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Isolation and Characterization of Zinc Solubilizing Bacteria from Kashmir Himalayas, India

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ABSTRACT

Keywords

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Colletotrichum capsici; Zinc solubilizing bacteria

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Rhizosphere soil samples from Brinjal crop were collected from the 12 different locations in north Kashmir districts with the objective to isolate the zinc solubilizing bacteria, their screening and characterization. Zinc carbonate was used as insoluble zinc source. Out of 80 Zn solubilizers, 10 most outstanding isolates were maintained for further screening for mineral solubilization (Zn and K). Among these $Z_nsb(Kup)M2$ which was identified as *Bacillus cereus* by molecular techniques, showed maximum solubilization index (S.I) and solubilization efficiency (S.E) with 2.4 and 340 % at 48 hrs and 3.8 and 480 % at 72 hr of incubation respectively. In broth containing zinc carbonate, the isolate *Bacillus cereus* { $Z_nsb(Kup)M2$ } released significantly highest zinc content of 12 ppm/ml 10 days after incubation. Four isolates also showed IAA activity, potassium and phosphate solubilization with highest index of 1.28 and 1.66 shown by *Bacillus cereus*, further the same isolate also possessed antagonism against *Colletotrichum capsici*.

Introduction

Zinc micronutrient plays active role in various metabolic processes in plants as well as animals. It is present as co- factor and metal activator in many enzymes. The role of zinc in the nutrition and physiology of both eukaryotic and prokaryotic organisms is widely studied. Such as the maintenance of structural and functional integrity of biological membranes and facilitation of

protein synthesis and gene expression. Among all metals, Zn is needed by the largest number of proteins. Zinc-binding proteins make up nearly 10 % of the proteomes in eukaryotic cells, and 36% of the eukaryotic Zn-proteins are involved in gene expression (Claudia *et al.*, 2006). Tolerance to environmental stress conditions has a high requirement for Zn to regulate and maintain the expression of genes needed to protect cells from the detrimental effects of stress

(Cakmak, 2008). The deficiency of zinc adversely affects the growth, development and yield of crop plants (Cakmak, 2008). The cultivation of crops and soil management practices reduce the large amounts of zinc from the pool of soil (Prasad, 2010). Zinc deficiency is widely prevalent world over, in India more than 50% of soils are deficient in zinc (Singh *et al.*, 2005). The zinc deficiency has been reported from the agricultural soils of Kashmir valley also (Wani *et al.*, 2013). The zinc deficiency in soils is reflected in crops which ultimately adversely affect the human and animal health who consume the food grains with poor zinc contents. Zinc deficiency is a critical nutritional and health problem in human beings, affecting nearly one third of the world population (Hotz and Brown, 2004). The Copenhagen Consensus concluded that zinc deficiency together with vitamin A deficiency as the top priority global issue and it was also recommended that eliminating zinc deficiency will result in immediate high impacts and high returns for humanity in the developing world.

Majority of the soils are containing high content of total zinc that exists in the unavailable forms in minerals like smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite (Zn_2SiO_4), and hopeite ($Zn_3(PO_4)_2 \cdot 4H_2O$) (Lindsay, 1979). It is therefore highly important to devise strategies and utilize the technological interventions for improving the zinc availability by mobilizing the already insoluble soil zinc reserves. Bio-geochemically very important numerous interactions occur in soils among various minerals and microbes which in most of the cases aid in unlocking of the otherwise trapped or occluded nutrient metal ions. Solubilization can be performed by a number of mechanisms, the most probable ones include excretion of metabolites such as organic acids, proton extrusion or production

of chelating agents, (Sayer and Gadd, 1997). Zinc bearing minerals are also solubilized by an array of soil borne bacterial and fungal isolates with special reference to *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Glucanacetobacter*, *Micrococcus* *etc* (Bapiri *et al.*, 2012). The present research was also conducted to screen some potential zinc solubilizing bacterial isolates for their further use in.

Materials and Methods

Collection of soil samples

A total of 36 rhizosphere soil samples were collected from brinjal rhizosphere from 12 locations of three districts (Bandipora, Kupwara, Baramulla) of Kashmir valley (four locations from each district and three samples from each location). After collection, a portion of each sample was immediately transferred to laboratory and stored at 4°C for microbial analysis while as the rest part of soil samples was shade dried and powdered and stored physical and chemical parameters.

