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Research Article

ISOLATION AND CHARACTERIZATION OF ZINC SOLUBILIZING BACTERIA FROM SOILS OF THOOTHUKUDI DISTRICT

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Abstract- Zinc is an important micronutrient and its adequate supply is considered indispensable for growth, development and normal functioning of plants. Interaction between soil microbes and minerals play a major role in environment cycling process, which leads to the mobilization of nutrient from soil component into available forms for biological uptake which enhances plant growth and yield. In this study ZSB was isolated from various crops rhizosphere soils from different regions of Thoothukudi district. The isolates were characterized biochemically and screened based on solubilisation potential of zinc. Among the bacterial isolates, ZSB 3 was found to be the best strain that showed maximum zinc solubilization potential.

Keywords- *Zinc solubilisation, Zinc solubilizing bacteria (ZSB), Isolation*

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Introduction

Globally micronutrient deficiency is considered as a limiting factor for crop production. Among the micronutrients Zn and its deficiency is considered to be the most severe abiotic stress in Asian countries and many parts of the Europe [1]. It has been estimated that more than 49 % per cent of Indian soils are zinc deficient and is predominant in the semi-arid tropical soils [2]. According to the soil survey on micronutrient, Zn has been reported to be deficient in all the districts of Tamil Nadu. The role of zinc in plant growth, yield and quality was well recognized. Zinc is the most relevant micronutrient to major enzymes present in biological systems [3] and also needed for structural integrity of proteins. In India major cause of increased zinc deficiency is the adoption of intensive cultivation, unbalanced nutrient application and low organic matter. However, Zn deficiency in agricultural lands has not only affects crop yield, but also shows its effect in nutritional quality and health problem in human biological system [4]. Many studies has been recommended the application of zinc as zinc sulphate through soil and foliar methods. The applied Zn fertilizers have been converted to unavailable forms by precipitation of carbonates, oxides, phosphates etc. It is vital to look for alternative to zinc fertilizer considering the huge cost of these fertilizers. Hence feasible bacterial based approach will solubilize these fixed forms of Zn to liable forms of Zn that enhances the Zn availability and subsequent uptake by plants [5]. The major objective of this study was to isolate bacteria from zinc deficient soils and to characterizer and screen the isolates for Zn solubilisation potential and to study the effect of efficient Zinc solubilizing bacterial culture in detail.

Materials and Methods

Location

The present investigation was carried out at the Agricultural Microbiology Laboratory, Department of Soil Science & Agricultural Chemistry, Agricultural College and Research Institute, Killikulam, 628 252, Tamil Nadu, India.

Isolation of zinc solubilizing bacteria

Collection of samples

Soil samples were gathered from Zn deficient areas of the district of Thoothukudi *viz*., Ottapidaram, Pottalurani, Eppothum Vendran, Thalavaipuram, Thattaparai, Vallinayakipuram, Keezhamudiman, Meentachipuram and Kattamaikanpatti. The soil samples were collected from the following plants such as Palm, Fodder sorghum, Banana, Senna, Sorghum, Indian abutilon, Noni and Celosia.

Isolation of Bacteria

Zinc Solubiliing Bacteria (ZSB) has been isolated from soil samples using serial dilution and plating methods. Bunt & Rovira agar medium was used to isolate ZSB. The plates were incubated at 28±2ºC. After the incubation period of 24-48 hours zinc solubilizing bacterial colonies were counted. The counts were expressed in colony forming units (cfu) per gram of the soil.

Maintenance of isolates

The pure cultures isolates of bacteria were maintained in Bunt & Rovira agar slants at 4°C and stored at 20°C in 30 % glycerol for further studies.

Morphological and biochemical characterization of the zinc solubilising bacterial isolates

Colony morphology

The bacterial isolates were observed for the morphological characteristics.

Gram staining

Thin smear of bacterial culture was made on glass slide are air dried, then smear was covered with crystal violet for 30 seconds, later slides are washed with distilled water. Again smear was covered with grams iodine solution for a minute and are washed with 95% ethyl alcohol followed by distilled water. Once again slide is wetted with Safranin for 30 seconds, which is washed with distilled water and blot dried. Later the slide is observed under the microscope.

