International Journal of Agriculture Sciences
ISSN: 0975-3710&E-ISSN: 0975-9107, Volume 8, Issue 56, 2016, pp.-3078-3081.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

ISOLATION AND CHARACTERIZATION OF ZINC SOLUBILIZING BACTERIA FROM STONE QUARRY DUST POWDER

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Received: October 29, 2016; Revised: November 06, 2016; Accepted: November 07, 2016; Published: November 18, 2016

Abstract- Substantial quantity of inorganic zinc applied to the soil is converted into unavailable form, but it can be converted back into available form to make it access to the plants by the intervention of zinc solubilizing bacteria. In this study, two zinc solubilizing bacteria were isolated from stone quarry dust powder, after purification and characterization, these isolates were identified as *Bacillus aerophilus* and *Enterobacter* sp by 16S rRNA gene sequencing. These two new isolates along with twenty two isolates including the reference strain of *Bacillus aryabhattai* obtained from microbiology lab, ICAR-IIHR, Bengaluru were then assessed for their ability of zinc solubilization in both solid and liquid basal media. The results indicated that among all isolates, *B. aryabhattai* showed significant increase in solubilization and produced larger clear halo zone on solid agar medium amended with 0.1% of zinc sources *viz.,* zinc oxide (42.1 mm), zinc carbonate (46.3 mm) and zinc phosphate (26.7 mm). Similarly, in liquid basal medium containing 0.1% of zinc sources, *B. aryabhattai* enhanced solubilization significantly and released the higher amount of zinc with zinc oxide (554.8 ppm), zinc carbonate (368.6 ppm) and zinc phosphate (576.5 ppm) after 15 days of incubation as compared with other isolates. The pH of the culture broth was found to be decreased in the range of 3.33 to 3.35. Among all isolates, *B. aryabhattai* was found as the most promising zinc solubilizing bacteria; hence it can be exploited as potential bio-fertilizer for sustainable crop production.

Keywords- Zinc solubilizing bacteria, Quarry powder, Zinc sources, *Bacillus aryabhattai*, Solubilization

Citation: Vidyashree D.N., et al., (2016) Isolation and Characterization of Zinc Solubilizing Bacteria from Stone Quarry Dust Powder. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 8, Issue 56, pp.-3078-3081.

Academic Editor / Reviewer: Prof P. J. George

Introduction

Zinc (Zn) is an essential element for the growth and development of humans, plants and animals. It is considered as one of the eight essential trace elements required for crop growth and production [1]. It is present on earth's crust in tune of 0.008%, and zinc has an important role in nutrition and metabolic activities of both eukaryotic and prokaryotic organisms and act as a cofactor or metal activator in various enzyme systems [2]. Both macro and micro nutrients are essentially needed for plant growth and reproduction. Application of these nutrients in the form organic or inorganic are taken by the plant roots along with water. Zinc is one of the imperative micronutrients required relatively in small concentrations in tissues for healthy growth and reproduction of plants. Zinc deficiency in plants found to reduce membrane integrity and carbohydrates synthesis, auxins, nucleotides, cytochromes, chlorophyll and also develops susceptibility to heat stress [3]. Excessive use of zinc fertilizers also poses problems to human being causing the impaired absorption of iron and copper.

Zinc deficiency is common in humans, animals and plants. More than 30 per cent of world's population suffers from Zn deficiency [4]. Zinc plays an important role in basic cellular functions in all the group of living organisms and is also play important role in human immune system. Zinc acts as a catalytic component in various kind of enzymes. The optimum dietary intake of zinc for human adults is 15 mg per day.

In soil, it undergoes a complex changes and precipitate with other element that is greatly influenced by pH and microflora [5-6], which ultimately affects their accessibility to roots for absorption. Medium requirement of zinc is needed, but in critical concentration and if the amount available is not adequate, plants and animals will suffer due to physiological stress brought about by the dysfunction of several enzyme systems and other metabolic functions [7]. Phosphorus is the important element that interferes on zinc uptake, as zinc uptake reduces by increasing phosphorus in soil [8].