Isolation of zinc solubilizing bacteria from collected soil samples

Serial dilution pour plate technique was used for isolation of zinc solubilizing bacteria and 1 gm of rhizosphere soil from each sample was used for serial dilution. The samples were serially diluted upto 10^{-5} dilution factor. The modified Pikovaskya's agar media containing (glucose-10.0 g; ammonium sulphate- 1.0 g; potassium chloride- 0.2 g; dipotassium hydrogen phosphate- 0.2 g; magnesium sulphate – 0.1 g; yeast extract- 0.2 g; distilled water- 1000 ml; pH– 7.0) given and 0.1% insoluble zinc carbonate as zinc source was used for the isolation of zinc solubilizing bacteria (Bapiri *et al.*, 2012). Sterilized medium was poured in to sterilized petri plates under aseptic conditions after

solidification of medium 0.5 ml of diluted sample suspension from 10^{-5} dilution was poured on these plates which were incubated at $28 \pm 2^{\circ}\text{C}$ for 72 hours in BOD incubator. A total of 80 isolates showed zinc solubilization. Pure cultures of these isolates were obtained by repeated streaking and were preserved for further studies (Bunt and Rovira, 1955).

Characterization of isolated zinc solubilizing bacteria

Preliminary characterization of the some outstanding zinc solubilizers was performed on the basis of colony features, morphological characteristics and biochemical tests like Gram's staining, Hydrogen sulphide test, catalase test, starch hydrolysis test, methyl red test, urease test, Voges-Proskauer test, casein hydrolysis test, gelatin liquification, growth at 7% NaCl, citrate utilization (Archana *et al.*, 2013). By morphological and biochemical characterization the selected isolates were identified up to genus level.

Screening of isolates for qualitative and quantitative zinc solubilization

For qualitative analysis plate assay was used and for quantitative analysis broth assay was used.

Plate assay

Isolated test organisms were inoculated on modified Pikovaskaya's media containing zinc carbonate as zinc source (ZnCO_3) and incubated at $28 \pm 2^{\circ}\text{C}$ for 72 hrs.

After incubation halo zones were observed around colonies which were measured after 48 hrs, and 72 hrs of incubation and zinc solubilization index and solubilization efficiency were calculated by using the following formula (Sunitha kumari *et al.*, 2016): $\text{S.I} = \text{Solubilization Index}$

$\text{S.I} = \text{halo zone diameter} - \text{colony diameter} / \text{colony diameter}$

Solubilization efficiency was calculated by using formula: $\text{S.E} = \text{Solubilization Efficiency}$

$\text{S.E} = \text{halo zone diameter} / \text{colony diameter} \times 100.$

Broth assay

For quantitative analysis broth assay was used. The modified Pikovaskaya's broth containing 0.1% insoluble zinc carbonate (ZnCO_3) as zinc component was prepared and split into 25 ml aliquots in 250 ml Erlenmeyer flasks and sterilized in an autoclave. Then flasks were inoculated with 1 ml suspension of test culture. Experiment was done in triplicates and control was also maintained. The quantity of zinc solubilized was estimated after 7th day and 10th day of incubation. A portion of the inoculated broth /culture was centrifuged at 10000 rpm for 10 minutes, the supernatant was filtered through Whatman no -1 filter paper and diluted 20 times with double distilled water, later on fed to Atomic Adsorption Spectrophotometer (AAS) for estimation of available zinc content (Sarvanan *et al.*, 2003)

Screening of zinc solubilizing bacteria for other beneficial plant growth promoting properties

The zinc solubilizing bacterial isolates were also screened for the plant growth promoting properties like potassium solubilization (Aleksandrov *et al.*, 1967), phosphate solubilization (Pikovskaya, 1948), antagonistic activity against plant pathogen *Colletotricum capsici* (collected from division of plant pathology, SKAUST-K, Wadura), indole acetic acid production and ammonia production (Archana *et al.*, 2013). For

potassium solubilization, Alexandrov medium used containing 0.2 % mica powder as potassium source and for phosphate solubilization, Pikovaskaya's agar medium was used. These two were done in plate assay method and after spot inoculation of cultures measured halo zone diameter, colony diameter and calculated S.I and S.E. For testing antagonistic activity against to plant pathogen *colletotricum capsici* potato dextrose agar medium used and tested by duel culture technique (Skidmore and Dickinson, 1976). Indole test was done by using medium (Gorden and Weber, 1951) containing peptone- 20.0 g; which was containing sufficient tryptophan, sodium chloride- 5.0 g; Distilled water- 1000 ml; and p^H – 7.4 and 0.2 ml of Kovac's reagent was used for 5ml of test culture medium. Positive test for indole production was formation of cherry red colour ring.

