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Isolation and characterization of zinc solubilizing bacteria from Western Maharashtra soils

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Abstract
The research was carried out at laboratory of plant pathology and Agricultural Microbiology, College of Agriculture, Pune. In the present investigation, efficient zinc solubilizing bacteria isolated from rhizospheric soils of Sangli and Satara districts of Western Maharashtra. Out of 45 samples, 9 bacterial isolates were obtained. Among the isolates, Pal-3 showed the highest efficiency, producing the largest zone of clearance (3.6 cm) on Bunt and Rovira medium. The zinc solubilization index among the isolates ranged from 2.59 to 3.57 while zinc solubilization efficiency varied between 129.41% and 257.14%. These values indicate significant variation in the zinc solubilizing capacity of the bacterial strains. Notably, Pal-3 exhibited both the highest solubilization index and efficiency, In addition to Pal-3, isolates Bor-1 and Pha-5 also showed strong zinc solubilizing ability. These three isolates were identified as the most efficient. Based on their cultural, morphological and biochemical characteristics, Pal-3 and Pha-5 were identified as *Bacillus* species whereas Bor-1 belonged to the *Pseudomonas* genus.

Keywords: Isolation, rhizospheric soil, zone of clearance, zinc solubilization index, zinc solubilization efficiency

Introduction

Current reports indicate that around half of the world's population is zinc deficient. Grain yield and nutritional quality are decreased when zinc availability is reduced in more than half of the world's cereal-producing soils. The majority of soils across the globe lack plant nutrients, especially micronutrients, which leads to low productivity in agricultural products. Among the micronutrients required for the growth and development of plants, animals and humans are manganese (Mn), molybdenum (Mo), iron (Fe), boron (B), chloride (Cl), copper (Cu) and zinc (Zn). One of the most important minerals for optimal plant growth is zinc.

The healthy development and reproduction of plants depend on zinc, a micronutrient that is required only in trace levels. Zinc affects many growth and metabolic processes, including photosynthesis, sugar production, protein synthesis, carbohydrate and auxin metabolism, fertilization and seed formation, growth regulation and disease resistance. Furthermore, it possesses potent antioxidant qualities. As a co-factor in the enzyme system, zinc is a metal activator of many different enzymes.

Many minerals, including sphalerite, olivine, hornblende, augite and biotite, contain zinc in natural soil conditions. Through activities including acidification, chelation and enzymatic activity, these bacteria enable the mobilization of zinc by dissolving complex mineral complexes and converting the otherwise insoluble forms into soluble, plant-accessible one. Insufficient zinc levels in the soil can seriously impair plant health and result in a variety of physiological conditions. Lowered growth rates, which frequently show up as stunted development and a conspicuous yellowing of the leaves (a condition known as chlorosis), are among the most frequently seen signs of zinc deficiency in plants.

According to Iqbal *et al*., (2010) [10], the widespread problem of zinc insufficiency that is seen in many crop species worldwide. The genera *Acinetobacter*, *Bacillus*, *Cyanobacteria*, *Gluconacetobacter*, *Pseudomonas* and *Serratia* have prominent examples of rhizobacterial strains that can solubilize zinc, according to plate assay techniques. Zinc-solubilizing microbial strains have demonstrated great promise for bioaugmentation in enhancing crop development, yield and quality.

A large amount of zinc is present in forms that are difficult for plants to access, even though the majority of agricultural soils contain enough of it to meet the nutritional needs of crops. The main reason for plants' low uptake of zinc is that it is present in insoluble or chemically bonded forms that are not absorbed by roots.

One such approach that shows promise is the use of zinc-solubilizing bacteria (ZSB), which provides an economical and ecologically friendly substitute for zinc biofortification. These advantageous microorganisms have the innate capacity to transform insoluble zinc compounds into forms that plants can easily absorb. This review seeks to clarify the magnitude of zinc shortage in agricultural systems, its low bioavailability in soils and the ensuing effects on crop output and plant growth in light of these complex roles. It also examines the methods used by ZSB to solubilize zinc, emphasizing their potential as an environmentally responsible and sustainable way to improve the nutritional value and yield of food crops, supporting the agricultural bioeconomy and ensuring food security.

