Application of Root Associated Zinc solubilizing *Bacillus proteolyticus* strain SH 25 on growth and augmentation of Zinc in Maize Plant

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Abstract

Zinc (Zn) stands out as a critical micronutrient crucial for the proper growth, development and functioning of plants. Recent studies indicate that zinc exists abundantly in soil, it predominantly persists in insoluble forms, hindering its uptake by plants. Zinc solubilizing bacteria (ZSB) emerge as pivotal agents in facilitating the transformation of insoluble zinc into soluble forms, thus aiding plant growth. To address this issue, the current study aimed at isolating, screening, identifying and characterizing efficient zinc solubilizing bacteria to enhance maize growth. Numerous bacteria were isolated from the maize rhizosphere in the Northern region of West Bengal by using the dilution plate technique. Screening involved the utilization of ZnO, zinc ores, with only four bacterial strains exhibiting notable zinc solubilizing capacity selected for further assessment. These strains underwent biochemical, morphological and plant growth-promoting (PGP) activity evaluations.

The selected bacterial isolates, capable of solubilizing Zn, underwent further screening for their ability to promote plant growth under sterile conditions. The identified bacterium was classified as Bacillus proteolyticus (SH 25/OM584287) by molecular identification through MALDI-TOF-MS analysis and 16S rDNA sequencing. This ZSB isolate demonstrated various growth-promoting attributes including the production of organic acids, contributing to its effectiveness in enhancing maize growth. $ZnSO_4$ served as the zinc source in this study. The impact of SH 25 on Zea mays (variety: SUPER DON-9533) was quantified using the Zinc Transformation Index (ZTI) measured via Atomic Absorption Spectroscopy (AAS).

Keywords: ZSB, Zn ores, Maize (*Zea mays*), 16S rDNA gene analysis, MALDI-TOF-MS, ZTI, AAS.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are mainly soil borne free living rhizospheric bacteria. When PGPRs are applied to seeds or crops, they stimulate the plant growth and increase yield production or reduce the fungal disease of soil borne plant pathogens biologically without any hazard of chemical fertilizer.[1-3] Rhizospheric soil consists a large-scale of various microbes which are dense in nutrient-rich soil regions including the upper layer of soil. That is why plants can benefit from rhizospheric soil microbes in several ways. Among all microbes, maximum rhizobacteria stimulate plant growth, enrich soils fertility, increase crop production or protect plants against pathogens.[4]

Zinc (Zn) is one of the most important micronutrients for healthy growth and development of plants, animals and humans. In plants, Zn deficiency can cause abnormalities like necrotic spots, chlorosis, bronzing of leaves, resetting of leaves, stunned plants and dwarf leaves etc.[5-8] Maize (*Zea maize* L.) is an essential crop cultivated worldwide, occupying an unique position in Indian agricuiture.[9] After rice and wheat, maize is the third and most important staple crop in India. Maize can be stored relatively very easily and is used in a variety of ways to consume as regular food.[10] Insufficient amounts of Zn can also adversely affect the quality and quantity of harvested products. Naturally little amount of insoluble Zn is present in soil but plants cannot uptake insoluble Zinc from soil but Zn solubilizing bacteria (ZSB) help to convert insoluble Zn to solubilizing form, transferred Zn from soil to plant.[11]

Due to this type of Zn deficiency problems, farmers used different types of chemical fertilizers commonly available in the market but by excess use of these, crop yield can decrease day by day. Therefore, it is very necessary to find out an easiest way to increase Zn uptake from soil to plant and to enhance crop varieties to tackle ailing health issues in our country.[5] In perspective on previous research, the current research concentrated on the impact of ZSB on growth and development of maize plants and also measured the Zn transformation index (ZTI) on maize plants by AAS. The objective of our research was to isolate and identify the Zn solubilizing plant growth promoting rhizobacteria from the Northern region of West Bengal and evaluate their PGP activity.

Material and Methods

Isolation and screening of ZSB: Root associated soil samples were collected from three different fields located in the radius of one kilometer growing in the same soil type and samples were identified as per location of the Northern region of West Bengal. Samples were put in the paper bags individually, labeled and transported to the lab. Then take one gram of soil in a sterile test tube from the different samples, serially diluted from 10-1 to 10-9 individually.