Results and Discussion

A total of 80 bacterial isolates showed halo zones on modified Pikovaskaya's medium supplemented with 0.1% zinc carbonate only top 10 best performers were selected based on halo zone diameter and were maintained in nutrient agar slants for further utilization. After morphological and biochemical characterization the isolates were identified up to genus level as out of 10, eight isolates were *Bacillus* type and 2 belonged to *Pseudomonas* genus.

The isolates were named as $Z_nsb(Kup)M2$, $Z_nsb(Band)S1$, $Z_nsb(Band)S1_1$, $Z_nsb(Band)S23$, $Z_nsb(Band)S24$, $Z_nsb(Band)S6_2$, $Z_nsb(Band)S7_{2-5}$, $Z_nsb(Band)S9_{2-1}$, $Z_nsb(Band)S9_{2-2}$, $Z_nsb(Band)S3_2$ were further characterized on biochemical basis (Table 1) (Plate 1 and 2). Among the screened 10 isolates $Z_nsb(Kup)M2$ was identified as *Bacillus cereus MH429978.1* by molecular techniques, showed maximum solubilization index (S.I) and solubilization efficiency (S.E)

with 2.4 & 340 % at 48 hrs and 3.8 & 480 % at 72 hr of incubation respectively, these were followed by $Z_nsb(Band)S1$ with 1.16 & 216.6% at 48 hrs and 1.66 & 166.6% at 72 hrs and least S.I and S.E was shown by $Z_nsb(Band)S24$ at 48 hrs and $Z_nsb(Band)S9_{2-2}$ at 72 hrs of incubation (Table 2). All 10 isolates significantly increased available zinc in broth containing zinc carbonate at 7 DAI, and 10DAI. Highest solubilization in broth media was shown by isolate $Z_nsb(Kup)M2$ (*Bacillus cereus MH429978.1*) with 12 ppm/ml, followed by $ZnS1_1$ (band) 11.006 ppm/ml and $ZnS1$ (band) 10.529 ppm/ml at 10 DAI (Table 3).

Screening of isolates for other beneficial effects

All the selected isolates were screened for other beneficial effects such as potassium and phosphate solubilization, Indole acetic acid production and antagonistic activity against *Colletotricum capsici* (Plate 3). Out of 10 only four isolates such as $Z_nsb(Kup)M2$ (*Bacillus cereus MH429978.1*), $Z_nsb(Band)S1$, $Z_nsb(Band)S1_1$, $Z_nsb(Band)S6_2$ showed potassium solubilization with solubilization index of -1.28, 0.66, 0.5 and 0.66 respectively (Table 4) and similarly four isolates $Z_nsb(Kup)M2$ (*Bacillus cereus MH429978.1*), $Z_nsb(Band)S1$, $Z_nsb(Band)S1_1$, $Z_nsb(Band)S24$ showed phosphate solubilization with solubility index of 1.66, 0.25, 0.5, 0.27 respectively (Table 5).

In addition to mineral solubilization, the isolates were screened for IAA production and antagonistic activity. Out of all four isolates $Z_nsb(Kup)M2$ (*Bacillus cereus MH429978.1*), $Z_nsb(Band)S6_2$, $Z_nsb(Band)S7_{2-5}$, $Z_nsb(Band)S9_{2-2}$ showed IAA production qualitatively and one isolate $Z_nsb(Kup)M2$ (*Bacillus cereus*) showed antagonistic activity against to *colletotricum capsici*.

Statistical analysis: Statistical analysis was done by using OP STAT software.