About 96 to 99% of the applied zinc is converted into different type of insoluble forms depending upon the soil types, physico-chemical reactions of the soil. The solubility of zinc is highly dependent on soil pH and moisture. Zinc occurs in soil as ores of augite, biotite, hornblende, olivine and sphalerite. However, availability of zinc from these sources is guided by many factors among which biochemical actions of rhizosphere microorganisms play an important role in converting such unavailable sources into available ones [9]. The zinc thus made unavailable can be reverted back to available form by application of bacterial strain capable of solubilizing it. Bacteria are known to immobilize zinc metal by precipitation and adsorption. The capability to dissolve immobilized zinc *i.e.* zinc carbonate, zinc oxide and zinc phosphate in appreciable quantity is not universal feature amongst the cultivable bacteria on soil surface. Few zinc solubilizing bacterial genera *viz.*, *Thiobacillus ferrooxidans, Thiobacillus thiooxidans*, *Acinetobacter* sp., *Bacillus* sp., *Gluconacetobacter* sp., *Pseudomonas* sp. and facultative thermophilic iron oxidizers were reported as zinc solubilizers [10]. As zinc is a limiting factor for crop production, this study on zinc solubilization by bacteria has an immense role in zinc nutrition to plants. In the present study, an attempt was made to isolate some

novel zinc solubilizing bacteria from quarry stone dust powder and assessed their potential for zinc solubilization and compared with reference strain *i.e. Bacillus aryabhattai.*

Material and Methods

Collection of stone quarry dust powder sample

The stone quarry dust powder was collected from a local quarry production unit near Hessaraghatta, Bengaluru, Karnataka and the sample was stored at 4°C for 48 hours and then used for isolation of zinc solubilizing bacteria.

Isolation and Identification of zinc solubilizing bacteria (ZSB) from quarry powder

Zinc solubilizing bacteria (ZSB) were isolated from stone quarry dust powder by serial dilution plate count method on petri plates containing basal medium containing 0.1 % insoluble zinc sources *viz.,* zinc oxide (ZnO), zinc carbonate ($ZnCO_3$) and zinc phosphate ($Zn_3(PO_4)_2$). The plates were incubated at room temperature (30±1°C) for 5 days and the colonies exhibiting larger zones of solubilization were selected, purified and the isolates were identified based on 16S r RNA gene sequencing.

Collection of zinc solubilizing bacteria

Twenty two bacterial strains including the reference strain of *B. aryabhattai* were obtained from Soil Microbiology Lab, Division of Soil Science and Agricultural Chemistry, ICAR-IIHR, Hessaraghatta, Bengaluru, India and screened for their ability in zinc solubilization of three different zinc sources as mentioned above. Based on the zinc solubilizing potential, among the 22 bacterial strains, five efficient strains *viz*., *Bacillus aryabhattai, Pseudomonas taiwenensis, Enterobacter oryzae, Bacillus* sp. (PAN-TM1) and *Enterobacter* sp.-1were selected for further study to compare with the two new ZSB isolates from quarry stone dust powder.

Screening of zinc solubilizing bacteria under *in vitro* condition

Qualitative estimation

The basal medium (glucose-10.0g; ammonium sulphate-1.0g; potassium chloride-0.2g, dipotassium hydrogen phosphate-0.1g, magnesium sulphate-0.2g, distilled water-1000ml, pH- 7.0) was prepared by supplementing 0.1% insoluble zinc sources separately (ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$), autoclaved at 121°C for 20min and then plated out in sterilized plastic petriplate. Then a 10 μL of 24 h old test cultures with a cell load of 1.2-1.7 x 10^9cfu /ml were spot-inoculated onto the agar plates and incubated at 30°C. The formation of clear zone around the bacterial colony was measured after 24 hours up to 7 days.

Quantitative zinc solubilization

50 ml basal broth medium containing different insoluble zinc salts (@ 0.1 % ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$) transferred into 100 ml Erlenmeyer flasks, autoclaved at 121^0 C for 20min. Then the flasks were inoculated with 1.0 ml suspension of the test culture with a cell load of 1.2-1.7 x 10^9cfu /ml. Experiments were conducted in triplicate along with an uninoculated control. The samples were withdrawn at 5, 10 and 15 days intervals, centrifuged to remove the debris and cells. Then the concentration of zinc in the supernatant was estimated by using atomic absorption spectrophotometer and the available zinc content was expressed in ppm.