Material and Methods

- **Collection of soil samples:** Soil samples were collected from two districts in the State of Maharashtra *viz*., Satara and Sangli. The soil samples were collected from the rhizospheric soil.
- **Isolation of Zinc solubilising bacterial strains:** Serial dilution pour plate technique was used for isolation of zinc solubilising bacteria, using Bunt and Rovira medium having Zinc oxide (ZnO) as a source of insoluble zinc (Nivaas *et al*. 2019) [12].
- **Qualitative zinc-solubilising assays:** The zinc-solubilising index and efficiency of the isolates was evaluated in Bunt and Rovira medium having zinc oxide as a source of insoluble zinc.

$$\text{Solubilization index} = \frac{\text{Halo zone diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

$$\text{Solubilization efficiency} = \frac{\text{Halo zone diameter}}{\text{Colony diameter}} \times 100$$

Identification of the isolates

By morphological and biochemical characterization, the selected isolates were identified up to genus level.

Results and Discussion

The study concentrated on evaluating several growth metrics and these bacteria's capacity to transform insoluble zinc into forms that plants can use. The results pertaining to these goals are shown below:

Isolation of zinc solubilising bacteria from different crops of rhizospheric soil collected from Satara and Sangli districts of Western Maharashtra: The robust and healthy plants of different crops were uprooted from the field and the soil that stuck to the roots and the soil surrounding the root zone was gathered in sterile, disinfected polythene bags. The necessary information was written on the bags' labels, Table 1 indicated, total of 45 rhizospheric soil samples were gathered from diverse locations in the Sangli and Satara districts as well as from a variety of crops, including sugarcane, groundnut, soybean, green gram, wheat, turmeric, maize, onion, and jowar. After being transported to the lab, the samples were kept at a low temperature of 4 °C in the refrigerator. The number of isolates derived from the soil samples, which were gathered from several sites of Sangli and Satara districts of Western Maharashtra. Following purification, these isolates were kept as pure cultures on Bunt and Rovira media slants and refrigerated at 4 °C for further research. (Gandhi and Muralidharan, 2016) [7].

Table 1: Soil samples collected from Sangli and Satara districts of Western Maharashtra

Sr. No.	Sampling site (Villages)	Soil type	Crop	Abbrivated name	Location
1	Palus	Black	Sugarcane	Pal-1	17.1007916, 74.4534056
2	Palus	Black	Groundnut	Pal-2	17.0992877, 74.4447028
3	Palus	Black	Soybean	Pal-3	17.0994248, 74.4470062
4	Palus	Black	Green gram	Pal-4	17.0877015, 74.4518640
5	Palus	Black	Wheat	Pal-5	17.0937427, 74.4540480
6	Miraj	Black	Wheat	Mir-1	16.8092662, 74.6459399
7	Miraj	Black	Soybean	Mir-2	16.7989412, 74.6528714
8	Kundal	Black	Sugarcane	Kad-1	17.1071656, 74.4113241
9	Islampur	Black	Groundnut	Kad-2	17.0569628, 74.2599610
10	Islampur	Black	Soybean	Kad-3	17.0730402, 74.2863023
11	Islampur	Black	Sugarcane	Is-1	17.0810727, 74.2807307
12	Sangli	Black	Sugarcane	San-1	16.8764937, 74.5689555
13	Walwa	Black	Sugarcane	Wal-1	17.0286471, 74.3663874
14	Walwa	Black	Sugarcane	Wal-2	17.0280018, 74.3642158
15	Tasgaon	Black	Sugarcane	Tas-1	17.0286124, 74.5963975
16	Tasgaon	Black	Soybean	Tas-2	17.0426220, 74.5941840
17	Tasgaon	Black	Sugarcane	Tas-3	17.0177388, 74.6117129
18	Borgaon	Black	Sugarcane	Bor-1	18.4508882, 75.2636272
19	Ashta	Black	Turmeric	Ash-1	16.9513134, 74.4032295
20	Ashta	Black	Sugarcane	Ash-2	16.9425777, 74.4274146
21	Kadegaon	Black	Sugarcane	Kor-1	17.3003787, 74.3281578
22	Kadegaon	Black	Maize	Kor-2	17.2922331, 74.3324061
23	Takari	Black	Wheat	Tak-1	17.1288855, 74.3590230
24	Karad	Black	Soybean	Kar-1	17.2867000, 74.1992626
25	Karad	Red	Sugarcane	Kar-2	17.3118565, 74.1963360
26	Patan	Black	Wheat	Pat-1	17.3732189, 73.8994570

