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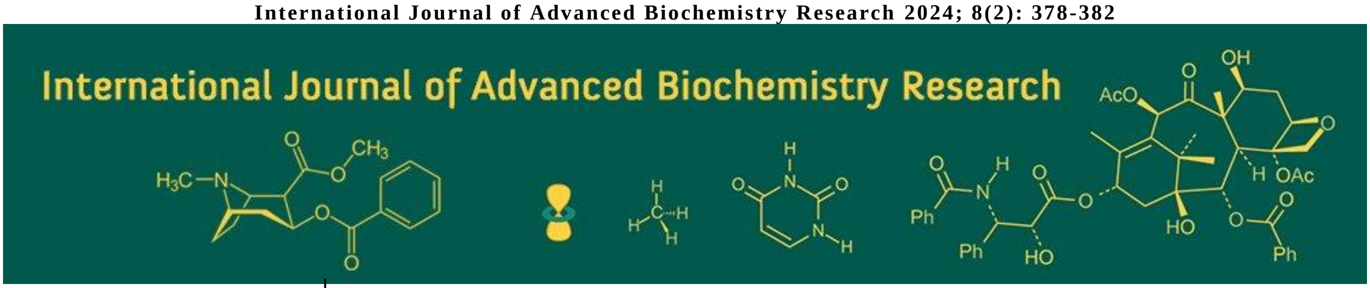

Assessment of zinc-solubilizing bacterial isolates for plant growth promoting traits and water stress tolerance behaviour

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Abstract

The present study elucidates the efficiency of zinc solubilization and the plant growth-promoting (PGP) traits exhibited by selected zinc-solubilizing bacterial (ZSB) strains. Qualitative analysis unveils distinct variations in solubilization efficiency, with isolate FMBR110 demonstrating exceptional proficiency in solubilizing zinc from various insoluble sources. PGP traits, encompassing phosphate solubilization, siderophore production, indole-3-acetic acid (IAA) synthesis, exopolysaccharide (EPS) generation, hydrogen cyanide (HCN) emission, and ammonia production are quantified, spotlighting the functional diversity among isolates. Notably, FMAR106 showed the highest phosphate solubilization zone value (2.20 cm), while FMBR110 exceled in siderophore production (4.37 cm halo zone), IAA synthesis (24.09 µg/ml), and stands out as the sole producer of HCN. Furthermore, isolate FMBR110, exhibited remarkable tolerance to water stress, even in high water saturated soil conditions. The study accentuates the importance of selecting ZSB strains tailored to specific water conditions in agriculture, offering promising solutions for sustainable crop production, particularly in high water-stressed regions or flooding conditions.

Keywords: Zinc solubilizing bacteria, moisture stress, flooding condition, zinc, plant growth promoting traits

Introduction

Zinc (Zn) deficiency in crops poses a widespread global challenge, endangering both food security and nutrition. This dearth, often referred to as "hidden-hunger," distresses more than two billion people worldwide (Lowe, 2021) [10], particularly those who rely on main crops like rice, wheat, and maize (Upadhayay *et al*., 2018) [24]. Zn is a crucial micronutrient essential for both plant growth and human health, playing a pivotal role in various physiological processes (Upadhayay *et al*., 2019; 2022a, 2022b 2022c, 2022d) [28, 20, 23, 26, 27]. Addressing Zn deficiency in crops demands ground-breaking agricultural strategies, and one promising approach involves harnessing the power of Zn-solubilizing bacteria (ZSB) (Khan *et al*., 2019, Upadhayay *et al*. 2021) [9, 25]. These outstanding microorganisms possess a unique ability to solubilize insoluble Zn compounds in the soil, thereby making this vital nutrient readily accessible to plants (Kamran *et al*. 2017; Singh *et al*., 2022) [6, 18]. By enhancing Zn uptake, ZSB have the potential not only to maximize crop yields but also to improve the nutritional quality of food, ultimately mitigating hidden hunger (Upadhayay *et al*., 2022d) [27]. Furthermore, ZSB exhibit various plant growth-promoting traits such as phosphate solubilization, production of IAA, siderophore, EPS, and more, exhibiting their promising application as bioinoculants (Bhatt and Maheshwari, 2020; Upadhayay *et al*., 2022b, 2023a, 2023b) [1, 23, 21, 22]. While the application of ZSB for Zn biofortification holds great promise, their performance under different environmental conditions, particularly varying moisture levels, is a critical aspect that requires thorough examination. Understanding how ZSB respond to varying moisture conditions is crucial for unlocking their full potential in addressing Zn deficiency and enhancing crop resilience in the face of water stress-related challenges. Therefore, there is a need to develop a robust methodology for assessing the water tolerance of selected ZSB strains.

