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Isolation and Characterization of Zinc Solubilizing Bacteria from Rhizosphere Soils of Paddy Grown in Tungabhadra Command Area

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A B S T R A C T

In our present study, we have isolated zinc solubilizing bacteria from rhizospheric soils of rice growing area surrounding Raichur and Koppal districts of Karnataka, India. Around 40 zinc solubilizing bacteria were isolated using TRIS-minimal agar medium (TMA) supplemented with 0.1 % ZnO. All the isolates were named after zinc solubilization such as MZSB-1 to MZSB-40 respectively. Under *in vitro* conditions, all the bacteria were able to grow in the TMA plates and solubilize Zinc. Among all the isolates, MZSB8 and MZSB6 showed a maximum zone of solubilization of 21 mm and 19 mm respectively. Based on the morphological and biochemical characterization the isolates were identified as *Pseudomonas* and *Bacillus sp.*

Introduction

Zinc is one of the important micronutrients which plays a vital role in plant growth and development, a component of enzymes that drive the metabolic reactions, component of the active catalytic center of the enzyme carbonic anhydrase. It puts a great effect on basic plant life processes such as N_2 metabolism and quality of protein; photosynthesis and chlorophyll synthesis, resistance to abiotic and biotic stresses and protection against oxidative damage (Potarzycki and Grzebisz, 2009). In rice, Zn deficiency causes multiple symptoms that usually appear 2 to 3 weeks after transplanting rice seedlings; leaves develop brown blotches and streaks that may fuse to cover older leaves entirely, plants remain stunted and in severe cases may die also. Zn deficiency is becoming a serious issue that is causing harm to nearly half of the world's population (Cakmak,

2009). This is possibly due to low Zn content of the crops grown in Zn deficient soils. According to Singh (2009), 48 % of soils in India are afflicted with Zn deficiency with much below the critical level of 1.5 ppm. To avoid these drawbacks, farmers apply Zn in the form of fertilizers like $ZnSO_4$, which in turn converted into different insoluble forms based upon the soil types, soil chemical reactions and becomes totally unavailable in the soil within few days of application (Rattan and Shukla, 1991).

Thus, proficient and efficient techniques to address Zn insufficiency must be formulated. Nowadays, bacterial based methodology was devised to take care of these micronutrient insufficiency issues (Anthoni Raj, 2002).

They play a prevalent role in the solubilization, transport of metals and minerals in the environment. Thus, microorganisms assume a noteworthy job in the change of inaccessible type of metal to accessible structure based on the reactions involved and the products (Lovely, 1991).

The discharge of natural acids has all the earmarks of being the useful metal resistance that chelates the metal particles extracellularly (Li *et al.,* 2008). Zinc deficiency being an important nutrient constraint, any approach to improve Zn uptake and its transport to grains have significant practical relevance.

One possible way is to increase crop productivity as well as food quality without creating environmental issues is by the use of plant growth promontory rhizobacteria (PGPR).

In the present study we aim at the selection of efficient zinc solubilizing bacterial isolates with multiple beneficial traits. Such isolates will increase the bioavailability of Zinc to rice plant.

Materials and Methods

Collection of soil sample

Soil samples were collected with the help of augur upto the depth of 15-20 cm from the rhizosphere of paddy grown in Tungabhadra command region in sterilized polythene bags. The Polythene bags were properly tied; labeled and at most care was taken to avoid contamination. The soil samples were preserved in a refrigerator at $4^{o}C$ for the isolation of zinc solubilizing bacterial isolates.

Physico-chemical analysis of collected soil samples

The soil samples collected from various regions were analyzed for their chemical properties like pH, EC, and organic carbon by following standard procedures mentioned by Piper (1966), Jackson (1973) and wet oxidation method of Walkley and Black (1934), respectively.

Media used for the experiment

TRIS-minimal agar medium containing 0.1 insoluble zinc compound was used for the isolation of zinc solubilizing bacteria. It serves as a selective medium for isolation of zinc solubilizers. Glucose (10.00 g), Zinc oxide (1.00 g), Ammonium sulphate (0.50 g), Potassium chloride (0.20 g), Yeast extract (0.50 g), Ferrous sulphate (0.01 g), Manganese sulphate (0.01 g), Dipotassium hydrogen phosphate (0.25 g), Agar (20.00 g), Double Distilled Water (1000 ml)

Isolation of zinc solubilizing bacteria

Bacteria were isolated from rhizospheric soil samples of paddy by serial dilution followed by agar plating on TRIS-minimal agar media containing 0.1 % insoluble zinc compound (ZnO) (Di Simine *et al.,* 1998).