27	Wathar	Black	Sugarcane	Wat-1	17.1783568, 74.1800422
28	Phaltan	Black	Soybean	Pha-1	17.9980376, 74.4177600
29	Phaltan	Black	Wheat	Pha-2	17.9879470, 74.4436383
30	Phaltan	Black	Wheat	Pha-3	17.9804759, 74.4136774
31	Phaltan	Black	Sugarcane	Pha-4	17.9839321, 74.4517490
32	Phaltan	Red	Sugarcane	Pha-5	18.0036633, 74.4363903
33	Rajmachi	Black	Sugarcane	Raj-1	17.3208101, 74.2362030
34	Wai	Black	Onion	Wai-1	17.9601817, 73.8841637
35	Wai	Black	Groundnut	Wai-1	17.9504848, 73.8758720
36	Rethare	Black	Sugarcane	Ret-1	17.1701202, 74.2103398
37	Rethare	Black	Maize	Ret-1	17.1688946, 74.2075945
38	Koregaon	Black	Sugarcane	Kor-1	17.6961828, 74.1515806
39	Koregaon	Black	Onion	Kor-2	17.6975895, 74.1723393
40	Satara	Black	Maize	Sat-1	17.7085602, 74.0111288
41	Satara	Red	Jowar	Sat-2	17.7077585, 74.0182028
42	Satara	Black	Sugarcane	Sat-3	17.6987607, 74.0348415
43	Malkapur	Black	Sugarcane	Mal-1	17.2635309, 74.1764098
44	Malkapur	Black	Sugarcane	Mal-2	17.2651616, 74.1637180
45	Atake	Black	Sugarcane	Ata-1	17.2045106, 74.2033040

Zinc solubilization activity of the isolates in Bunt and Rovira media by plate assay method

In this study, a plate assay method was used to assess the bacterial strains' capacity to solubilize zinc. Bunt and Rovira agar medium supplemented with 0.1% zinc oxide (ZnO) as the zinc source was used to inoculate the isolates. Both the colony diameter and the diameter of the halo zone that developed surrounding the colonies were measured in order to calculate the solubilization efficiency. As indicated in Table 2 and Figure 1, the isolates' levels of zinc solubilization varied.

Out of 45 soil samples collected from Sangli and Satara districts of Western Maharashtra. Only 9 isolates *viz*, Bor-1, Wat-2, Mir-1, Pal-3, Pha-5, Ash-2, Is-1, Ret-2 and Sat-3 showed better performance to zinc solubilising index (2.59 to 3.57) and zinc solubilising efficiency (129.41% to 257.14%).

The investigated nine bacterial isolates showed a range of halo zones from 1.6 cm to 3.6 cm, indicating different capacities to solubilize insoluble ZnO. Isolate Pal-3 had the largest solubilization zone, measuring 3.6 cm, while isolate Pha-5 had the smallest, measuring 1.6 cm. With a solubilization efficiency of 257.14%, Pal-3 surpassed the other isolates, followed by Pha-5 with 228.57%. This suggests that while Pal-3 was the most efficient isolate, all of them had the potential to solubilize zinc. As the most effective isolate for zinc solubilization, Pal-3 once again had the greatest solubilization index value (3.57) followed by Pha-5 (3.28). These results were corroborated by Senthil Kumar *et al*., (2004) [14].