Table-1 *Colony morphology of Zinc solubilizing bacterial isolates*

Isolates	Gram staining	Colony shape	Colony size	Colony margin	Pigment	Elevation	Polysaccharide production
ZSB 1	Negative	Circular	Moderate	Undulate	White	Flat	-ve
ZSB 2	Negative	Circular	Moderate	Undulate	White	Flat	-ve
ZSB 3	Negative	Circular	Small	Sharply defined	Dull white	Flat	+ve
ZSB 4	Negative	Circular	Moderate	Undulate	White	Flat	-ve
ZSB 5	Negative	Circular	Small	Sharply defined	White	Flat	+ve
ZSB 6	Negative	Circular	Moderate	Sharply defined	White	Convex	+ve
ZSB 7	Negative	Circular	Moderate	Undulate	White	Flat	-ve
ZSB 8	Negative	Circular	Moderate	Undulate	White	Flat	-ve

Biochemical characterization
IMVIC test (Indole production, MR, VP, Citrate utilization)

Indole production test
In peptone water, bacterial isolates were inoculated and incubated for three days. The reagent of Kovack was added after incubation and the pink ring formation in the upper layer of the broth was observed [6].

Methyl red test
In the broth, bacterial isolates were inoculated and three days incubated. Methyl red indicator was added after incubation and the color change of methyl red is observed [8].

Voges proskauer test
In the broth, bacterial isolates were inoculated and three days incubated. Twelve drops of VP reagent -1 (Napthol solution) and 2-3 drops of VP reagent- II (40 percent KOH) were added after incubation and observed for colour change [6].

Citrate utilisation test
On Simon citrate agar, bacterial isolates have been streaked and incubated for three days. Change in colour after incubation was observed [6].

Ammonia production
In order to determine ammonia production, bacterial isolates were inoculated in peptone water. Then for 5 days test tubes were incubated and observed for colour change.

Enzyme activity
Amylase test
On starch agar, bacterial isolates were streaked and incubated at 30±1°C for 48 hours. Petri plates were flooded and drained with Lugol's iodine solution for 30 sec after the incubation period [7].

Catalase test
The bacterial isolates were streaked on the agar plates of Bunt & Rovira and incubated at 30±1°C for 48 hours. After incubation, a few drops of 30 percent hydrogen peroxide (H2O2) were added to the culture grown plates and observed for the appearance of effervescence [8].

Mineral solubilisation
Phosphate solubilisation
The bacterial isolates phosphate solubilisation was determined by streaking the isolates on the medium of Pikovskaya. Then plates have been incubated for 3 days at 30±1°C. Clear halo area around the colony was noted [9].

Potassium solubilisation
Spot inoculation of isolates on the Alexandro medium determined the potassium solubilisation of the bacterial isolates. Plate was incubated for 24-48 hours at 30±1°C [10].

Antimicrobial activity
HCN Production
The bacterial isolates were streaked on Bunt & Rovira agar medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed inside the lid of each Petriplate. The plates were then sealed air-tight with Parafilm and incubated at 30±1°C for 48 h. A change in colour of the filter paper from deep yellow to reddish-brown indicates the production of Hydrogen cyanide (HCN) [11].

Qualitative estimation of zinc solubilizing potential
All eight bacterial isolates were screened for solubilisation index in the qualitative research using the Modified Bunt and Rovira agar medium comprising 0.1% of ZnO as an insoluble Zn source. Plates for 48h were incubated at 30ºC. To calculate the Zn solubilisation index, the diameter of clear zone and colony growth was measured.

$$Solubilization\ index\ (SI) = \frac{Colony\ diameter\ +\ Halozone\ diameter}{Colony\ diameter}$$

Data analysis
Data obtained from each parameter are mean ± SD were done in triplicate and each data were subjected to one way analysis of variance (ANOVA) with least significant difference level at ($p<0.05$).

Results
Morphological characterization of isolated bacterial strains
All the isolated bacterial strains are observed for morphology and based on cell colony shape, size, colony margin, pigment, elevation and polysaccharide production the results were tabulated in [Table-1]

Gram staining
Gram staining was performed for the bacterial isolates. All the eight bacterial isolates were found to be gram negative [Table-1]

Biochemical tests
The biochemical tests were conducted in accordance with Sherman & Cappuchino (2007), and the findings of the IMVIC test are as follows [Table-3]

Indole production test
Kovac's reagent was used to treat the inoculated test tubes. In strains found to be positive, a red colored ring formation was observed.

MR test
Methyl red indicator is used for treating test tubes with bacterial cultures. The formation of red color indicates positive and negative yellow remains.