The soil samples were serially diluted to 10^{-3}, 0.1 ml of an aliquot from diluted sample was spread on the media plates and incubated at room temperature (30±1˚C) for 3 days. The distinct colonies exhibiting clear zones were selected, purified by a four-way streak plate method, and isolates were preserved on nutrient agar slants.

Characterization of isolates

All the selected isolates were examined for the colony morphology, cell shape, and gram reaction as per the standard procedures given by Anonymous (1957) and Barthalomew and Mittewer (1950). The biochemical characterization of the isolates was carried out as per the procedures outlined by Cappuccino and Sherman (1992).

Results and Discussion

Collection of soil samples

Four soil samples from each site were collected up to 15-20 cm deep from the rhizosphere of paddy grown in different parts of the TBP command area in sterilized polythene bags. Rhizosphere contains plenty of useful microbes which supports their growth and survival.

Thus, rhizosphere soil serves every purpose of the microbiologist who works on the isolation of soil microorganisms. The soil samples were stored in the refrigerator at 4°C to arrest the biological activity.

Physico-chemical analysis of soil samples

Zinc solubilization in the soil is a function of various factors including population densities and action of zinc solubilizing microorganisms, zinc bioavailability and soil parameters such as pH, soil moisture availability and temperature. Soil analysis has been extensively carried out in agriculture and horticulture to examine the soil health and provides beneficial information for imposing significant soil and water management strategies to boost crop productivity.

Variability in pH was studied for all the 40 soil samples and it was found to be in the range of a minimum of 5.95 to a maximum of 8.88. The maximum pH was exhibited by MNV-3 sample of Manavi site and the minimum pH was observed in YRG-2 sample of Yeragera site. Electrical conductivity was found to range from a minimum of 0.21dsm^{-1} to a maximum of 0.56 dsm^{-1}. Organic carbon percentage was found to range between 3.18 %and 6.75 %.All the samples were black soils with fine texture.

Isolation of zinc solubilizing bacteria

Forty zinc solubilizing bacterial isolates were isolated from different rhizosphere soils of rice grown in TBP command area. After 2-3 days of incubation at 30 ^{o}C, observed hollow zone around the bacterial colonies which indicates solubilization of inorganic Zinc on TRIS minimal agar plates. The results are supported by Sunitha *et al.,* (2016), Muhammad *et al.,* (2015), Gandhi *et al.,* (2014), Kajal and Pratibha (2014), who isolated zinc solubilizing bacteria from rhizosphere soils of different agricultural crops.

Characterization of zinc solubilizing bacteria

The morphological characterization revealed that the zinc solubilizing bacteria were both gram-negative and positive. The biochemical characterization of forty zinc solubilizing bacterial isolates revealed that all bacterial isolates were found positive for starch hydrolysis, catalase activity, citrate utilization, gas production, and denitrification tests.

Whereas, negative for urease test, methyl-red test, and indole test and variation was observed in H_2S production, gelatine liquefication, Voges-Proskauer test, and casein hydrolase test (Table 1–3).

Table.1 Chemical properties of paddy rhizospheric soil samples collected from Tungabhadra command area