After that, serial dilution agar plating techniques were performed for the isolation of individual colonies by the help of Luria Bertani (LB) (HiMedia, M1151) agar plate. Picked the colonies and purify them by retreating[9]. Then purified colonies were grown on Modified Pikovskaya's agar (HiMedia, M520) media with 0.1% of ZnO for the screen of ZSB. After incubation (37 ºC for 24-36 h), potential ZSB isolates showed a clear holo zone around colonies and this zone diameter was calculated by Zn solubilization index (ZSI) with three biological replicates:[12]

ZSI = (Colony diameter + halo zone) / colony diameter

Phosphorus (P) and potassium (K) Solubilizing ability: Selected ZSB isolates were spot inoculated on Pikovskaya agar plates and incubated at 37 ºC for 5–7 days to check the P and K solubilizing ability.[13] Clear holo zones around the colonies showed positive results and this experiment was performed with three biological replicates.

Antibiotic resistance: Four different types of antibiotic discs such as azithromycin (Himedia, SD204), oxacillin (Biogram, C30-1813), chloramphenicol (Biogram, OX1-1807) and rifampicin (Himedia, SD128) were used for the antibiotic resistances of ZSB. According to the Vincent method[30], ZSB were inoculated on bacteria containing LA plates spread by swab and incubated at 37 ºC overnight.[14,15] Based on the zone diameter, ZSB strains were classified as highly or moderately resistant or susceptible.

Plant growth-promoting and extracellular hydrolytic enzyme activity of ZSB: Four selected ZSB were screened for *in vitro* plant growth-promoting (PGP) activities including indole acetic acid (IAA) production, production of hydrogen cyanide (HCN), ammonia production, siderophore production and phosphate solubilization.[16] For extracellular hydrolytic enzyme activity check, a qualitative assay of protease production[17], chitinase production[18], amylase production[19] as well as cellulase production was carried out.[20]

Morphological, Biochemical and Molecular Identification: Based on solubilizing capacities, four potent ZSB isolates were selected and identified morphologically and biochemically. At first, colony morphology was checked under the microscope and Gram staining was performed for the morphological characterization.[21] Then methyl red (MR) test, Voges Proskauer (VP) test, oxidase test, production of acid and gas from carbohydrate, citrate utilization, nitrate reduction, coagulase test, urease test and catalase test were carried out for biochemical analysis.[22] After morphological and biochemical identification, only one potential ZSB was selected for molecular identification depending on MALDI-TOF and 16S rDNA sequencing.

The genomic DNA of the SH 25 was extracted by the CTAB extraction technique and PCR sequences were subjected to BLAST analyses in NCB.[23,24] Then the sequences were deposited to NCBI GenBank through Bankit procedure and the phylogenetic analyses were conducted in MEGA-6.0 software.[25] Identification by using MALDI-TOF biotype relies primarily on mass spectrometry of bacterial ribosomal protein profiles and the mass spectral pattern of protein expression was compared with reference pattern in a database. Then the target was ready for loading to MALDI-Biotyper.[26] According to Kurli et al[12], formic acid extraction protocol was followed to extract proteins for the sample's preparation for MALDI-TOF analysis of SH 25 identification.[27]

***In vitro* evaluation of ZSB on maize:** The soil was collected from the experimental field of the Department of Botany, University of Gour Banga, Malda. For this experiment, at first the physical and chemical characteristics of soil were measured and then used. On the other side, a fresh bacterial sample was prepared for seed bacterization. Soil preparation, pot filling and seed bacterization process were discussed in earlier studies.[28] All treated pots were properly watered with sterile distilled water and put in sunlight with the natural environment (Temperature 25-30 ºC, 11 h photoperiod and proper aeration) for 30 days.

Impact of ZSB on treated maize Plants: The PGP activity was standardized by the germination percentage of seeds, shoot length, root length, leaves count, shoot dry weight and root dry weight after 30 days of germination but only after 48–72 h of seed bacterization, seeds germination percentage was measured.

Extraction and estimation of total proteins, chlorophyll and carbohydrate: Extraction and estimation of total proteins, chlorophyll and total soluble sugar were calculated by standard protocol.[28] For extraction and estimation of soluble protein, whole plant parts were collected and were homogenized in a pre-chilled mortar pestle containing 50 mM sodium phosphate buffer (pH 7.2) and polyvinyl pyrrolidone (PVP) under ice-cold condition. Then centrifuge at 4 ºC for 15 min with 10,000 rpm and the supernatant was collected. For chlorophyll estimation, at first collect fresh leaves, homogenize in 80% acetone, filter using Whatmann no. 1 filter paper and collect in a sterile test tube. Then the absorbance was measured in UV–VIS spectrophotometer at 645 nm and 663 nm respectively.