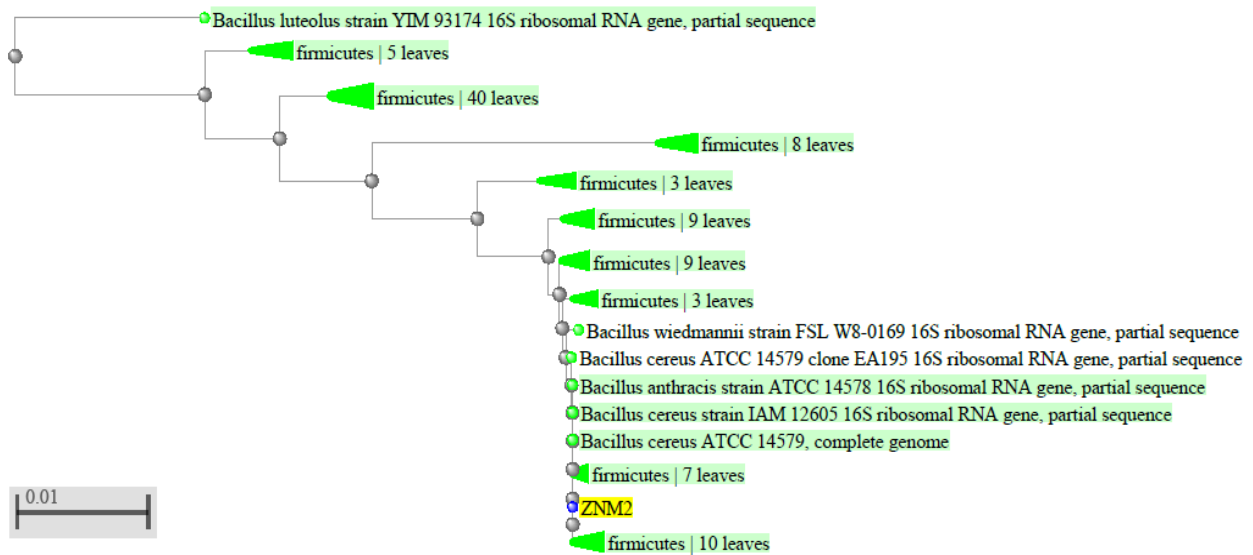
Molecular analysis

Out of all isolates the most efficient strain was molecularly identified up to species level

by using 16 S rRNA sequencing. For the molecular analysis efficient strain Z_nsb (Kup)M2 was sent to Agarkar Research Institute, Pune, Maharashtra. The isolate Z_nsb(Kup)M2 was identified as *Bacillus cereus* MH429978.1 by 16S rRNA sequencing.

>ZNM2

CAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTC
ACTTATGGATGGACCCGCGTTCGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACCTCTGTTGTTAG
GGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAACTGGGAGACTTGAGTGCAGAAGAGGAA
AGTGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGG
CGAAGGCGACTTTCTGGTCTGTAACCTGACACTGAGGCGCGAAAGCGT



Soil is a habitat for different types of beneficial and harmful microorganisms, which are involved in natural cycles like carbon, nitrogen, phosphorus, potassium and micronutrients. The multiple benefits harvested from this microbial wealth includes

improving nutrient availability and controlling plant pathogens besides producing plant growth promoting hormones for better crops. Plants absorb zinc as divalent (Zn⁺²) cation from soil solution.

Table.1 Morphological and biochemical characters of selected isolates of zinc solubilizing bacteria and probable genus

Isolates	Colony Characters	1	2	3	4	5	6	7	8	9	10	11	Cell shape	Gram reaction	Probable Genus
Z _n sb(Kup)M2	Yellow, rough, large, circular, raise	+	+	-	+	+	++	+	++	+	+	+	Rods	+ve	<i>Bacillus cereus</i>
Z _n sb(Band)S1	smooth, creamy bluish white, raise	+	-	-	+	+	++	+	++	-	+	+	Short rods	-ve	<i>Pseudomonas sp</i>
Z _n sb(Band)S1 ₁	white, rough, small, flat surface	+	-	++	+	-	++	+	+	+	+	+	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S23	White, rough, circular	+	-	++	+	+	++	+	+	+	+	-	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S24	White, rough, circular	+	-	++	+	-	++	+	+	+	+	-	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S6 ₂	Sky blue, Smooth raised	+	-	+	+	+	-	+	-	-	+	+	Short rods	-ve	<i>Pseudomonas sp</i>
Z _n sb(Band)S7 ₂₋₅	Wheat white color, rough, flat	+	-	++	+	+	++	-	-	-	+	-	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S9 ₂₋₁	Light pinkish, rough, flat	+	-	++	+	-	++	+	-	+	+	-	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S9 ₂₋₂	White, rough, flat	+	-	++	+	+	++	+	-	-	+	-	Rod	+ve	<i>Bacillus sp</i>
Z _n sb(Band)S3 ₂	white, rough, flat	+	-	++	+	+	++	+	+	+	+	-	Rod	+ve	<i>Bacillus sp</i>