Table 2: Estimation of qualitative zinc solubilization ability of ZSB isolates

Sr. No.	Isolate name	Halo zone diameter (cm)	Colony zone diameter (cm)	Solubilization index	Solubilization efficiency (%)
1	Pal-1	1.9	1.2	2.53	158.33
2	Pal-2	2.2	1.5	2.46	146.66
3	Pal-3	3.6	1.4	3.57	257.14
4	Pal-4	3.1	1.7	2.8	182.35
5	Pal-5	1.6	0.7	3.28	228.57
6	Mir-1	2.9	1.6	2.81	181.25
7	Mir-2	1.9	1.3	2.46	146.15
8	Kad-1	2.8	2.3	2.22	121.73
9	Kad-2	2	1.2	2.66	166.66
10	Kad-3	1.8	1.3	2.38	138.46
11	Is-1	2.0	1.2	2.66	166.66
12	San-1	2.4	1.5	2.6	160.00
13	Wat-1	1.7	1.2	2.41	141.66
14	Wat-2	2.4	1.5	2.6	160
15	Tas-1	2.1	1.6	2.3	131.25
16	Tas-2	1.6	1.1	2.45	145.45
17	Tas-3	0	0	0	0
18	Bor-1	2.55	1.2	3.1	212.5
19	Ash-1	0	0	0	0
20	Ash-2	2.95	1.85	2.59	159.45
21	Kor-1	1.6	1.3	2.23	123.07
22	Kor-2	1.5	1.2	2.25	125
23	Tak-1	2.1	1.7	2.24	123.53
24	Kar-1	2.4	1.7	2.41	141.17
25	Kar-2	1.6	1.2	2.33	133.33
26	Pat-1	0	0	0	0
27	Wal-1	2.3	1.5	2.53	153.33
28	Pha-1	1.9	1.3	2.46	146.15
29	Pha-2	2	1.4	2.42	142.85

30	Pha-3	1.3	0.4	4.25	325
31	Pha-4	1.8	1.4	2.28	128.57
32	Pha-5	1.6	0..7	3.28	228.57
33	Raj-1	0	0	0	0
34	Wai-1	2.3	1.8	2.27	127.77
35	Wai-2	1.6	0.7	3.28	228.57
36	Ret-1	1.8	1.5	2.2	120
37	Ret-2	2.2	1.7	2.94	129.41
38	Kor-1	2.1	1.55	2.35	135.48
39	Kor-2	0	0	0	0
40	Sat-1	1.9	1.5	2.26	126.66
41	Sat-2	1.8	1.3	2.38	138.46
42	Sat-3	2.25	1.4	2.61	160.71
43	Mal-1	1.6	1.1	2.45	145.45
44	Mal-2	0	0	0	0
45	Ata-1	2.1	1.7	2.235	123.52

