO	4.10 ± 0.30	3.93 ± 0.31	3.37 ± 0.32
	$ZnCO_3$	4.63 ± 0.47	4.20 ± 0.36	3.47 ± 0.12
	$Zn(PO_3)_4$	4.03 ± 0.31	3.97 ± 0.25	3.63 ± 0.55
ZSB-23	ZnO	6.10 ± 0.30	5.80 ± 0.40	4.13 ± 0.29
	$ZnCO_3$	4.67 ± 0.15	4.00 ± 0.26	4.07 ± 0.15
	$Zn(PO_3)_4$	4.63 ± 0.55	4.43 ± 0.61	3.97 ± 0.31
SEd			0.33665	
CD ($P < 0.05$)			0.66882	

Values are mean ± SD of three samples in each group.

Table 5. Production of indole-3-acetic acid (IAA) by zinc solubilizing bacteria

Treatment	IAA (mg/l)
Control	0
ZSB-1+ZnO	16.97 ± 0.25
ZSB-1 alone	7.00 ± 0.50
ZSB-10+ZnO	6.93 ± 0.12
ZSB-10 alone	5.60 ± 0.56
ZSB-13+ZnO	9.00 ± 0.40
ZSB-13 alone	6.53 ± 0.35
ZSB-22+ZnO*	23.93 ± 0.40
ZSB-22 alone	16.10 ± 0.36
ZSB-23+ZnO	6.33 ± 0.57
ZSB-23 alone	6.10 ± 0.46
SEd	0.0923
CD ($P < 0.05$)	0.2736

Values are mean ± SD of three samples in each group.

other isolates. Similar observations were also made by several other workers[10,34,35], who reported that gluconic acid is the major organic acid in the solubilization of insoluble minerals. Hence the mineral solubilizing ability of the organisms may be due to the secretion of gluconic acid.

Zinc forms an important metalloprotein which is responsible for the synthesis of tryptophan, which in turn acts as a precursor for the production of IAA. Efficiencies of the best isolates on the production of IAA in the presence and absence of ZnO in the Bunt and Rovira liquid medium supplemented with 0.1% of tryptophan were estimated. The results reveal that all the isolates produce IAA in the medium supplemented with tryptophan, and that there is further enhancement in IAA production by the isolates due to the addition of Zn source (ZnO). This may be due to the induction of high Zn solubilizing efficiency of the isolates, which results in the stimulation of IAA synthesis. Among the five isolates, *P. aeruginosa* (ZSB-22) was found to produce more IAA (23.93 mg/l) in the presence of Zn than in its absence compared to the other isolates (Table 5).

Conclusion

There is plenty of zinc in the soil to support crop growth, but the crops exhibit zinc deficiency due to the presence of unavailable fractions of zinc. Exogenous application of zinc in the form of fertilizers also becomes unconventional because of the transformation into unavailable fractions soon after application and accumulation in the soil. Thus identification of an elite strain capable of transforming unavailable forms of Zn into available forms will be an alternative tool to alleviate zinc deficiency in plants. The above experimental study reveals that *P. aeruginosa* (ZSB-22) has such a potential to solubilize the insoluble forms of zinc and can be used as a bioinoculant to overcome Zn deficiency in plants.

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