Similarly, fresh leaves were crushed with 95% ethanol and alcoholic fraction was evaporated off in a boiling water bath. Then centrifuge at 4 ºC and 10,000 rpm for 10 min and collect the supernatant. Total soluble sugar contents was measured by using the anthrones method by Plummer.[14]

Plant Zn Content and Zn Translocation Index (ZTI): The Zn content of experimental plants shoot and root portion was carried out commercially by AAS (Atomic Absorption Spectroscopy) in the State Soil Testing Laboratory, Malda, West Bengal. Zn translocation index (ZTI) was calculated by using the formula.[30,31]

ZTI = [Zn concentration in plants / Zn concentration in shoot] × 100

Statistical analysis: All experimental results are demonstrated as mean ± SE (Standard error) with Anova.

Results

Approximately seventy-two bacterial isolates were obtained from the rhizospheric soil of agricultural areas. Following initial screening, only four zinc solubilizing bacteria (ZSB) were identified based on factors such as solubilization zone, solubilization index and the quantity solubilized, demonstrating the ability to solubilize zinc sources like ZnO. These strains were designated as SH 11, SH 25, SH 30 and SH 39.

ZSB Screening: The strain SH 25 demonstrated the largest solubilization zone, measuring 2.9 cm followed by SH 11 with measurements of 2.4 cm, SH 30 with measurements of 1.8 cm and SH 39 with measurements of 1.2 cm in the medium supplemented with ZnO.

Gram staining for morphological characterization: During Gram staining, three strains of bacteria, namely SH 11, SH 25 and SH 39, were found to be Gram positive as they retained the colour of safranin. On the other hand, the SH 30 strain was identified as Gram negative because it retained the colour of crystal violet.

Biochemical characterization of ZSB: All selected ZSB showed promicing results as in table 1.

Table 1
Biochemical characterization

Biochemical characterization	Result of ZSB isolates			
	SH 11	SH 25	SH 30	SH 39
Catalase activity	++	++	+	_
Coagulase test	_	+	_	_
Methyl red test	+	_	+	+
Voges-Proskauer test	_	++	+	_
Production of acid gas	+	+	+	+
Nitrate test	1	++	+	+
Oxidase test	+	++	+	+

[#](+) indicate positive result and (-) indicate negative result.

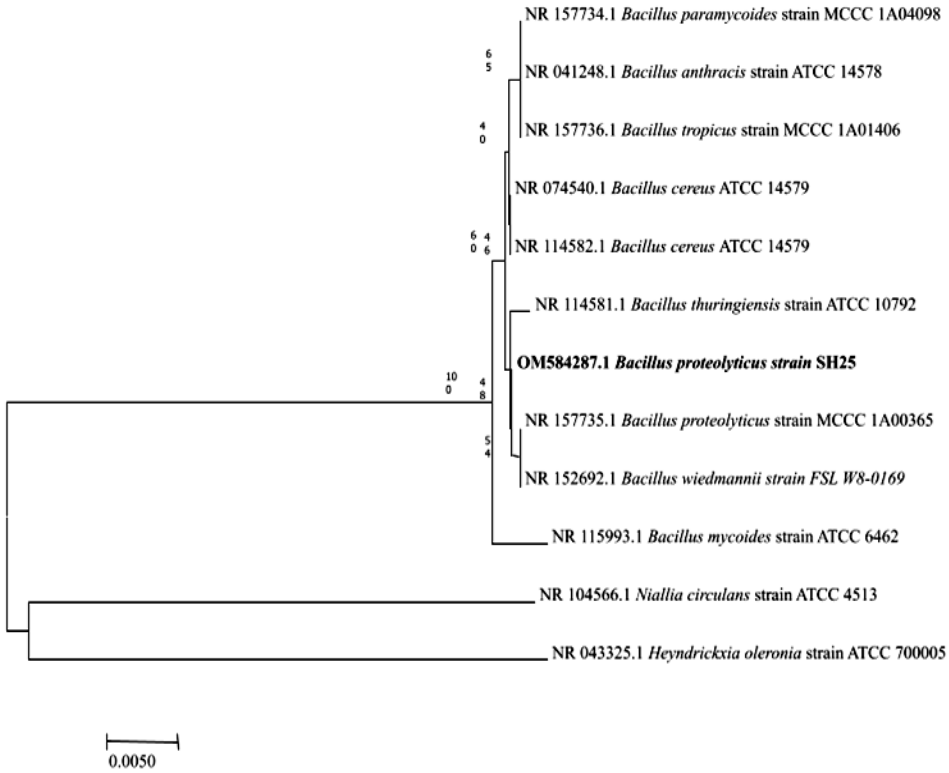

Figure 1: Phylogenetic hierarchy of SH 25 isolates based on 16S rDNA gene sequencing