VP test
In the test tubes, bacterial cultures were inoculated and added with reagents from Barritt and the color change was noted and the outcomes were reported.

Citrate utilization test
Inoculation of bacterial cultures on Simmon's citrate agar medium and 37 degree Celsius incubation, the medium's green colour will turn out to be Prussian blue in ZSB (1), ZSB (2), ZSB (5), ZSB (7) and ZSB (8) strains.

Qualitative analysis of Amylase, catalase, HCN production and mineral solubilisation
After the period of incubation, the plates with bacterial strains are flooded with.

Lugol's solution, clear zone was observed around the strains indicates positive and blue in colour shows negative starch hydrolysis. [Table-4]. Effervescence on bacterial isolates when 30% hydrogen peroxide is dropped, that indicates positive [Table-4]. After 2 days of incubation culture grown plates turns filter paper from deep yellow to reddish-brown which, indicates positive [Table-4]. P and K solubilisation is observed when halo zone formation around the isolates, indicates positive in nature [Table-4].

Table-2 *Zinc solubilisation potential of Zinc solubilizing bacterial isolates*

Isolates	Zone diameter (mm)		Solubilisation index
	Solubilisation zone	Culture diameter	
ZSB 1	8.5 ± 0.5	5.33 ± 0.7	2.6 ± 0.19
ZSB 2	9.16 ± 0.28	7 ± 0.7	2.3 ± 0.064
ZSB 3	21 ± 1.73	5.83 ± 0.35	4.66 ± 0.74
ZSB 4	12.16 ± 1.04	8.83 ± 0.35	2.37 ± 0.01
ZSB 5	9.33 ± 0.57	3.66 ± 0.57	3.58 ± 0.38
ZSB 6	14.3 ± 1.52	7 ± 0.707	3.05 ± 0.092
ZSB 7	6.33 ± 0.288	4.66 ± 0.57	2.36 ± 0.11
ZSB 8	15.6 ± 2.08	6.33 ± 1.41	3.51 ± 0.27

Table-3 *Biochemical tests for ZSB isolates*

Isolates	1	2	3	4	5	6
ZSB 1	+	-	-	+	+	+
ZSB 2	+	-	-	+	+	+
ZSB 3	-	-	-	-	+	+
ZSB 4	+	+	-	-	+	+
ZSB 5	+	+	-	+	+	+
ZSB 6	-	+	-	-	+	+
ZSB 7	+	+	-	+	+	+
ZSB 8	+	+	-	+	+	+

(+) -Positive, (-) -Negative, 1-Indole test, 2- MR test, 3- VP test, 4-citrate utilization, 5- N free Malic acid, 6- NH3 production

Table-4 *Qualitative analysis of amylase, catalase, HCN production, mineral solubilisation*

Isolates	1	2	3	4	5
ZSB 1	+	+	-	-	-
ZSB 2	+	+	-	-	-
ZSB 3	+	+	-	-	-
ZSB 4	+	+	-	-	-
ZSB 5	+	+	-	-	+
ZSB 6	-	+	-	+	+
ZSB 7	+	+	-	-	+
ZSB 8	+	+	-	-	+

(+) - Positive, (-) - Negative, 1- Amylase, 2- Catalase, 3- HCN production, 4- Potassium solubilisation, 5- Phosphorus solubilisation

Qualitative estimation of zinc solubilizing potential

In this study, the bacterial strains were tested using plate assay. The bacterial strains were inoculated in the Bunt & Rovira agar medium amended with ZnO of Zn at 0.1%. The solubilisation index of the isolates was calculated by measuring the diameter of the colony growth and the solubilisation zone. Zinc solubilizing potential varied with different zinc solubilizing isolates [Table -2]. The isolated zinc solubilizing bacterial isolates were screened for solubilisation of insoluble zinc oxide (ZnO) as a source of zinc. The solubilisation zone is ranged from 6.33mm to 21mm, where higher solubilisation was found in strain ZSB 3 of 21 ± 1.73mm and least Solubilisation was 6.33 ± 0.288mm for ZSB 7. Among all the bacterial isolates, strain ZSB 3 of 4.66 ± 0.74 was recorded higher solubilisation index followed by ZSB 5 of 3.58 ± 0.38. The data indicated that all bacterial strains are capable of solubilizing ZnO. However, ZSB 3 recorded significantly higher solubilisation. Among all the isolates obtained, the bacterial strain ZSB 3 solubilized more zinc oxide in plate assay. Here by we can conclude that isolates can used as a good Zinc solubilizer for sustainable solution for Zinc uptake in plant nutrition and production.