Such a methodology is crucial for gaining insights into how these bacteria perform under different moisture regimes and for identifying strains that exhibit exceptional water tolerance. This article sets the stage for the exploration of potential water stress-tolerant ZSB. The findings from this study can potentially guide the application of ZSB in water-stressed agricultural systems, ultimately contributing to enhanced crop yields, improved nutritional content, and global food security.

Materials and Methods

Isolation of Bacteria

Soil samples were collected from three hilly regions in Uttarakhand: Almora, Bhowali, and Ranichauri. These samples were taken from the rhizospheres of finger millet and barnyard millet plants. The collected soil was stored in sterile polythene bags at 4 °C. For bacterial isolation, 1g of soil from each rhizosphere was mixed with 10 ml of sterile saline solution and diluted (ranging from 10^{-1} to 10^{-5}). Aliquots (100µl) from each dilution were plated on nutrient agar and incubated at 28±2 °C until distinct colonies formed. Selected colonies were purified using the 'four-way-streaked procedure' on nutrient agar plates.

Zinc Solubilization Bioassay

The study screened potential ZSB isolates by following procedure of Ramesh *et al.* (2014) [14]. Mineral salt medium was prepared with insoluble zinc compounds (ZnO, $Zn_3(PO_4)_2$, $ZnCO_3$), autoclaved, and poured into Petri plates. Overnight-grown test isolates were spot-inoculated on mineral salt media plates supplemented with insoluble zinc compounds (ZnO, $Zn_3(PO_4)_2$, $ZnCO_3$), and plates were sealed with parafilm and incubated at 28 °C for a week. The appearance of a halo zone around bacterial colonies indicated positive results. Zinc solubilization efficiency (S.E) was calculated using the formula:

Zn S.E. = diameter of solubilization halo/diameter of the colony×100

In vitro Screening of Plant Growth Promoting Traits

The phosphate solubilization potential of the bacterial isolates was quantitatively assessed following the method described by Nautiyal (1999) [12]. The synthesis of the plant hormone indole-3-acetic acid (IAA) by the bacterial strains was analyzed following the procedure of Patten and Glick (2002) [13]. Siderophore production, which involves the production of iron-chelating agents, was estimated using 'Chrome-Azurol S (CAS) medium' following the method of Schwyn and Neilands (1987) [16]. CAS agar plates were spot-inoculated with bacterial culture, and after 4 days of incubation at 28±2 °C, siderophore production was confirmed based on the measurement of the yellow-orange halo zone that appeared around the bacterial colony. The EPS production ability of bacterial isolates was performed by following method of Siddikee *et al.* (2011) [17]. After 5 days of incubation at 28±2 °C, the supernatant was mixed with chilled ethanol for precipitation, and the dry residue weight was measured in mg/ml. For ammonia production, bacterial cultures were incubated in peptone water, and ammonia was detected using Nessler's reagent following method of Cappuccino and Sherman (1992) [2].

Water stress tolerance assay

In this study, we conducted an experiment to evaluate the water tolerance of selected zinc-solubilizing bacterial (ZSB) strains under a range of moisture conditions. The soil used for this experiment was collected from the Crop Research Centre (CRC) at G.B. Pant University of Agriculture and Technology, Pantnagar. To ensure its sterilization, the soil underwent a thorough sterilization process over three consecutive days. For the experiment, plastic disposable glasses with a capacity of 300 mL each were employed. These glasses were cleaned with 70% ethanol and then subjected to sterilization under UV light for two hours. Each of the glasses was filled with 100 grams of the sterilized soil. To create different moisture conditions, five distinct treatments were established by adding distilled water (DW) to each cup as follows:

1. **Glass 1:** The soil in the first cup was saturated by adding 28ml of distilled water.
2. **Glass 2:** The soil in the second cup was maintained at 60% moisture level by adding 9.3ml of water to achieve this level.
3. **Glass 3:** The soil in the third cup was saturated with water, and the cup was filled with DW from the soil's upper surface to a height of 2 cm.
4. **Glass 4:** The soil in the fourth cup was saturated with distilled water, followed by filling the cup with DW from the soil's upper surface to a height of 5 cm.
5. **Glass 5:** The soil in the fifth cup was first saturated with sterilized distilled water, followed by filling the cup with DW from the soil's upper surface to a height of 10 cm.

To introduce the ZSB strains, a 4 mL aliquot of an overnight-grown bacterial culture was added to each glass. To prevent contamination, each glass was covered with parafilm and aluminum foil and then incubated at room temperature. Sampling was conducted on the 7th, 14th, and 21st days of the experiment. From each glass, 1 gram of soil was sampled and subjected to Colony-Forming Unit (CFU) counting using the standard pour plate method on nutrient agar media. The moisture tolerance of the selected ZSB strains was assessed based on the number of colonies, categorized as less than 30, between 30-300, or more than 300 colonies, at three different dilutions (10^{-3}, 10^{-5}, and 10^{-7}) on various days (7th, 14th, and 21st days) under different soil moisture conditions. This comprehensive methodology allowed us to evaluate the water tolerance of these ZSB strains, shedding light on their performance, particularly in water-stressed environments.

Results and Discussion

The results, summarized in the table 1, reveal variations in solubilization efficiency, indicating the extent to which each isolate mobilized zinc. Isolate FMAR106 displayed the largest halo zones for ZnO (1.47±0.12 cm) and $ZnCO_3$ (1.8±0.21 cm), and isolate FMAR119 for $Zn_3(PO_4)_2$ (1.1±0.17 cm). Conversely, BMBR260 showed the smallest halo zones for all three zinc sources. Several prior investigations, reflecting similar trends, have identified a variety of microorganisms capable of zinc solubilization, characterized by the formation of a distinct halo zone around bacterial colonies (Gontia-Mishra *et al.*, 2017; Kamran *et al.*, 2017; Khan *et al.*, 2023) [3, 6, 7]. Zinc plays a crucial role in plant growth, and the ability of soil microorganisms to solubilize this essential nutrient is a critical trait. These microorganisms employ various mechanisms to convert zinc into a soluble form, making it readily available to crop plants (Upadhayay *et al*., 2018) [24]. The functional traits of the selected bacterial isolates, including phosphate solubilization, siderophore production, indole-3-acetic acid (IAA) production, quantitative exopolysaccharide (EPS) production, hydrogen cyanide (HCN) production, and ammonia production are depicted in table 2. The results unveiled a spectrum of functional diversity among the isolates. Notably, FMAR106 displayed a larger phosphate solubilization halo zone (2.20±0.20 cm). Furthermore, FMBR110 stood out with the highest siderophore production (halo zone diameter: 4.37±0.57) and IAA production (24.09±0.71 µg/ml). In contrast, FMBR206 exhibited relatively high EPS production (2.36±0.26 mg/ml). Interestingly, hydrogen cyanide production was solely detected in FMBR110. Ammonia production trait was evident in FMAR106, FMBR110, FMAR119, and FMBR206, while absent in the other isolates. The production of siderophores by these isolates indicates their capacity to enhance the solubilization of iron (Jaiswal *et al*., 2023) [5], making them valuable as bio-inoculants for iron-fortified crop production (Upadhayay *et al*., 2018) [24]. Additionally, their ability to solubilize phosphate is crucial for crop revitalization. This aspect is consistent with earlier findings, such as those by Bhatt and Maheshwari *et al*. (2020) [1], which also identified phosphate-solubilizing properties in ZSB. This variation in IAA production aligns with previous studies involving bacterial isolates from different sources (Singh *et al*., 2020; Khan *et al*., 2022) [19, 8]. Additionally, the isolates were assessed for exopolysaccharide (EPS) production, which supports efficient root colonization and nutrient absorption. Prior research has demonstrated that ZSB isolates capable of producing EPS have been used to enrich crops with zinc (Nandal and Solanki, 2021) [11]. Lastly, the isolates displayed characteristics conducive to plant growth, including ammonia production, a well-documented trait of plant growth-promoting bacteria (Roshni *et al*., 2020) [15]. These diverse traits highlight the potential of these isolates to and make them promising bioinoculants to enhance nutrient availability, promote plant growth, and improve soil health.