Determination of pH

The pH (ELICO PE. 136) of the culture filtrate in both inoculated and uninoculated samples was determined at 5, 10 and 15 days intervals after inoculation.

Statistical analysis

The data were analyzed using Web Agri Stat Package version WASP2.0, and subjected to one way analysis of variance (ANOVA). Treatment difference was evaluated using least significant difference (LSD) at p = 0.05.

Results and Discussion

Qualitative analysis of the selected isolates for zinc solubilization abilities were examined on basal medium supplemented with 0.1% of different zinc sources and the results are given in [Table-1, Fig-1 & Fig-5]. Among all the selected ZSB isolates, *B. aryabhattai* produced significantly larger solubilization zone in medium supplemented with different zinc sources *viz*., zinc oxide (42.1 mm), zinc carbonate (46.3 mm) and zinc phosphate (26.7 mm) after seven days of incubation which was significantly differed from all other ZSB isolates [Table-1]. The two zinc solubilizing bacteria isolated from quarry stone dust powder, identified as *Bacillus aerophilus* and *Enterobacter* sp-1, have also shown lower zinc solubilizing ability as compared to *B. aryabhattai*. The isolates of *Enterobacter* sp.-1 (2.4-18.6 mm) and *E. oryzae* (4.5-6.8 mm) observed to be poor in zinc solubilizing ability as compared to other isolates.

Table-1 *Zinc solubilization potential of different bacterial isolates on agar medium supplemented with 0.1% of different zinc sources (7 days after inoculation)*

Bacterial isolates	Zinc Solubilization zone formation (mm)		
	Zinc oxide	Zinc carbonate	Zinc phosphate
B. aryabhattai	42.1	46.3	26.7
P. taiwenensis	06.3	22.6	06.3
E. oryzae	04.5	06.8	06.2
Bacillus sp.(PAN-TM1)	12.1	10.3	08.1
*Enterobacter*sp.-1	04.5	14.2	02.4
Bacillus aerophilus	08.6	20.2	10.4
*Enterobacter*sp.-2	18.7	08.6	16.4
S.Em±	0.46	0.53	0.54
CD at 5%	1.39	1.59	1.61

SEM-Standard error means
CD (p=0.05)-Critical difference at 5 % level

The quantitative estimation of zinc solubilizing efficiency of different bacterial isolates was also assessed in liquid basal medium supplemented with different sources of zinc @ 0.1 per cent *viz.,* zinc oxide, zinc carbonate and zinc phosphate. The results indicated that the amount of zinc solubilized from zinc oxide, zinc carbonate and zinc phosphate by all the bacterial isolates increased with increase in incubation time and at the same time the pH was found decreased [Tables-2, 3 & 4]. The data indicated that all the ZSB isolates were capable of solubilizing zinc oxide, zinc carbonate and zinc phosphate in varying levels. However, *B. aryabhattai* recorded significanly higher zinc solubilization *viz*., zinc oxide (554.8ppm), zinc carbonate (368.6ppm) and zinc phosphate (576.5ppm) when compared to other isolates after 15 days of incubation [Fig-2,3,4].

Table-2 *Zinc oxide solubilization and pH of the culture broth of different bacterial isolates under in vitro condition at different time intervals*

Bacterial isolates	Solubilization of Zinc oxide (ppm)					
	5 Days	pH	10 Days	pH	15 Days	pH
B. aryabhattai	380.9	4.14	469.8	3.93	554.8	3.35
P. taiwenensis	147.4	5.45	201.1	4.96	277.4	3.97
E. oryzae	54.3	6.74	83.6	6.69	91.6	6.61
Bacillus sp. (PAN-TM1)	143.3	5.61	196.2	5.23	274.3	4.10
*Enterobacter*sp.-1	31.7	6.81	40.2	6.74	78.8	6.36
Bacillus aerophilus	62.9	6.71	91.4	6.54	121.2	6.06
Enterobacter sp.-2	106.6	6.10	127.3	5.64	169.9	5.12
Un inoculated Control	4.0	7.02	7.0	7.09	11.10	7.12
S.Em±	3.84	0.11	10.79	0.05	49.0	0.06
CD at 5%	11.44	0.33	32.18	0.14	146.02	0.17