Sl. No.	Sample code	pH	EC (dS m^{-1})	OC (g kg^{-1})
1	MLB- 1	7.85	0.48	5.73
2	MLB-2	7.24	0.41	5.93
3	YRG- 1	7.60	0.43	5.70
4	YRG- 2	5.95	0.21	4.63
5	MNT- 1	6.70	0.37	4.97
6	MNT- 1	7.30	0.41	6.36
7	MNT- 2	7.47	0.42	4.45
8	MNV- 1	7.60	0.46	6.75
9	MNV- 2	7.56	0.45	4.30
10	MNV- 3	8.88	0.56	3.96
11	KLM- 1	7.82	0.48	3.92
12	KLM- 2	7.70	0.45	4.50
13	KLM- 3	8.00	0.51	4.75
14	SRV- 1	7.34	0.41	3.97
15	SRV- 2	7.70	0.46	4.22
16	SRV- 3	7.78	0.47	4.75
17	KVT- 1	7.73	0.46	3.65
18	KVT- 2	7.64	0.45	3.35
19	KVT- 3	7.65	0.46	3.49
20	KLR- 1	7.57	0.45	3.77
21	KLR- 2	7.75	0.46	4.91
22	KLR- 3	8.60	0.54	3.75
23	KPG- 1	7.91	0.48	3.56
24	KPG- 2	7.55	0.37	4.25
25	KPG- 3	8.10	0.51	5.30
26	NMV- 1	8.10	0.52	5.16
27	NMV- 2	8.04	0.49	3.95
28	TNP- 1	7.67	045	3.65
29	TNP- 2	7.70	0.46	3.50
30	TNP- 3	7.47	0.43	3.18
31	TNP- 4	8.02	0.50	4.41
32	SND- 1	7.85	0.47	3.97
33	SND- 2	7.96	0.48	4.00
34	SND- 3	7.82	0.41	4.95
35	GVT- 1	8.52	0.53	3.54
36	GVT- 2	8.12	0.50	3.69
37	GVT- 3	8.21	0.51	5.10
38	GVT- 4	8.03	0.49	3.44
39	SDP- 1	8.23	0.52	4.85
40	SDP- 2	7.98	0.48	4.31

Table.2 Morphological characteristics of Zinc solubilizing isolates isolated from rhizosphere soil of paddy grown in Tungabhadra command area

Sl. No.	Isolate	Morphological characters		Motility
		Colony character	Gram reaction	
1	MZSB1	Creamy white, smooth, small, slimy	-ve, rod	Motile
2	MZSB2	White, small, round	-ve, rod	Motile
3	MZSB3	Creamy white, smooth, circular	-ve, rod	Motile
4	MZSB4	White, small, irregular, slimy	-ve, rod	Motile
5	MZSB5	Yellow, large, irregular	-ve, rod	Motile
6	MZSB6	White, small, round, slimy	-ve, rod	Motile
7	MZSB7	Light yellow, small, round	-ve, rod	Motile
8	MZSB8	White, small, round, slimy	-ve, rod	Motile
9	MZSB9	Yellow, small, irregular	-ve, rod	Motile
10	MZSB10	Creamy white, large, irregular	+ve, rod	Motile
11	MZSB11	White, small, irregular, spreading	-ve, rod	Motile
12	MZSB12	White, large, irregular	+ve, rod	Motile
13	MZSB13	Yellow, large, irregular	-ve, rod	Motile
14	MZSB14	Creamy white, smooth, circular	-ve, rod	Motile
15	MZSB15	White, small, round	-ve, rod	Motile
16	MZSB16	Creamy white, small, slimy	-ve, rod	Motile
17	MZSB17	Dull white, large, irregular	+ve, rod	Motile
18	MZSB18	Yellow, small, irregular	-ve, rod	Motile
19	MZSB19	Light yellow, small, round	-ve, rod	Motile
20	MZSB20	White, small, irregular, slimy	-ve, rod	Motile
21	MZSB21	White, small, round, slimy	-ve, rod	Motile
22	MZSB22	Creamy, dull wrinkled	+ve, rod	Motile
23	MZSB23	Creamy white, small, slimy	-ve, rod	Motile
24	MZSB24	Dull white, irregular, dry	+ve, rod	Motile
25	MZSB25	White, large, irregular umbonate	+ve, rod	Motile
26	MZSB26	Creamy white, large, irregular	-ve, rod	Motile
27	MZSB27	White, large, irregular	+ve, rod	Motile
28	MZSB28	White, small, round, slimy	-ve, rod	Motile
29	MZSB29	Creamy white, large, irregular	+ve, rod	Motile
30	MZSB30	White, small, irregular	-ve, rod	Motile
31	MZSB31	White, large, irregular umbonate	+ve, rod	Motile
32	MZSB32	Dull white, irregular, dry	+ve, rod	Motile
33	MZSB33	Yellow, large, irregular	-ve, rod	Motile
34	MZSB34	Yellow, small, irregular	-ve, rod	Motile
35	MZSB35	Creamy white, small, slimy	-ve, rod	Motile
36	MZSB36	White, small, round, slimy	-ve, rod	Motile
37	MZSB37	Creamy white, large, irregular	+ve, rod	Motile
38	MZSB38	Yellow, large, irregular	-ve, rod	Motile
39	MZSB39	White, small, irregular, slimy	-ve, rod	Motile
40	MZSB40	Creamy white, large, irregular	-ve, rod	Motile

