Research Article

Isolation and Characterization of Zinc Solubilizing Bacteria from Rice Rhizosphere of Kashmir-J&K, India

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Abstract

A soil study was conducted in the year, 2019 to isolate and evaluate the solubilizing potential of ZnSBs isolated from the rice rhizosphere soil of the Kashmir region of Jammu & Kashmir, India. Thirty (30) samples were collected from the ten (10) districts of the Kashmir region with three samples from each district. The ZnSB isolates were isolated (90) using modified Pikovskaya agar medium (amended with 0.1% of various zinc sources viz; ZnO, $ZnCO_3$, $ZnSO_4$, $Zn(PO_3)_4$ and Chelated-Zn). These Isolates were screened (qualitatively and quantitatively), based on their zinc solubilisation efficiency. The isolates (KZSB16, KZSB21 KZSB13, KZSB18 & KZSB11) produced high clear halo zones. KZSB16 exhibited the highest solubilization zone on zinc oxide with the diameter (13.19 mm), zinc phosphate (9.89 mm), and zinc carbonate (7.93 mm) followed by zinc sulphate and Chelated-Zn during plate assay. Similarly, In broth assay, KZSB16 showed the highest solubilization range in zinc oxide (33.27 mg Zn/L), Zinc phosphate (31.02 mg Zn/L) and zinc carbonate (29.39 mg Zn/L) followed by zinc sulphate, Chelated-Zn, respectively. The isolates showed positive results for the catalase test, methyl red test and citrate utilization test and negative results for the oxidase test. The KZSB16 isolate, found to be the most effective isolate owing to its high solubilization capacity of zinc, exhibited in both qualitative and quantitative assays. The isolate can be developed as a bio-fertilizer to increase the availability of zinc in soil for better growth and development of rice crops.

Keywords: KZSB, Rhizosphere, Chelated-Zn, Pikovskaya, Methyl red, bio-fertilizer

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Introduction

Plants require a variety of nutrients viz; macro and micronutrients for their growth and development. Zinc is one of the most important micronutrients required for normal healthy plant growth and reproduction. Zinc deficiency in plants causes the development of various types of abnormalities such as stunted growth, chlorosis, smaller leaves and spikelet sterility. Zinc deficiency can also harm harvested product quality; plant becomes susceptible to injury from high light or temperature intensity, as well as infection by fungal diseases [1, 2]. It undergoes complex changes in soil and precipitates with other elements, which are greatly influenced by pH and microflora [3] which affects their availability to roots for absorption. Zinc is required at a very low concentration and if the amount available is insufficient plants and animals will suffer from physiological stress caused by the malfunction of several enzyme systems and other metabolic functions [4]. Zinc solubilizing bacteria (ZnSBs) can solubilize zinc from both the organic and inorganic pools of total soil zinc, increasing availability of zinc to plants. Fungi have also been extensively studied for their ability to solubilize insoluble zinc compounds in vitro and in vivo. However, only a few *Acinetobacter, Bacillus, Gluconacetobacter, and Pseudomonas* bacterial species have been reported previously by [5]. Zinc is essential for the growth and development of rice. In recent years, the zinc solubilizing bacteria (ZnSBs) importance has grown extensively for improving the availability of Zn to host plants for increasing the crop growth, yield and quality without affecting the environment. Alternatively, roots which are associated with several

microorganisms can boost plant growth and productivity [6], by increasing the mineral nutrient availability in the soil with low mobility such as P, Zn and Cu [7]. The various bacterial strains that have been reported to exhibit solubilisation of zinc on a laboratory scale are *Pseudomonas aeruginosa* [8], *Gluconacetobacter diazotrophicus* [9], *Bacillus spp., Pseudomonas striata, Pseudomonas fluorescence, Burkholderia cenocepacia* [10], *Serratia liquefaciens, Serratia marcescens and Bacillus thuringiensis* [11].

Materials and Methods

Collection of soil samples

Rice rhizosphere samples (90) were collected from ten (10) districts of the Kashmir region **Table 1**. The samples were collected in polythene bags and transported to the laboratory right away. The soil samples were air-dried in a room, crushed in pestle and mortar and sieved through a 2 mm sieve before being stored at 4°C for isolation of ZnSBs.