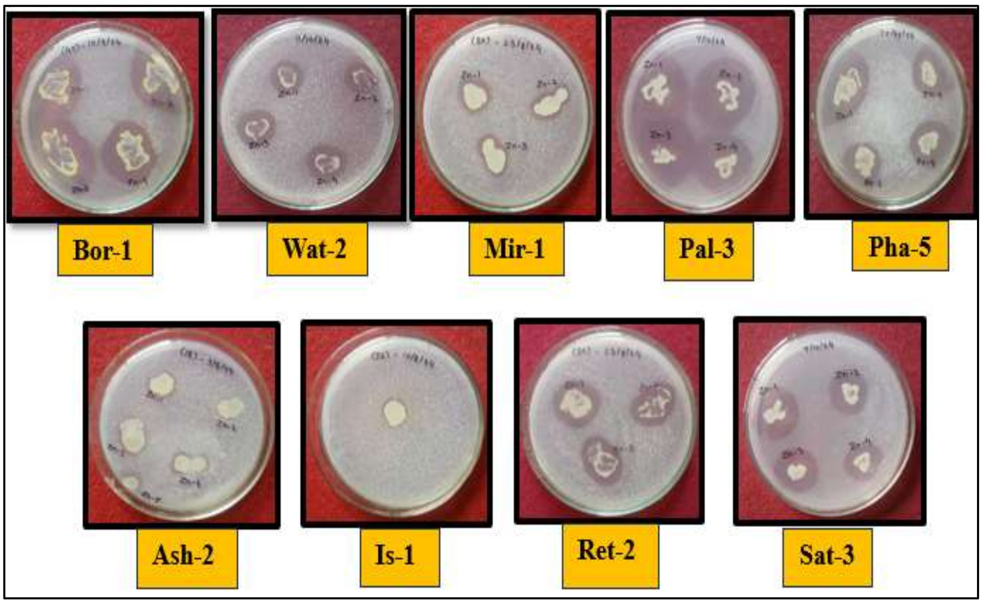

Fig 1: Halo zones (cm) of selected ZSB isolates

Characterization of zinc solubilising bacterial isolates

The isolated zinc-solubilizing bacteria were characterized using biochemical tests in addition to their morphological and cultural characteristics. Following a 3-4 days incubation period, these observations were made on petri plates.

Morphological characteristics of ZSB isolates

The morphological characteristics of the zinc-solubilizing bacterial isolates, including their pigment synthesis, cell shape and Gram staining reaction were investigated in the current study. According to the results, which are shown in Table 3, every isolate was Gram-positive. The generation of pigments, which varied in colour from creamy white to yellowish, was another analysis of the isolates. Bor-1 was the only isolate showing yellow pigment, while other exhibit white pigmentation. The morphological analysis revealed that all zinc solubilising isolates showed rod shape and Gram positive reaction.

These results align with previous studies. For example, *Bacillus species*, are usually rod-shaped, Gram-positive and have white pigmentation (Jagana *et al*., 2019; Iqbal *et al*., 2010) [11, 10]. The Gram-positive, short, yellowish-pigmented rods that are seen in *Pseudomonas species* also match the findings published by Desai *et al*., (2012) and Chibuogwu and Ezeobi (2011) [5, 3].

Table 3: Morphological characteristics of ZSB isolates

Sr. No.	Isolates name	Cell shape	Pigment	Gram staining
1	Bor-1	Rods	Yellow	+
2	Wat-2	Rods	White	+
3	Mir-1	Rods	White	+
4	Pal-3	Rods	White	+
5	Pha-5	Rods	White	+
6	Ash-2	Rods	White	+
7	Is-1	Rods	White	+
8	Ret-2	Rods	White	+
9	Sat-3	Rods	White	+

Colony characteristics of the zinc solubilizing isolates

In this research, colony features, including size (small, medium), shape (circular, irregular), form, elevation (flat, slightly raised) and colour (yellow, creamy white and dull white) were examined on culture media in petri plates following a two-days incubation period. Small size ranged from 0.5 to 2 mm and medium size from 2 to 4 mm. According to the findings, which are compiled in Table 4, the colony characteristics of the nine isolates (Bor-1, Wat-2, Mir-1, Pal-3, Pha-5, Ash-2, Is-1, Ret-2 and Sat-3) were comparable. Every colony of bacteria that solubilized zinc was round in shape except Pal-3, Ret-2 and Sat-3. Bor-1, Wat-2, Mir-1 and Ash-2 produced smaller colonies than Pal-3, Pha-5, Is-1, Ret-2 and Sat-3 which developed medium sized colonies. While Wat-2, Mir-1, Pal-3, Pha-5, Ash-2, Is-1 and Sat-3 had a higher height and Bor-1 and Ret-2 colonies had flat elevations. Regarding colour, only Bor-1 was yellowish in hue, while Wat-2, Mir-1, Pal-3, Pha-5, Ash-2, Is-1, Ret-2 and Sat-3 were all white.