Molecular chareterization: Based on 16S rDNA sequence analysis and MALDI-TOF-MS, the most potential ZSB strains (SH 25) was identified as *Bacillus proteoliticus.*

ZSB's ability to enhance plant growth: Four specifically chosen ZSB isolates exhibited positive outcomes on PGP activity assessments. Furthermore, an assay was conducted to measure the activity of extracellular hydrolytic enzymes such as protease, cellulase, chitinase and amylase. Out of the 4 ZSB isolates, SH 25 exhibited the highest positive result in table 2.

Antibiotic sensitivity assay: Various antibiotics exhibited diverse effects on isolates by creating distinct zones. All bacterial strains exhibited susceptibility to chloramphenicol and azythromycin antibiotics (Table 3).

Impact of ZSB on plant growth and development: The impact of SH 25 treatment on the growth parameters of maize plants including shoot and root length, shoot and root fresh weight, as well as dry weight and leaf count, was assessed 30 days after seed sowing. Furthermore, a Zn untreated pot was used as a blank while a commercially available $ZnSO_4$ treated pot was used as a control (Figure 2).

Impact on Carbohydrate Levels: Various plant treatments resulted in fluctuating carbohydrate concentrations. The highest carbohydrate content was observed in SH 25 (Figure 3).

Effect on chlorophyll content: Different treatments also showed the varying effects on chlorophyll contents.

Total protein estimation of treated and untreated plant: It has been observed that the total soluble protein content increased significantly in all the treatments in comparison to the control and maximum increased in SH 25 treated plant as shown in figure 5.

Estimation of Zn Translocation Index (ZTI) of treated plant: The effect of ZSB on the total Zn transformation on grain and ZTI is shown in table 4.

Table 2
Plant growth-promoting attributes and extracellular hydrolytic enzyme activity

Plant growth-promoting activities	**Result of ZSB**			
	SH 11	**SH 25**	**SH 30**	**SH 39**
IAA production	- ve	+ ve	+ ve	+ ve
Ammonia production	+ ve	+ ve	+ ve	- ve
Phosphate solubilization	+ ve	+ ve	+ ve	+ ve
HCN production	+ ve	+ ve	+ ve	+ ve
Siderophore production	+ ve	+ ve	+ ve	- ve
Extracellular enzymes				
Protease activity	+ ve	+ ve	- ve	+ ve
Amylase activity	- ve	+ ve	+ ve	+ ve
Cellulase activity	+ ve	+ ve	- ve	+ ve
Chitinase activity	- ve	+ ve	+ ve	- ve

[#] '+ ve' means positive and '- ve' means negative.

Table 3
Effect of antibiotics on isolates (zone of inhibition in cm)

Treatment	**SH 11**	**SH 25**	**SH 30**	**SH 39**
Oxacillin	0.7	2	-	-
Azythromycin	2	2.1	1.4	1.1
Chloranphenicol	1.2	2.2	1	0.9
Rifampicin	0.9	1.3	0.8	-

"-" Resistant

Table 4
Zn Translocation Index (ZTI) of ZSB isolates

Treatment code	**ZTI**
Control	123.75±3.1
Control + Zn	125±3.3
SH 11	133±4.2
SH 25	150±5
SH 30	144±3.8
SH 39	140±3.5