Table 1 List of ZnSB isolates obtained from rice rhizosphere fields of the Kashmir region

S. No	Total no. of isolates	Effective Isolates	Locations
1	12	4	Baramullah
2	08	2	Bandipora
3	10	3	Kupwara
4	11	3	Anantnag
5	06	2	Srinagar
6	09	2	Budgam
7	07	2	Shopian
8	13	4	Kulgam
9	08	3	Pulwama
10	06	2	Ganderbal
Total	90	27	10

Isolation and Enumeration of Zn solubilizing bacteria

This was accomplished through the use of a serial dilution plate count method in which 1.0 g of soil transferred to a dilution tube containing 9.0 mL sterilised water (10^{-1}), shaken for 10 minutes. After that, dilution tubes were left alone for 30 minutes to allow the suspension to settle in the tube. Following the same for other tubes as well, 1.0 mL of the bacterial suspension from the 10^{-1} dilution to another dilution tube containing 9.0 mL of sterilised water (10^{-2}). This was repeated up to a dilution (10^{-6}) before being pour-plated on Nutrient Agar (NA), incubated for 48 hours at 28 °C. The bacterial population [colony forming units (CFU/gm)] was estimated using (10^{-4}-10^{-6}) dilution, and bacterial colonies were sub-cultured individually on Nutrient Agar [12].

The zinc solubilizing bacteria (ZnSBs) present in soil samples were enumerated by the plate count method. The addition of 0.1% of modified Bunt and Rovira agar medium with different zinc sources viz; (ZnO, $ZnCO_3$, $ZnSO_4$, $Zn(PO_3)_4$ and Chelated-Zn) aided in the bacterial enumeration. The agar plates were incubated in an incubator for two days at 30°C for colony development. The colonies developed after two days with the clearing halo zone were thought to be the zinc solubilizers, were counted, isolated, and purified. The twenty seven (27) most prevalent, morphologically and biochemically distinct bacterial colonies were chosen for qualitative and quantitative analysis and were labelled as KZSB-1, KZSB-2 … KZSB-27.

Qualitative estimation

All the twenty seven (27) isolates of ZnSBs were tested for solubilization efficiency using plate assay on a modified Bunt and Rovira agar medium amended with 0.1% ZnO, $ZnCO_3$, $Zn(PO_3)_4$, $ZnPO_4$ and Chelated Zn as zinc sources in the qualitative study. After that, plates were incubated at 30 ^{0}C for 48 hours. The zinc solubilization potential was determined by measuring the diameter of the clear halo zones and colony growth.

Quantitative estimation

The five (5) bacterial isolates were grown in 50 ml of Bunt and Rovira broth supplemented with 0.1% ZnO, $ZnCO_3$, $Zn(PO_3)_4$, $ZnPO_4$ and Chelated-Zn in 100 ml Erlenmeyer flasks to determine the amount of solubilized zinc in the flask and un inoculated controls were also kept for comparison. The treatments were replicated thrice. For the

estimation of soluble Zn, the bacterial cultures were removed after the sixth and eighth days of incubation at 30 ^{0}C. The cultures were then centrifuged at 15,000 rpm for 20 minutes, there after supernatant was passed through a 0.2 m membrane filter to obtain filtrates containing only metal soluble forms. The samples fed into an atomic absorption spectrometer (AAS) to determine the available zinc concentration. The minimum detection limit of instrument for zinc is 0.2 mg/kg and linearity over the concentration range is 0.9952 (fit factor).

Morphological and biochemical characterization

Morphological characterization

All of the isolated zinc-solubilizing isolates were tested for their purity before colony morphology examination. The cell shape and gram reaction of the isolates were also recorded by standard procedures provided by [13].

Biochemical characterization of zinc solubilizing bacterial isolates

Catalase test

The catalase test was carried out in bacterial colonies to investigate the presence of catalase enzyme. Pure isolates (24 hours old) were placed on glass slides and one drop of H_2O_2 (30%) was poured on it. The appearance of a gas bubble on the slide indicated, presence of catalase enzyme [14].

Oxidase test

The overnight isolate's, spotted on plates already poured with Trypticase Soy Agar (TSA) and were incubated for 24 hrs. at 30 ^{0}C. After incubation 2-3 drops of N, N, N', N'-tetramethyl-p-phenylenediamine Dihydrochloride (Wurster's reagent) were applied on to the surface of bacteria. The isolates that changed colour with reagent to maroon were identified as oxidase positive [15].

Methyl red test

The test culture was inoculated into sterilised glucose-phosphate broth tubes incubated at 30 ^{0}C for 48 hours. Five (5) drops of methyl red indicator were added after incubation and shaken into each tube. Red colour production was considered positive for this test, production of yellow colour was considered negative [16].