Table 4: Colony characteristics of the zinc solubilizing isolates

Sr. No.	Isolate Name	Colony size (mm)	Colony shape	Colour	Elevation	Surface	Margin
1	Bor-1	1.10	Circular	Yellow	Flat	Smoothy shine	Irregular
2	Wat-2	1.26	Circular	Creamy white	Slightly raised	Shine	Regular
3	Mir-1	1.16	Circular	Creamy white	Slightly raised	Smoothy shine	Irregular
4	Pal-3	3.00	Irregular	Dull white	Slightly raised	Smoothy shine	Irregular
5	Pha-5	3.26	Circular	Creamy white	Slightly raised	Shine	Irregular
6	Ash-2	1.20	Circular	Creamy white	Slightly raised	Smoothy shine	Regular
7	Is-1	3.26	Circular	Creamy white	Slightly raised	Shine	Irregular
8	Ret-2	3.10	Irregular	Creamy white	Flat	Smoothy shine	Irregular
9	Sat-3	3.36	Irregular	Creamy white	Slightly raised	Smoothy shine	Regular
	SE (m)±	0.30					
	CD at 1%	0.90					

Biochemical characterization of zinc solubilizing bacteria

A variety of biochemical assays, such as the catalase, IAA production, motility test, oxidase test and starch hydrolysis were performed on each of the bacterial isolates in this investigation.

All isolates demonstrated positive results for the IAA (Indole Acetic Acid) production test and motility test as indicated in Table 4. Different isolates produced different results for catalase test, oxidase test and starch hydrolysis; some tested positive while others tested negative.

The investigation's findings are shown in Table 5, which unequivocally show that, the catalase test revealed that six of the nine isolates (Bor-1, Wat-2, Mir-1, Pal-3, Ash-2, and Ret-2) produced catalase enzyme while Is-1, Sat-3 and Pha-5 were the other three isolates that tested negative. All isolates exhibited positive IAA (indole-3-acetic acid) production data, indicating their potential as rhizobacteria that promote plant growth (PGPR). Only Mir-1 was non-motile, whereas eight isolates were motile. Isolate Mir-1, Ash-2, Is-1 and Sat-3 did not exhibit oxidase activity, whereas other eight isolates were oxidase-positive. The amylase activity of five isolates *viz*, Bor-1, Wat-2, Pal-3, Pha-5 and Ret-2 was confirmed by positive starch hydrolysis. The four isolates that remained tested negative.

Table 5: Different biochemical tests of the zinc solubilizing bacterial strains

Sr. No.	Isolate name	Catalase test	IAA production test	Motility test	Oxidase test	Starch hydrolysis
1	Bor-1	+	+	+	+	+
2	Wat-2	+	+	+	+	+
3	Mir-1	+	+	-	-	-
4	Pal-3	+	+	+	+	+
5	Pha-5	-	+	+	+	+
6	Ash-2	+	+	+	-	-
7	Is-1	-	+	+	-	-
8	Ret-2	-	+	+	+	+
9	Sat-3	-	+	+	-	-

Note: Positive: (+), Negative: (-)