SEM-Standard error means | CD (p=0.05)-Critical difference at 5 % level

The pH of culture broth inoculated with *B. aryabhattai* was found drastically reduced from 7.12-7.30 to 3.33-3.91, which indicates that the above culture might have produced organic acids that played a major role in dissolution of insoluble zinc in liquid broth [Fig-2.1, 3.1, 4.1]. It was observed that the zinc solubilizing ability of *P. taiwenensis* (ZnO: 277.4 ppm, ZnCO3:288.4ppm and $Zn_3(PO_4)_2$:313.1 ppm) and *Bacillus* sp. (PAN-TM1) (ZnO: 274.3 ppm, $ZnCO_3$:264.6ppm and $Zn_3(PO_4)_2$: 298.3ppm) were found significantly at par with each other. The control broth recorded lowest zinc solubilization in different zinc compounds *viz*., ZnO-11.10

ppm, ZnCO 3-8.5ppm and $Zn_3(PO_4)$ 2-11.11ppm, after 15 days of incubation, wherein the pH was found unaffected (7.12- 7.26). Out of twenty four isolates examined, five isolates recorded higher concentration of zinc with greater than 100 ppm from different sources of zinc (zinc oxide, zinc carbonate and zinc phosphate).

Table-3 *Zinc carbonate solubilization and pH of the culture broth of different bacterial isolates under in vitro condition at different time intervals*

Bacterial isolates	Solubilization of Zinc Carbonate (ppm)					
	5 Days	pH	10 Days	pH	15 Days	pH
B. aryabhattai	264.7	4.98	314.4	4.22	368.6	3.91
P. taiwenensis	154.3	5.13	198.2	4.87	288.4	4.06
E. oryzae	31.4	6.93	47.1	6.78	59.8	6.69
Bacillus sp.(PAN-TM1)	161.2	5.4	232.5	4.98	264.6.	4.26
*Enterobacter*sp.-1	36.7	6.88	49.7	6.81	66.2	6.76
Bacillus aerophilus	57.7	6.84	93.3	6.61	114.3	6.01
*Enterobacter*sp.-2	84.4	5.45	128.7	5.85	147.8	5.25
Un inoculated Control	4.3	7.10	6.7	7.16	8.5	7.20
S.Em±	2.10	0.05	2.92	0.05	2.92	0.05
CD at 5%	6.25	0.17	8.70	0.17	8.70	0.17

SEM-Standard error means
CD (p=0.05)-Critical difference at 5 % level

Table-4 *Zinc phosphate solubilization and pH of the culture broth of different bacterial isolates under in vitro condition at different time intervals*

Bacterial isolates	Solubilization of Zinc Phosphate (ppm)					
	5 Days	pH	10 Days	pH	15 Days	pH
B. aryabhattai	389.1	4.41	482.4	3.93	576.5	3.33
P. taiwenensis	221.9	4.81	267.3	4.52	313.1	4.32
E. oryzae	58.4	6.86	84.6	6.71	104.6	6.31
Bacillus sp. (PAN-TM1)	183.6	5.03	241.5	4.17	298.3	4.17
*Enterobacter*sp.-1	48.8	6.8	66.8	6.54	97.7	6.54
Bacillus aerophilus	98.2	6.51	130.7	5.98	180.8	5.08
*Enterobacter*sp.-2	166.1	5.3	173.	5.54	224.7	4.94
Un inoculated Control	3.3	7.16	10.5	7.24	11.11	7.26
S.Em±	4.10	0.57	5.40	0.50	4.6	0.06
CD at 5%	12.2	1.72	16.1	1.49	13.8	0.19