Note: Positive: (++) ; weakly positive (+); Negative: (-)

1. Catalase test, 2. Urease test, 3. Methyl red test, 4. Voges-Proskauer test, 5. Starch hydrolysis, 6. Casein hydrolysis, 7. H₂S production, 8. Gelatin liquefaction, 9. Growth at 7% NaCl, 10. Dextrose utilization, 11. Citrate utilization.

Table.2 Qualitative estimation of Zinc carbonate solubilization through Solubilization index and Solubilization efficiency

Isolate	48 hr of incubation				72 hr of incubation			
	C.D (mm)	H.D (mm)	S.I	S.E (%)	C.D (mm)	H.D (mm)	S.I	S.E (%)
Z _n sb(Kup)M2 (<i>Bacillus cereus</i> MH429978.1)	5	17	2.4	340	5	24	3.8	480
Z _n sb(Band)S1	6	13	1.16	216.6	6	16	1.66	266.6
Z _n sb(Band)S1 ₁	7	10	0.42	142.8	7	14	1	200
Z _n sb(Band)S23	6	9	0.5	150	6	11	0.83	183.3
Z _n sb(Band)S24	5	6	0.2	120	5	8	0.6	160
Z _n sb(Band)S6 ₂	8	13	0.62	162.5	8	15	0.87	187.5
Z _n sb(Band)S7 ₂₋₅	7	11	0.57	157.1	7	12	0.71	171.4
Z _n sb(Band)S9 ₂₋₁	7	11	0.57	157.1	7	13	0.85	185.7
Z _n sb(Band)S9 ₂₋₂	7	10	0.42	142.8	7	11	0.57	157.1
Z _n sb(Band)S3 ₂	6	11	0.83	183.3	6	12	1	200

C.D = Colony diameter; H.D = Halo zone diameter; S.I = Solubilization index; S.E = Solubilization efficiency

Table.3 Quantitative estimation of Zinc carbonate solubilization

Isolate	7 DAI(ppm/ml)	10 DAI(ppm/ml)
Control	0.020	0.020
Z _n sb(Kup) M2 (<i>Bacillus cereus</i> MH429978.1)	9.232	12.202
Z _n sb(Band)S1	8.640	10.529
Z _n sb(Band)S1 ₁	8.426	11.006
Z _n sb(Band)S23	5.503	6.557
Z _n sb(Band)S24	8.890	9.453
Z _n sb(Band)S6 ₂	9.771	10.005
Z _n sb(Band)S7 ₂₋₅	5.465	6.220
Z _n sb(Band)S9 ₂₋₁	7.645	8.543
Z _n sb(Band)S9 ₂₋₂	4.922	5.254
Z _n sb(Band)S3 ₂	8.222	9.224
C.D(p≤0.05)	0.035	0.077
SE(m)	0.012	0.026

Table.4 Qualitative estimation of Potassium solubilization

Isolate	At 72 hrs of incubation			
	C.D (mm)	H.D (mm)	S.I	S.E (%)
Z _n sb(Kup)M2(<i>Bacillus cereus</i> MH429978.1)	7	16	1.28	228.5
Z _n sb(Band)S1	6	10	0.66	166.6
Z _n sb(Band)S1 ₁	6	9	0.5	150
Z _n sb(Band)S6 ₂	6	10	0.66	166.6

C.D = Culture Diameter; H.D = Halozone Diameter; S.I = Solubilization index; S.E = Solubilization Efficiency

Table.5 Phosphate solubilization of isolates

Isolate	At 72 hrs of incubation			
	C.D (mm)	H.D (mm)	S.I	S.E (%)
Z _n sb(Kup)M2 (<i>Bacillus cereus</i> MH429978.1)	6	16	1.66	266.6
Z _n sb(Band)S1	8	10	0.25	125
Z _n sb(Band)S1 ₁	10	15	0.5	150
Z _n sb(Band)S24	11	14	0.27	127.7