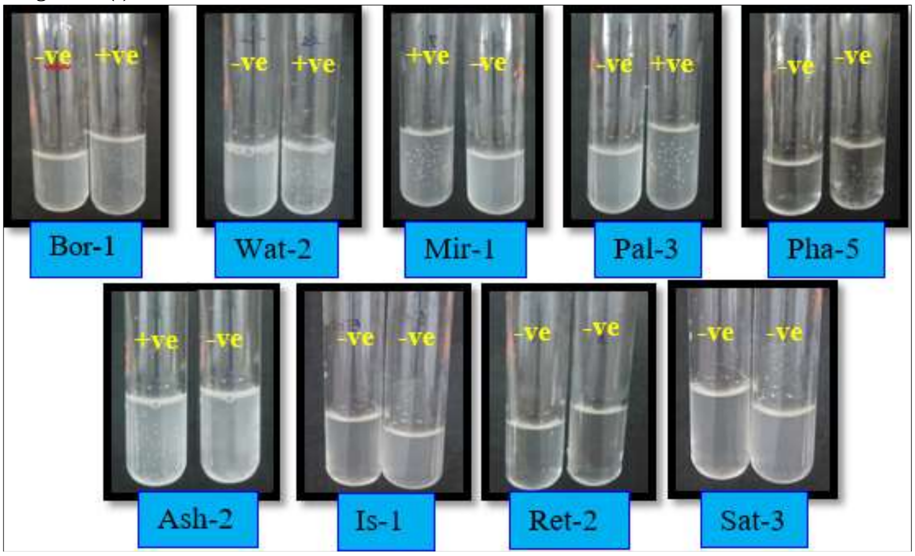

Fig 2: Biochemical characterization of ZSB isolates – Catalase test

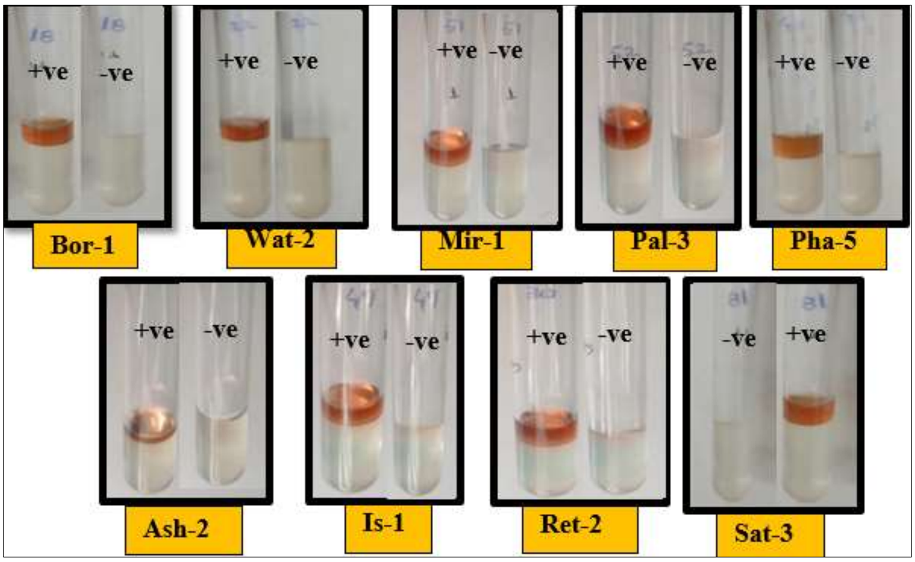

Fig 3: Biochemical characterization of ZSB isolates – IAA Production test

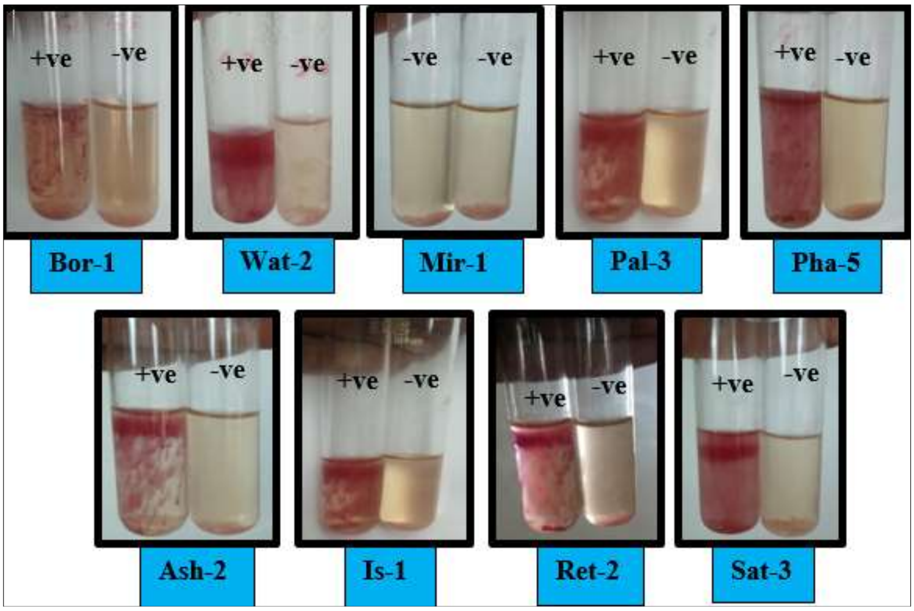

Fig 4: Biochemical characterization of ZSB isolates – Motility test

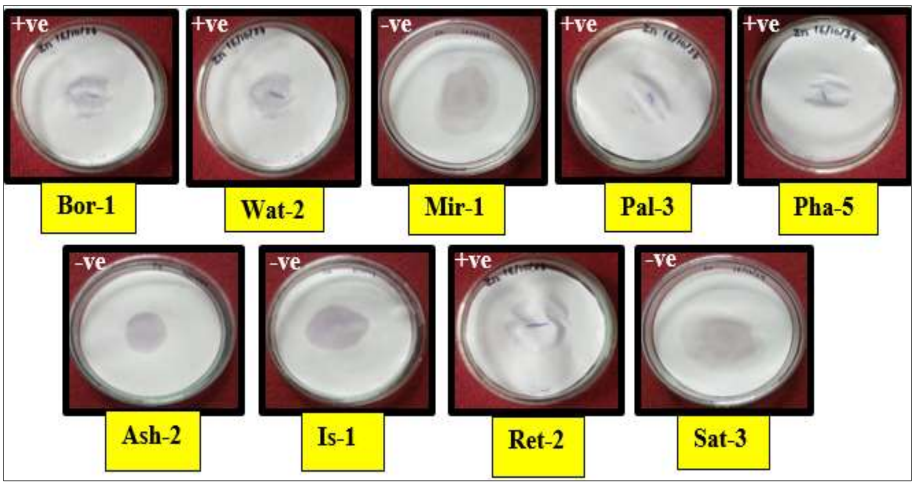

Fig 5: Biochemical characterization of ZSB isolates – Oxidase test

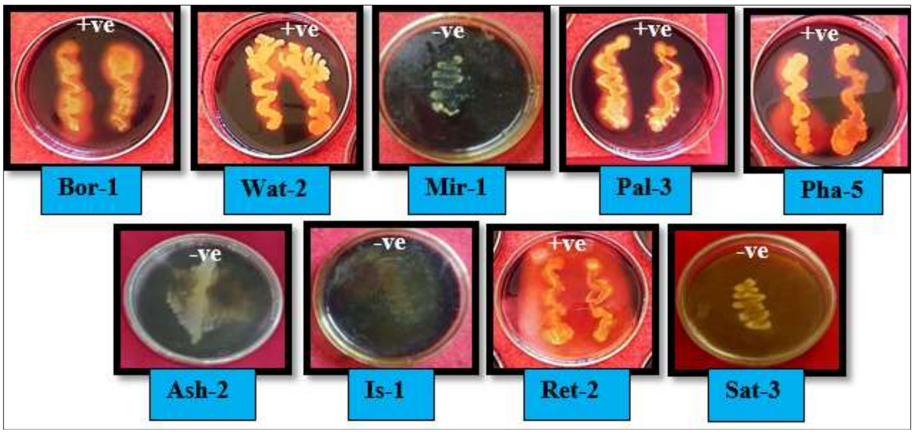

Fig 6: Biochemical characterization of ZSB isolates – Starch hydrolysis test

Conclusion

The study found that ZSB, especially Pal-3, Bor-1 and Pha-5 had a lot of potential for releasing soluble zinc into the soil and allowing plants to use it by producing acid.

Based on cultural, morphological and biochemical characteristics, it was concluded that Pal-3 and Pha-5 were *Bacillus* species while Bor-1 was *Pseudomonas* species.

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