JASEM ISSN 1119-8362

Full-text Available Online at
www.ajol.info and
www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *Sept.*, 2012
Vol. 16 (3) 295 - 298

Evaluation of Zinc solubilization potential by different strains of Fluorescent Pseudomonads

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ABSTRACT: Zinc solubilizing ability of *Pseudomonas fluorescent* was evaluated using zinc oxide, zinc carbonate and zinc sulphide in both plate and broth media assays. Forty bacterial strains and 0.1% of each chemical source in six replications were used. Colony and halo diameters were measured after incubating the plates for 48h in incubator. Zn solubilizing ability of 40 mentioned strains in three replications was studied with ZnO and $ZnCO_3$ solutions in broth assay. The soluble zinc and pH were measured after five days. The results showed, only 8 of 40 strains could form clearing zone in plate assay. Halo diameter, ratio of the halo diameter to the colony diameter and area respectively for zinc oxide and zinc carbonate were as following, respectively: 0.60-1.32 cm, 1.20-2.64 and 0.95-2.60 cm^2, 0.13-1.70 cm, 0.27-2.99 and 0.31-4.10 cm^2. There were no halos observed in zinc sulphide. The concentration of soluble Zn for ZnO was 28-625 mg/l and pH was shifted from 7.0-7.2 to 3.90-6.50 and for $ZnCO_3$ was 247-753 mg/l and pH was shifted from 7.0-7.2 to 3.5-6.3 after 5 days of inoculation in 28$^{\circ C}$. @JASEM

Key words: Zinc, *Pseudomonas fluorescent*, Zinc solubilizing bacteria, clearing zone.

Zinc is an essential micronutrient for microorganisms and plants. This element is present in the enzyme system as co-factor and metal activator of many enzymes (Parisi et al., 1969). The role of zinc in the nutrition and physiology of both eukaryotic and prokaryotic organisms, especially its importance for activity of many enzymes is widely studied (Hughes and Poole, 1989). Many bacterial enzymes contain zinc in the active center or in a structurally important site (Clarke and Berg, 1998). Bacteria can contribute to metal immobilization by several processes such as precipitation and adsorption. In anoxic environments, the formation of highly insoluble metal sulphide bioprecipitates is a consequence of the activities of sulphate-reducing bacteria (Gadd, 1996; White et al., 1997). Zinc solubilizing potential of few bacterial genera has been studied. Hutchins et al., (1986), reported that *Thiobacillus thioxidans*, *Thiobacillus ferroxidans* and facultative thermophilic iron oxidizers solubilize zinc from sulphide ore (sphalerite).

A few fungal genera possess immense potential of solubilizing zinc tolerating a high zinc level *Aspergillus niger* was found to grow under 1000 mg Zn and this fungi is used to quantify zinc in soils containing low zinc (2 mg kg^{-1} available zinc) (Bullen and Kemila, 1997).

Zinc deficiency in fungi and bacteria is accompanied by impairment of the formation of pigments such as melanin, chrisogenin, prodigiosin, subtilin and others (Chernavina, 1970). Exogenous application of soluble zinc sources, similar to fertilizer application, has been advocated to various crops. This causes transformation of about 96-99 percent of applied available zinc to various unavailable forms.

This zinc thus made unavailable can be reverted back to available form by inoculating a bacterial strain capable of solubilizing it (Saravanan et al., 2003). Since zinc is a limiting factor in crop production in alkaline and calcareous soils of the world. This study on zinc solubilization by bacteria has an immense importance in zinc nutrition to plants in calcareous soils of Iran and the world.

MATERIALS AND METHODS

Forty *Pseudomonas fluorescent* bacterial isolates were selected from microbial culture collection of soil microbiology research department of Soil and Water Institute (SWRI) (Table 1). Bacteria were isolated from rhizosphere of wheat and brassica. Isolates were screened for efficient zinc solubilizing isolates and tested on different insoluble zinc compounds. The solubilization potential was evaluated both qualitatively and quantitatively under *in-vitro* condition.

Plate assay: The isolates were inoculated into modified PKV medium (Pikovskaya, 1948) (ingredients gr l^{-1}), (glucose-10.0 g; ammonium sulphate-1.0 g; potassium chloride-0.2 g; dipotassium hydrogen phosphate-0.2 g; magnesium sulphate-0.1

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g; Yeast-0.2 g; distilled water -1000 ml, pH 7.0) containing 0.1% insoluble zinc compounds (ZnO, $ZnCO_3$ and ZnS). The test organisms were inoculated on these media and incubated at 28℃ for 48 hours. The diameters of the colony and clearing zones around the colonies were measured.