Table 1: Qualitative zinc solubilization by selected bacterial isolates using three different insoluble zinc sources (zinc oxide, zinc carbonate and zinc phosphate)

Bacterial Isolates	**Zinc sources**					
	ZnO (Zinc Oxide)		**$ZnCO_3$ (Zinc Carbonate)**		**$Zn_3(PO_4)_2$ (Zinc phosphate)**	
	Zone (cm)	**Solubilization efficiency (%)**	**Zone (cm)**	**Solubilization efficiency (%)**	**Zone (cm)**	**Solubilization efficiency (%)**
FMBR75	1.1±0.20	133.69	1.3±0.26	140.37	0.9±0.12	100.78
FMAR106	1.47±0.12	286.19	1.8±0.21	244.44	0.8±0.15	131.58
FMBR110	1.37±0.35	533.33	1.6±0.05	408.33	0.9±0.20	209.30
FMAR119	1.4±0.17	422.22	1.5±0.20	292.62	1.1±0.17	183.33
BMRR128	0.83±0.15	195.00	1.2±0.10	258.33	0.7±0.05	175.00
FMBR206	1.3±0.10	371.11	1.7±0.20	468.89	1.0±0.30	188.68
BMBR260	0.63±0.12	177.78	0.9±0.15	240.00	0.6±0.21	121.43

*Data are represented by mean value using three replicates

Table 2: Plant growth promoting characteristics of zinc solubilizing bacterial isolates

S. No.	**Bacterial isolate**	**Phosphate solubilization**	**Siderophore production**	**IAA Production (µg/ml)**	**Quantitative EPS production (mg/ml)**	**HCN production**	**Ammonia production**
		Halo zone Diameter (cm)					
1.	FMBR75	1.30±0.10	2.77±0.12	7.21±0.69	1.13±0.09	-	-
2.	FMAR106	2.20±0.20	2.63±0.15	6.92±1.45	1.02±0.07	-	+
3.	FMBR110	1.30±0.10	4.37±0.57	24.09±0.71	1.80±0.13	+	+
4.	FMAR119	1.10±0.10	2.53±0.12	5.15±1.17	1.52±0.17	-	+
5.	BMRR128	2.17±0.21	2.57±0.12	7.38±0.9	1.23±0.13	-	-
6.	FMBR206	1.03±0.5	1.67±0.42	10.77±0.63	2.36±0.26	-	+
7.	BMBR260	0.90±0.10	2.43±0.21	10.40±0.56	0.94±0.09	-	-

*Data are represented by mean value using three replicates

The valuable insights were extracted through the systematic analysis of selected ZSB isolates, elucidating their tolerance to diverse moisture conditions across various levels of moistened soil over a 21-day timeframe (representing conditions of water stress). The results were analyzed by determining colony-forming units (CFU) at dilutions (10^{-3}, 10^{-5}, and $10^{-7)}$ on days 7, 14, and 21 to assess the performance of bacterial isolates (Table 3). Strain FMBR110 showed striking water tolerance behaviour, especially when the soil was saturated with water up to 10 cm from the surface. Even at the dilution of 10^{-5}, the appearance of bacterial after plating indicated that this isolate thrived throughout the entire 14-day period, indicating its ability to adapt to very moist conditions. While FMAR106, BMRR128, FMBR206, and BMBR260 displayed colony counts of fewer than 30 when the soil was saturated with water up to a depth of 10 cm and studied at the 7th day with a 10^{-5} dilution. This resilience suggests that FMBR110, FMAR106, BMRR128, FMBR206, and BMBR260 could be valuable candidates for environments susceptible to water stress. This potential makes them promising candidates for improving agriculture in areas facing high water stress related challenges or flood conditions.