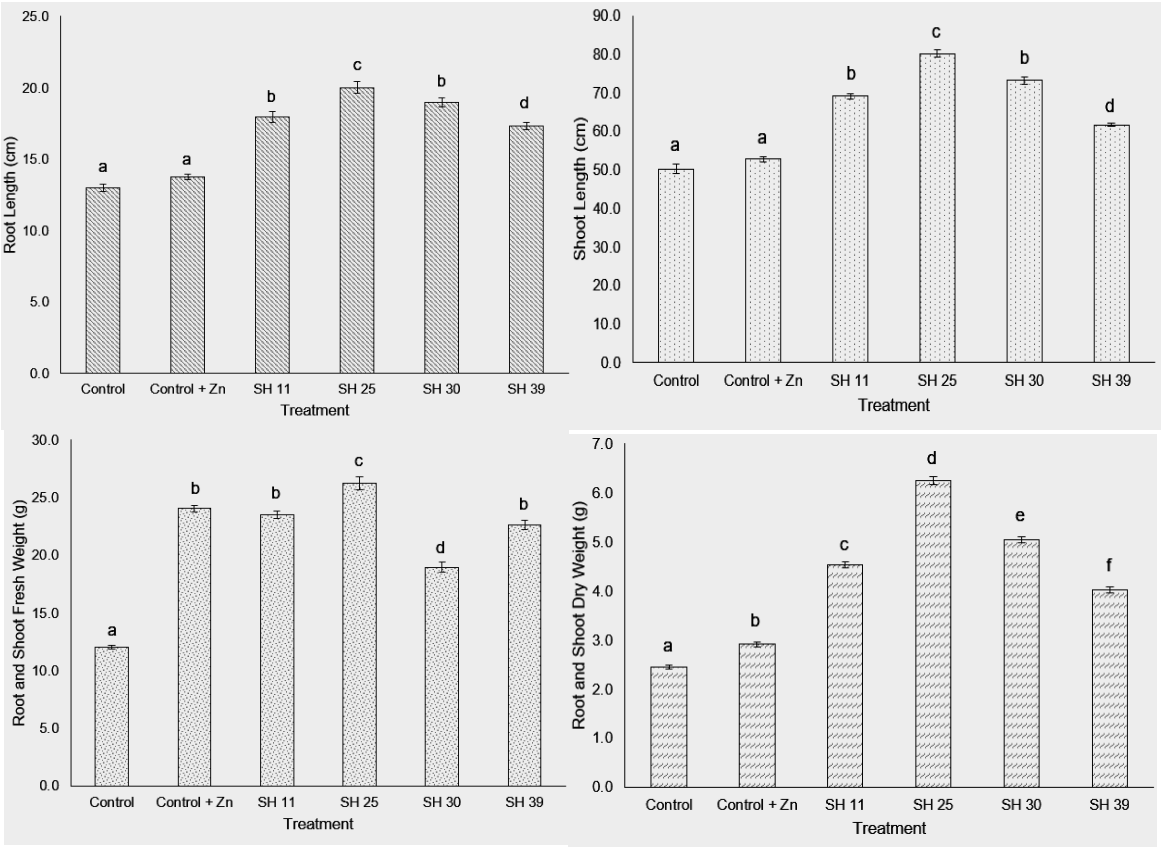

Figure 2: The effects of ZSB on plant growth after 30 days

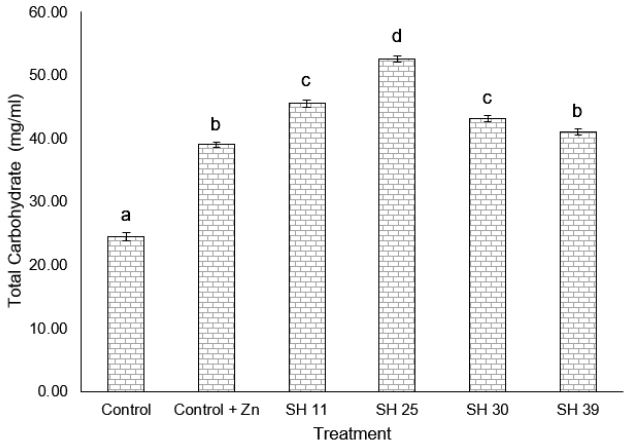

Figure 3: Total carbohydrate content (mg/mL) of treated plant.

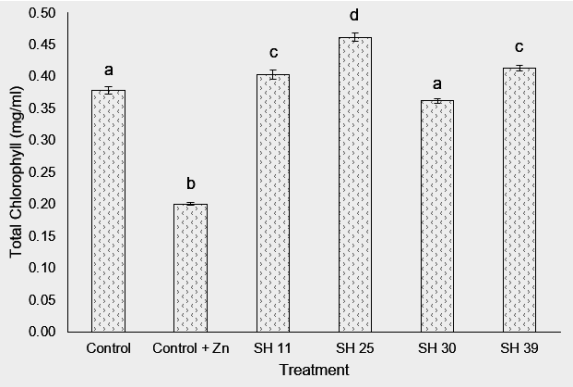

Figure 4: Total Chlorophyll Content (mg/mL) of treated plant.