Table.3 Biochemical characteristics of Zinc solubilizing isolates isolated from rhizosphere soil of paddy grown in Tungabhadra command area

Sl. No.	Isolate	Biochemical characterization												Tentative genus
		1	2	3	4	5	6	7	8	9	10	11	12	
1	MZSB1	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
2	MZSB2	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
3	MZSB3	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
4	MZSB4	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
5	MZSB5	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
6	MZSB6	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
7	MZSB7	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
8	MZSB8	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
9	MZSB9	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
10	MZSB10	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
11	MZSB11	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
12	MZSB12	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
13	MZSB13	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
14	MZSB14	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
15	MZSB15	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
16	MZSB16	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
17	MZSB17	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
18	MZSB18	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
19	MZSB19	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
20	MZSB20	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
21	MZSB21	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
22	MZSB22	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
23	MZSB23	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
24	MZSB24	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
25	MZSB25	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
26	MZSB26	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
27	MZSB27	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
28	MZSB28	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
29	MZSB29	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
30	MZSB30	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
31	MZSB31	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
32	MZSB32	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
33	MZSB33	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
34	MZSB34	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
35	MZSB35	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
36	MZSB36	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
37	MZSB37	+	+	-	-	+	+	+	-	+	+	+	+	*Bacillus sp.*
38	MZSB38	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
39	MZSB39	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*
40	MZSB40	+	+	-	-	-	+	+	-	-	+	+	+	*Pseudomonas sp.*

1 - Starch hydrolysis, 2 - Catalase test, 3 - Urease activity, 4 - Methyl red test,
5 -Voges-Proskauer test, 6 - Citrate utilization test, 7 - Denitrification test, 8 - Indole test,
9 - H_2S production, 10 - Casein hydrolysis test, 11 - Gas production, 12 – Gelatin liquefaction

The clear zone around the colony indicates starch degradation due to the production of amylase and in this investigation, there was a clear zone around the colonies after the addition of iodine and reported as positive for the starch hydrolysis. In citrate utilization test, isolates were streaked on Simmon's citrate agar. Change in color from green to blue occurs as bacteria metabolize citrate. The ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue. In gelatin liquefication test, it remains solid below 22^{o}C while the degraded form of gelatin *i.e.* amino acids and peptides remain liquid. Thus, the tubes with liquid form were scored as positive. In the casein hydrolysis test, clear zone around the colony was observed against creamy white background. This is due to the fact that casein imparts white color to the media, which upon degradation by the caseinase enzyme, media loses color and becomes hallow. Thus, colonies with hallow zones were scored as positive. In H_2S production test, the bacterial isolates were inoculated into test tubes containing 5 ml of sterile SIM agar medium, the formation of a black ring in the medium due to conversion of ferrous sulfate to ferrous sulfide was taken as positive for H_2S production. Depending on biochemical tests, the isolates were tentatively identified as *Pseudomonas* and *Bacillus sp.*

Similar results were obtained by many researchers. Bhagobaty and Malik (2008) reported four bacterial isolates belonging to genus *Pseudomonas,* which tested positive for oxidase, negative for indole, RA-3 and RA-20 showed a negative test for methyl red and only RA-5 was found positive for Voges-Prosekeur test. Similarly, Dilfuza (2005) isolated the organisms from the rhizosphere of different crops and identified them as *Pseudomonas* species based on the biochemical characterization. A *Pseudomonas* strain PsA15 showed positive results for Gelatine liquefaction, Citrate utilization, Oxidase, Catalase tests and it showed negative results for Casein hydrolysis and urease tests.

Forty isolates efficient in zinc solubilization under *in vitro* conditions were isolated based on the diameter of hallow zone. Isolates were characterized morphologically and biochemically and tentatively identified as *Pseudomonas* and *Bacillus sp.* They can assist in remediating the lack of Zinc and ensure the soil health and fertility by solubilizing the fixed form of zinc.

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