Discussion

The aim of this study was to isolate and characterize the Zinc solubilizing bacterial species that could be used as a potential Zn solubilizer. A total of eight bacterial strains were isolated from different regions of Thoothukudi district. Similarly, Vandana Nandal and Manu Solanki [12] isolated a total of 10 zinc solubilizing bacterial isolates spotted on BR agar medium plates by serial dilution, which is being incubated at 30 ± 2ºc for 2 days. From the soils of Thoothukudi, eight zinc solubilizing bacterial cultures been isolated. Same kind of investigation is carried out by Kritika *et al.*[13] who reported ZSB-14 (Enterobacter cloacae) a strain isolated from rhizosphere of rice is capable of solubilizing insoluble zinc sources such as ZnO, ZnCO3 and Zn3(PO4)2. In total of 8 bacterial isolates, 7 isolates hydrolyses starch, when it is screened through qualitative analysis i.e., amylase starch hydrolysis test. Similarly, Alariya *et al.* [14] reported that seven bacterial isolates are assessed for amylase producing bacteria and after screening by starch iodine test, only 4 bacterial strains showed amylase activity. Eight bacterial isolates were found to outperform Zn solubilisation in vitro, with variable degrees of solubilisation as indicated by variation in diameter of halo zone formation. This is corroborated with earlier reports differ in their ability to solubilize. Saravanan *et al.* [15] identified the bacterial isolates Bacillus sp. (ZSB-O-1) isolated from zinc ore sphalerite and pseudomonas sp. (ZSB-S-2 and ZSB-S-4) isolated from paddy soil which was efficient zinc solubilizing isolates exhibited highest zinc solubilizing potential with clear zone. The Solubilizing efficiency will be different from one bacterium to another, such results were observed by Ramesh *et al.*, [16] who indicated that zinc solubilizing ability of B. aryabhattai is higher. Among eight ZSB cultures, ZSB 3 isolate solubilizes halo zone of clearance with mean of 21 ± 1.73, followed by other isolates and with least of minimum zone of clearance 6.3 ± 0.28 mm in Zinc oxide. Similarly, Dinesh *et al.* observed ZnSB2 solubilizes maximum zone of clearance with 14.6mm in ZnO as a Zn source. Gandhi and Muralidharan, [17] obtained 143 zinc solubilizing bacterial colonies and screened to 15 isolates based on higher zinc solubilisation potential, to that the isolate AGM-3 showed significantly high zinc solubilizing halo zone of 13.21 mm and 10.71 mm followed by isolate AGM-9 forms halo zone of 11.74mm and 7.90mm. The bacterial isolates are good plant growth promoters which results better solubilisation capacity that gains insight of nutrient availability to plant that nourishes nutrition. Archana *et al.* [18] reported that active bacteria in such solubilisation process is an effective mechanism to enhance labile form of nutrient elements in soil.

Conclusion

Zinc solubilizing bacteria was isolated from rhizosphere region of plants from different regions of Thoothukudi district of Tamil Nadu, where it is further characterised and studied for their Zn solubilisation potential. Out of many bacterial isolates obtained, ZSB 3 was found to be the most potent strain capable of higher zinc solubilisation and organic acids production. Further studies were done to study the potential Zn solubilizing isolate against various factors that determines the availability of zinc in soil.

Application of research: In zinc deficient state, the availability of Zn persisted through cost-effective zinc fertilization, in order to minimize such situation, ZSB isolates can be used as useful solubilizer, that mobilizes nutrients and increases the supply of micronutrients to plant and its yield. This could assist famers to mitigate zinc deficient condition.

Research Category: Agriculture Microbiology

Abbreviations:

ZSB - Zinc Solubilizing Bacteria
mm - millimeter
BR - Bunt and Rovira medium
% - percentage
SI - Solubilisation Index, MR- Methyl Red, VP- Voges-Proskuer

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Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Soil samples were collected from Ottapidaram, Pottalurani, Eppothum Vendran, Thalavaipuram, Thattaparai, Vallinayakipuram, Keezhamudiman, Meentachipuram and Kattamaikanpatti regions of Thoothukudi district.

Cultivar / Variety name: Nil

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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