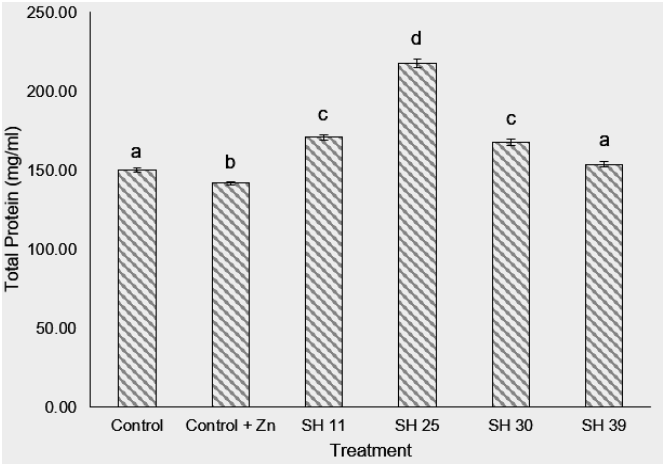

Figure 5: Estimation of total protein (mg/mL) of treated plant.

Discussion

Zinc plays a pivotal role in facilitating the growth and development of plants. Within enzymes, cysteine, histidine and glutamate or aspartate are the primary sites where zinc exerts its crucial binding effects. Zinc plays an important role in cellular functions, protein metabolism, chlorophyll biosynthesis, photosynthesis and seed maturation. However, zinc deficiency poses a common challenge, resulting in reduced crop yields. To address this issue, various methods such as fertilizer application, genetic engineering and transgenic approaches are employed. Nonetheless, the use of zinc-containing fertilizers has significant implications for soils, human health and the environment.

In this study, zinc solubilizing bacteria (ZSB) were isolated from agricultural fields in in the Northern region of West Bengal. Following initial screening, four proficient ZSB strains were identified based on the formation of clear zones around colonies when exposed to various zinc ores. These selected strains underwent morphological, biochemical and plant growth-promoting (PGP) tests, along with molecular identification via 16S rDNA sequencing. Zinc solubilizing bacteria aid in converting insoluble zinc into a form accessible to plants, aiming to identify strains suitable for use as zinc bio-fertilizers. All selected ZSB isolates were characterized as Gram-positive bacteria and exhibited positive results for carbohydrate, protein and indole acetic acid (IAA) production.

IAA plays a vital role in plant cell division, expansion, differentiation, root elongation, seed germination and flowering, ultimately influencing photosynthesis. Additionally, certain strains such as SH 25 demonstrated positive results for methyl red and Voges-Proskauer tests, as well as for nitrate and oxidase tests. Some strains including SH 11, SH 25 and SH 30 produced siderophore which acts as a solubilizing agent for iron and other heavy metals under iron-limiting conditions.

Moreover, strains SH 11, SH 25 and SH 39 exhibited phosphate solubilization capabilities, forming clear zones by solubilizing suspended tricalcium phosphate (TCP) due to the release of organic acids. Among all strains, SH 25 displayed the highest levels of carbohydrate and protein production. Potted plants treated with bacterial strains exhibited longer shoot and root lengths compared to controls and those treated with zinc fertilizer. The enhanced growth performance suggests that bacterial strains facilitate nutrient uptake and promote plant growth.

Furthermore, the four selected strains were found to produce indole acetic acid, which aids in elongating and multiplying plant cells, thereby enhancing shoot and root length. Zinc solubilization mechanisms include the production of chelating agents and organic acids. All strains tested positive for hydrogen cyanide production which can inhibit phytopathogens and can disrupt cellular energy supply, ultimately leading to organism death.

Conclusion

Zinc solubilizing bacteria offer a promising solution to numerous challenges faced in modern agriculture. Particularly in developing nations like India, the necessity of chemical fertilizer for crop production has increased tremendously to meet the nutritional demands of crops. However, the excessive use of fertilizers can lead to detrimental consequences such as soil fertility depletion and adverse environmental impacts, along with abnormalities in plant growth. To address these concerns and to reduce the over dependency on chemical inputs, alternative approaches that foster eco-friendly agricultural practices, have emerged.

The *Bacillus proteolyticus*SH 25 strain, possessing zinc solubilizing capabilities, successfully transformed insoluble zinc sources into soluble forms, demonstrating its proficiency in enhancing the growth of maize plants.

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