Plate.1 Zinc solubilization by zinc solubilizing isolates

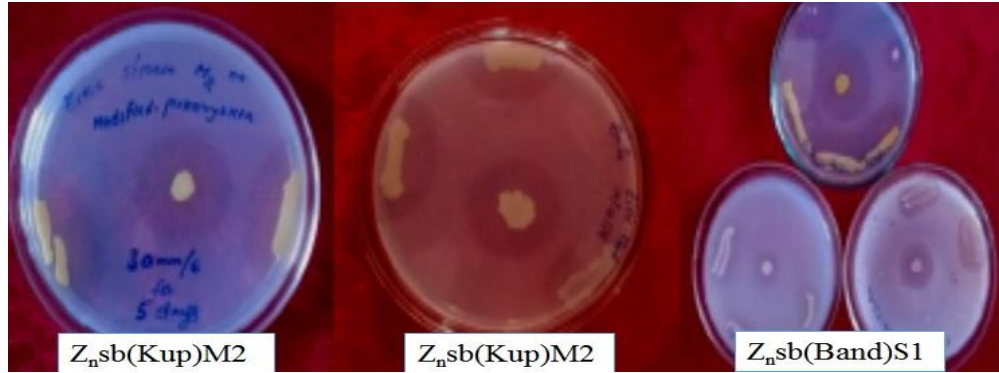
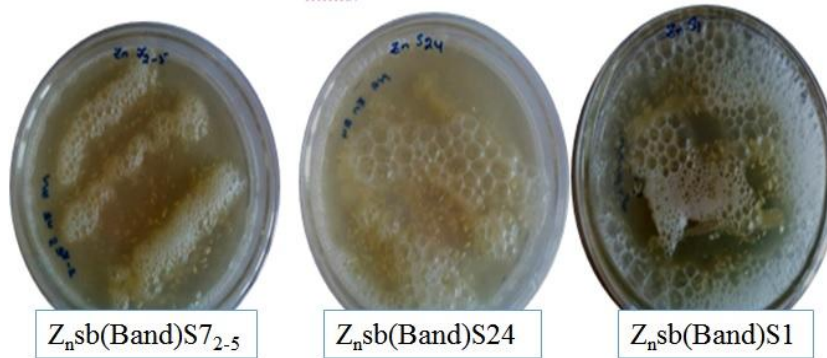
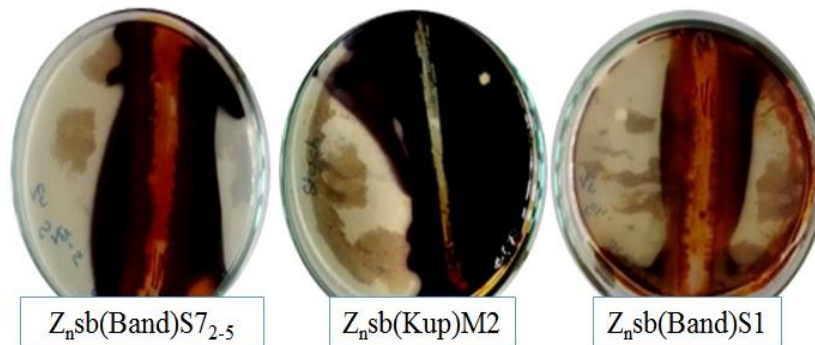


Plate.2 Biochemical tests of zinc solubilizing isolates

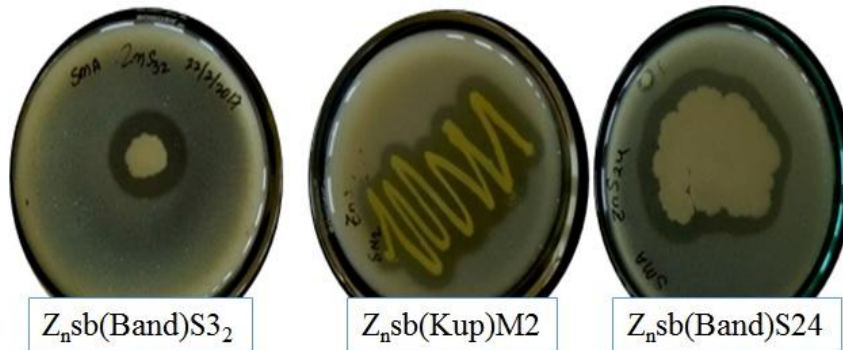
a. Catalase activity of zinc solubilizing bacteria