Citrate utilization

The bacterial isolates were streaked on to Simmon's citrate agar slants and incubated for 24 hours at 30 ^{0}C. Changing of colour from green to blue indicates a positive reaction to citrate utilisation [17].

Results and Discussions

Isolation and biochemical characterization of zinc solubilizing bacteria (ZnSB)

Using a liquid salt medium amended with sources of zinc viz; ZnO, $ZnCO_3$, $Zn(PO_3)_4$, $ZnSO_4$ and Chelated-Zn, ninety (90) zinc solubilizing isolates were obtained. On liquid salt agar medium, five (5) ZnSB isolates with a high solubilisation halo zone were chosen. Biochemical tests on bacterial isolates (catalase, methyl red, citrate utilisation and oxidase test) revealed positive results for catalase, methyl red, and citrate utilisation, but negative results for the oxidase test **Table 2**. These findings are in line with the results of [18] who isolated the bacterial spp. of *Pseudomonas* from the fifteen rhizosphere samples of various regions.

Zinc solubilization efficiency of ZSB

In both plate and broth assays **Table 3** all of the isolated ZnSB were found to be effective in solubilizing zinc sources (ZnO, $ZnCO_3$, $Zn(PO_3)_4$, $ZnSO_4$, and Chelated-Zn) (**Figure 1**). KZSB16, KZSB21, KZSB13, KZSB18, and KZSB11 isolates produced a highly clear halo zone of zinc solubilization. During plate assay, KZSB16 exhibited the highest solubilization on zinc oxide with a diameter of (13.19 mm), zinc phosphate with a diameter of (9.89mm), zinc carbonate with a diameter of (7.93 mm) etc. In broth assay, KZSB16 showed a high solubilization range in zinc oxide, zinc phosphate and zinc carbonate 33.27 mg Zn/L, 31.02 mg Zn/L and 29.39 Zn/L etc. respectively Figure 1. Similar results were observed in the discovery of [19]. They found the highest levels of halo zone in amended medium of zinc oxide and zinc carbonate inoculated with *Bacillus spp.*, [20] discovered that the solubilization potentiality of *Pseudomonas spp.* was higher in zinc oxide than in zinc sulphate supplemented medium. Zinc can be dissolved

through a variety of mechanisms, including the excretion of the metabolites viz; organic acid production, proton extrusion and production of chelating agents [21]

Table 2 Morphological & Biochemical characteristics of effective ZnSB isolates

S.No.	Morphological & Biochemical characteristics	ZSB Isolates				
		KZSB18	KZSB11	KZSB21	KZSB16	KZSB13
1	Halo zone diameter ZnO (mm)	7.54	6.62	9.89	13.19	7.93
2	Citrate utilization test	+	+	+	+	+
3	Methyl red test	+	+	+	+	+
4	Catalase test	+	+	+	+	+
5	Oxidase test	-	-	-	-	-

Table 3 Effect of zinc solubilizing organisms on the amount of solubilized zinc present in the filtrates (mg/L)