Table 3: Tolerance behavior of different zinc solubilizing bacterial strains for different moisture levels

Soil Conditions	Dilution	Bacterial isolates																				
		FMBR75			FMAR106			FMBR110			FMAR119			BMRR128			FMBR206			BMBR260		
		7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
WSS	10^{-3}	H	M	L	H	M	L	H	H	H	H	M	M	H	L	L	H	M	M	M	L	L
	10^{-5}	M	L	-	H	L	-	M	M	M	M	L	-	M	L	L	H	M	L	L	-	-
	10^{-7}	-	-	-	L	-	-	-	L	L	-	-	-	-	-	-	-	-	-	-	-	-
MS	10^{-3}	H	M	L	H	M	L	H	H	H	H	H	L	H	M	L	H	M	L	M	M	M
	10^{-5}	M	L	L	M	L	-	M	M	M	H	L	L	H	M	-	H	M	-	M	M	-
	10^{-7}	L	-	-	L	-	-	-	L	-	L	-	-	-	-	-	L	-	-	-	-	-
Wfg2	10^{-3}	H	L	-	M	L	-	M	M	M	H	L	-	H	M	-	H	M	L	L	-	-
	10^{-5}	L	-	-	M	L	-	L	L	L	L	-	-	M	L	-	M	L	-	-	-	-
	10^{-7}	-	-	-	-	-	-	L	L	-	-	-	-	-	-	-	-	-	-	-	-	-
wfg5	10^{-3}	M	L	-	M	L	-	M	M	L	L	L	-	M	L	-	H	L	-	M	M	M
	10^{-5}	L	-	-	M	L	-	L	L	-	L	-	-	M	-	-	M	-	-	-	-	-
	10^{-7}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wfg10	10^{-3}	M	-	-	M	L	-	M	M	L	L	-	-	M	L	-	M	L	L	M	M	L
	10^{-5}	-	-	-	L	-	-	L	L	-	-	-	-	L	-	-	L	-	-	L	-	-
	10^{-7}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

WSS: water saturated soil, **MS**: 60% Moistened soil, **Wfgw:** water filled glass upto 2 c.m, **wfg5:** water filled glass up to 5 cm, **wfg10:** water filled galss upto 10 cm, **H:** colonies exceeding 300, **M:** colonies between 30 and 300, **L:** colonies fewer than 30, **'-':** absence of colony formation

The strategic selection of ZSB strains has a profound impact on nutrient accessibility to plants, with the potential to significantly enhance crop growth and productivity. This influence is particularly noteworthy in regions where water stress is a prevalent concern, specifically in flooded soils. Our findings underscore the paramount importance of meticulously choosing ZSB strains that align with the prevailing moisture conditions in a given agricultural environment, emphasizing this significance, especially in paddy fields. Notably, previous research has shown that tomato plants grown from seeds inoculated with *Enterobacter* and *Pseudomonas putida* strains expressing ACC deaminase exhibited resistance to the negative effects of flooding stress (Grichko and Glick, 2001) [4]. While our study sheds light on the water stress tolerance of these bacterial strains, further research is necessary to understand the exact mechanisms by which they adapt to different moisture levels. Additionally, field trials are needed to assess how practical and effective these strains are in real-world agricultural situations.

Conclusion

In conclusion, our study demonstrates the effectiveness of selected bacterial strains in solubilizing Zn and their diverse plant growth promoting properties (PGP). Furthermore, the bacterial isolate FMBR110, followed by FMAR106, BMRR128, FMBR206 and BMBR260, showed remarkable tolerance to water stress, especially in highly water-saturated soil conditions. The variable degrees of moisture tolerance among these strains underscore their potential to fortify crop resilience, especially in regions susceptible to flooding. Additionally, ZSB strains with high water tolerance can be a valuable asset for sustainable agriculture, especially in areas struggling with excessive moisture. To fully unlock the practical benefits of these bacteria in augmenting crop growth and productivity in water-stressed environments or flood conditions, additional research and on-field trials are imperative.

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