b. Starch hydrolysis activity of zinc solubilizing bacteria



c. Casein hydrolysis activity of zinc solubilizing bacteria



d. Phosphate solubilization of zinc solubilizing bacteria

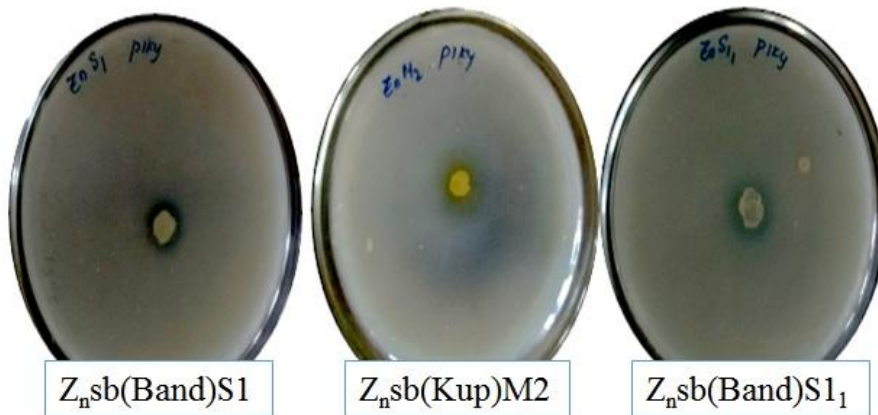


Plate.3 Biocontrol activity of Znsb(Kup)M2(*Bacillus cereus* MH429978.1) against to *colletotricum capsici*



However, major part of the zinc is present in rocks and minerals in unavailable form. It is reported that a variety of microorganisms plays significant role in increasing zinc availability to plants by solubilization of zinc from minerals (Sentil Kumar *et al.*, 2004). Apart from zinc solubilization microbes play

important role in solubilization of phosphorus and potassium minerals. Morphologically out of 10, eight isolates were *Bacillus* type and 2 belonged to *Pseudomonas* genus and all showed distinct Zn solubilization efficiency in both qualitatively and quantitatively. These results are supported by the findings of Nazia

Jamil *et al.*, (2010), Desai *et al.*, (2012), who also isolated *Bacillus* sp. and *Pseudomonas* sp. as zinc solubilizers from rhizosphere soils. All isolates showed significant difference in solubilization of mineral ($ZnCO_3$) in broth medium. By increasing the incubation period solubilization of zinc mineral also increased quantitatively. Out of all 10 isolates one isolate $Zn_{sb}(Kup)M2$ (*Bacillus cereus* MH429978.1) showed highest solubilization qualitatively by means of solubilization index (2.4 at 48 hrs and 3.8 at 72 hrs) and solubilization efficiency (340 % at 48 hrs and 480 % at 72 hrs) followed by $Zn_{sb}(Band)S1$ (*Pseudomonas* sp.) showed highest S.I (1.16 at 48 hrs and 1.66 at 72 hrs) and S.E (216.6 % at 48 hrs and 266.6 % at 72 hrs) and so on. Also $Zn_{sb}(Kup)M2$ (*Bacillus cereus* MH429978.1) showed highest solubilization of zinc mineral in broth medium with 9.232 ppm/ml at 7 days after incubation and 12.202 ppm/ml at 10 days after incubation. These are significantly differentiate with others. These finding were similar to that of earlier findings, who also reported *Bacillus cereus* and *Pseudomonas* sp. have the ability to good solubilization of zinc minerals (Muhammad shakeel *et al.*, 2015).

The mechanisms of zinc solubilization and other mineral solubilization might be a consequence of proton extrusion, production of organic acids like gluconic acid, oxalic acid, citric acid etc. and other chelating metabolites (Agnihorti, 1970) of microbial origin possibly in a non-specific way leading to solubilization of zinc and other minerals.

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