SEM-Standard error means
CD (p=0.05)-Critical difference at 5 % level

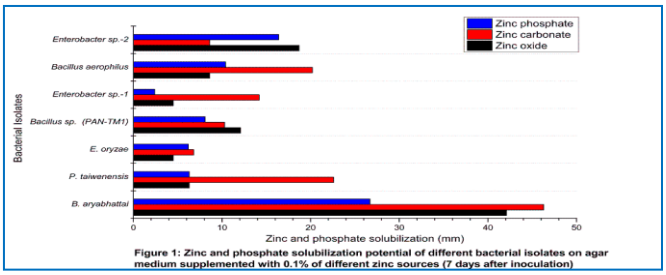

Fig-1 Zinc solubilization potential of different bacterial isolates on agar medium supplemented with 0.1% of different zinc sources (7 days after inoculation)

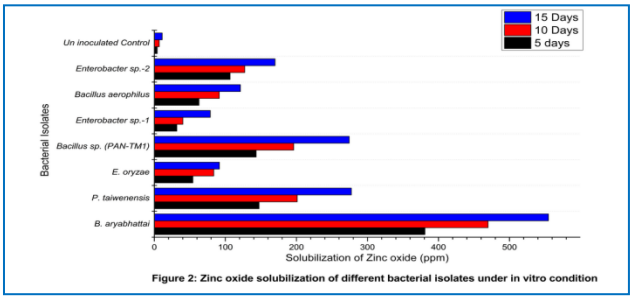

Fig-2 Zinc oxide solubilization and pH of the culture broth of different bacterial isolates under *in vitro* condition

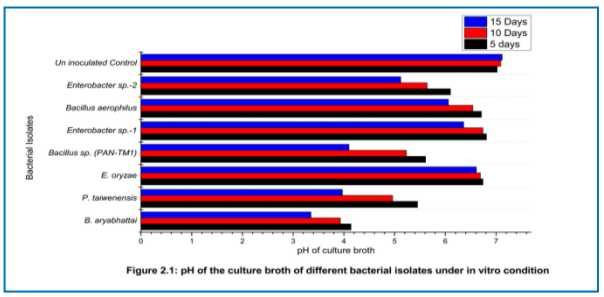

Fig-2.1 pH of the culture broth of different bacterial isolates under *in-vitro* condition

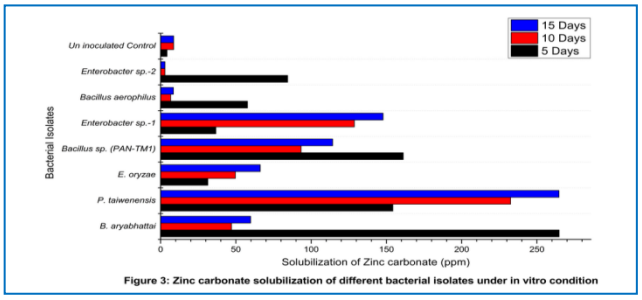

Fig-3 Zinc carbonate solubilization and pH of the culture broth of different bacterial isolates under *in vitro* condition

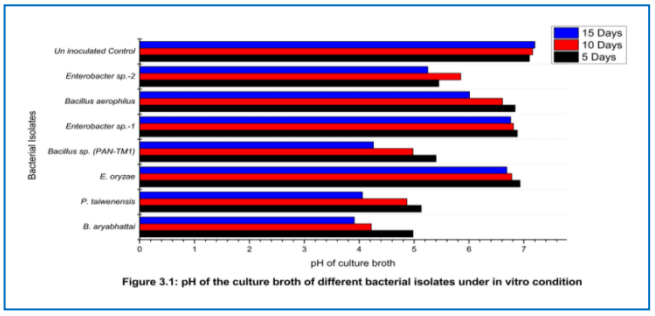

Fig-3.1 pH of the culture broth of different bacterial isolates under *in-vitro* condition

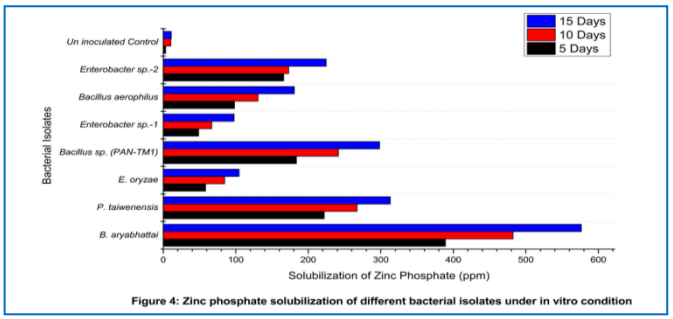

Fig-4 Zinc phosphate solubilization and pH of the culture broth of different bacterial isolates under *in vitro* condition