Isolate	Amount of zinc present in the cultural filtrates (mg/L)		
	Zn source in the medium	6 DAI	8 DAI
KZSB18	ZnO	28.02 ± 0.29^{a}	29.18 ± 0.47^{b}
	$ZnCO_3$	20.84 ± 0.15^{abcd}	21.11 ± 0.38^{cd}
	$Zn(PO_3)_4$	18.11 ± 0.24^{ac}	19.7 ± 0.30^{cde}
	$ZnSO_4$	7.75 ± 0.58^{ab}	7.75 ± 0.29^{d}
	Chelated Zn	3.15 ± 0.24^{c}	3.15 ± 0.21^{c}
KZSB11	ZnO	24.71 ± 0.08^{abc}	25.54 ± 0.56^{ac}
	$ZnCO_3$	18.49 ± 0.58^{ac}	19.05 ± 4.67^{cde}
	$Zn(PO_3)_4$	17.10 ± 0.57^{ac}	18.35 ± 0.77^{cde}
	$ZnSO_4$	7.54 ± 1.34^{ab}	7.57 ± 2.41^{d}
	Chelated Zn	3.11 ± 0.40^{c}	3.11 ± 2.98^{c}
KZSB21	ZnO	29.2 ± 2.70^{e}	31.02 ± 0.19^{i}
	$ZnCO_3$	23.79 ± 0.15^{c}	24.38 ± 0.19^{ac}
	$Zn(PO_3)_4$	20.62 ± 1.15^{abcd}	21.74 ± 1.62^{cd}
	$ZnSO_4$	8.12 ± 1.72^{ab}	8.17 ± 0.57^{d}
	Chelated Zn	4.45 ± 0.50^{c}	4.46 ± 0.73^{c}
KZSB16	ZnO	32.25 ± 2.82^{h}	33.27 ± 1.53^{h}
	$ZnCO_3$	25.8 ± 0.38^{abc}	26.89 ± 0.77^{abc}
	$Zn(PO_3)_4$	22.26 ± 0.65^{i}	24.11 ± 1.15^{ac}
	$ZnSO_4$	9.75 ± 0.83^{cd}	9.81 ± 1.70^{abcd}
	Chelated Zn	4.78 ± 0.57^{c}	4.81 ± 0.40^{c}
KZSB13	ZnO	28.56 ± 0.19^{a}	29.39 ± 1.34^{b}
	$ZnCO_3$	21.73 ± 0.82^{i}	22.91 ± 0.50^{cd}
	$Zn(PO_3)_4$	19.15 ± 1.36^{abcd}	21.2 ± 1.58^{cd}
	$ZnSO_4$	7.89 ± 0.51^{ab}	7.90 ± 0.65^{cde}
	Chelated Zn	3.56 ± 0.19^{c}	3.56 ± 0.82^{c}

Data are represented as mean± standard error (n=5), Mean values do not differ significantly in each column with the same superscript(s) by Duncan post hoc multiple comparison tests ($P\leq 0.05$).
**DAI, Days after incubation*
T_1-Control, T_2-ZnSB, T_3- $ZnSO_4$ T_4-$ZnCO_3$, T_5-$Zn(PO_3)_4$,T_6-ZnO, T_7-Ch-Zn, T_8-$ZnSO_4$+KZSB16, T_9-$ZnCO_3$+KZSB16, T_{10}-$Zn(PO_3)_4$+KZNSB16, T_{11}-ZnO+KZNSB16 T_{12}-Ch-Zn+KZNSB16

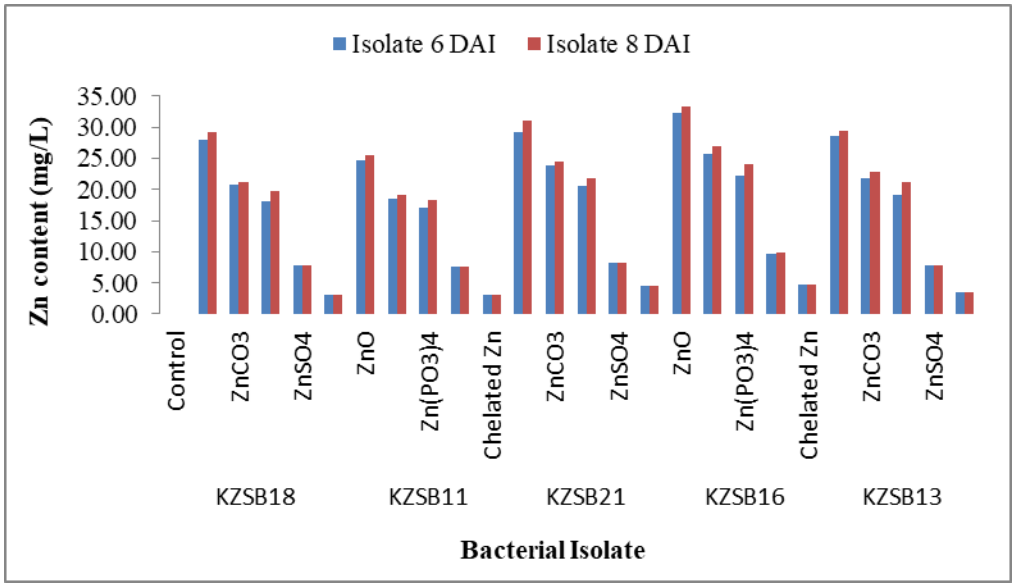

Figure 1 Effect of zinc solubilizing organisms on the amount of solubilized Zinc present in the filtrates (mg/L)

Conclusion

Thus, it was concluded that the isolate, KZSB16 either alone or along with an insoluble source of zinc showed better performance on growth and yield of rice compared to control and can help in minimizing the dependence on chemical zinc fertilizer in rice cultivation.

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