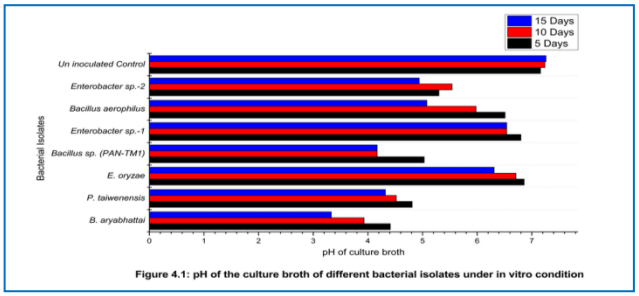

Fig-4.1 pH of the culture broth of different bacterial isolates under *in-vitro* condition

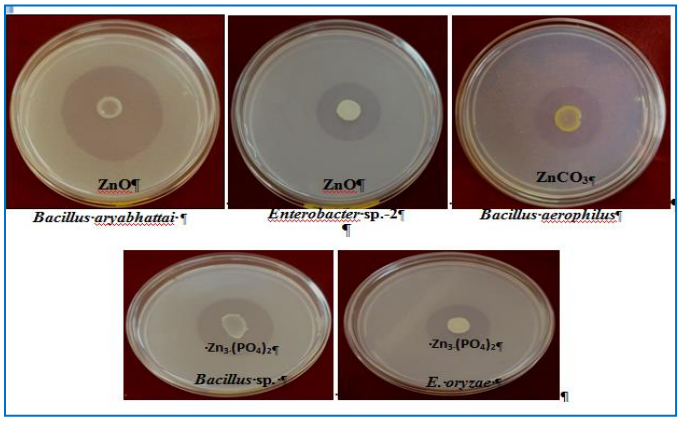

Fig-5 Zinc solubilization potential of different zinc solubilizing bacterial isolates under *in vitro* condition (7 DAI)

Solubilization potential was varied among the different bacterial isolates, which might be due to the inherent properties of organisms. The zinc solubilizing efficiency will vary from one bacterium to another bacterium. Similar kind of results was observed by Ramesh *et al*. [11], who reported the zinc solubilizing ability of *B. aryabhattai*. They have also reported that a significant decline in pH of the medium inoculated with *B. aryabhattai* over un-inoculated control and acidification of medium seemed to be the main strategy for zinc solubilization. Saravanan *et al*. [12] reported the potential of *Pseudomonas* spp. and *Bacillus* sp. in zinc oxide and zinc carbonate solubilization. Similarly, Panneerselvam *et al*. [13] investigated zinc solubilization potential of mycorrhiza helper bacteria *viz.*, *P. putida* and *B. parabrevis* in different zinc compounds. It may be attributed that zinc solubilization ability of ZSB isolates may be due to production of organic acids in the culture broth which might have helped in the solubilization of the zinc salts. Also, the solubilization of zinc by bacteria might be due to other mechanisms which include proton extrusion and production of chelating agent [11].

Conclusion

Soil may have sufficient zinc, but in some time, it may not be available to the plants due to fixation. Under this situation, application of *B. aryabhattai* could be useful to alleviate zinc deficiency. The results also indicated that selection and inoculation of such zinc solubilizing bacteria in soils may inherently provide rich native zinc or inoculation of these strains with insoluble zinc compounds may augment the availability of zinc to plants thereby saving the additional cost of input.

Acknowledgements

The authors gratefully thank the Director, Indian Council of Agricultural Research - Indian Institute of Horticultural Research for the support and infrastructure facilities.

Abbreviations

IIHR: Indian Institute of Horticultural Research, ICAR: Indian Council of Agricultural Research, mm: millimeter, ppm: parts per million, %: percentage, *viz:* videlicet (namely), rRNA: ribosomal ribonucleic acid. DAI: days after inoculation, ZSB: zinc solubilizing bacteria

Conflict of Interest: None declared

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