Table 1. Some physiological features of *Pseudomonas fluorescent* isolates (microbial collection of soil microbiology research department of Soil and Water Institute).

Isolates	Auxin	Sidrophore	HCN	Inorganic Phosphate solubility in plate assay	Inorganic Phosphate solubility in broth assay
P. putida strain P1	+	+	-	+	+
P. putida strain P2	+	+	-	+	+
P. putida strain P3	+	+	-	+	+
P. putida strain P19	+	+	-	+	+
P. putida strain R69	+	+	-	+	+
P. fluorescens strain R187	+	+	-	+	+
P. aeruginosa strain GRP3	+	+	+	+	+
P. aeruginosa strain MPFM	+	+	-	+	+

Broth assay: The bacterial isolates were inoculated separately to basal medium supplemented with 0.1% insoluble zinc compounds. The solubilization of zinc from laboratory grade ZnO, $ZnCO_3$ and ZnS by isolates was assessed. Modified PKV medium was prepared, splitted in 25 ml aliquots in 50 ml Erlenmeyer flasks and 0.1% of these chemicals were added and steam sterilized for 30 minutes in autoclave. Then the flasks were inoculated with 0.1 ml suspension of the test culture. Three flasks were maintained with an uninoculated control for each treatment. Experiments were done in triplicate. The samples were withdrawn after 5 days, centrifuged to remove the debris and cells. One ml of this solution was directly fed to Atomic Absorption Spectrophotometry (AAS) to determine the available zinc content.

Determination of pH: The pH of the isolates culture filtrates and the uninoculated samples were determined after 5 days of inoculation. The culture was filtered using Whatman No.42 filter paper. The pH was measured using pH meter.

Statistical analysis: All statistical tests were done in SAS and graphs drowned by Microsoft office excel 2003**.** Letters in the figures represent mean values of soluble zinc and pH values. Means with different letters shown in figures are significantly different (Duncan, $p < 0.01$).

RESULTS AND DISCUSSION

The results showed that only 8 strains (20%) of the 40 strains could form clearing zone in plate assay. There were no halos observed in zinc sulphide. Zinc solubilizing potential varied with each isolate (Table 2).

Table 2. Zinc solubilizing potential of *Pseudomonas fluorescent* isolates with different insoluble compounds.

Isolates	Auxin	Sidrophore	HCN	Inorganic Phosphate solubility in plate assay	Inorganic Phosphate solubility in broth assay
P. putida strain P1	+	+	-	+	+
P. putida strain P2	+	+	-	+	+
P. putida strain P3	+	+	-	+	+
P. putida strain P19	+	+	-	+	+
P. putida strain R69	+	+	-	+	+
P. fluorescens strain R187	+	+	-	+	+
P. aeruginosa strain GRP3	+	+	+	+	+
P. aeruginosa strain MPFM	+	+	-	+	+

The *Pseudomonas putida* strain R69 obtained the highest potential in zinc oxide (ZnO) containing medium, producing a clearing zone of 1.32 cm, halo diameter/colony diameter of 2.64, and a zone area of 2.60 cm^2. Its performance in zinc carbonate was 0.75 cm, halo diameter/colony diameter of 1.38 and zone area of 1.33 cm^2. MPFM *(P. aeruginosa)* obtained the highest potential in zinc carbonate ($ZnCO_3$) containing medium, producing a clearing zone of 1.70 cm, halo diameter/colony diameter of 2.99 and a zone area of 4.10 cm^2. Its performance in zinc oxide was 1.20 cm, halo diameter/colony diameter of 2.00

and zone area of 2.54 cm^2. The P2 (*P. putida*) produced minimum clearing zone of 0.60 cm, halo diameter/colony diameter of 1.20 and zone area of 0.95 cm^2 with zinc oxide and zinc carbonate. The findings were confirmed in the broth assay using the same chemicals. The results revealed higher Zn solubilization by the isolates MPFM with 625 mg/l of zinc in the ZnO, and 753 mg/l in the $ZnCO_3$. The minimum available Zn revealed by P2 with 28 mg/l in the zinc oxide and 247 mg/l in the zinc carbonate containing medium (Fig 1, 2). All cultures showed a shift in pH after growth in the broth. After 5 days, the pH of the broth was acidic in some of cultures. The pH shifted from 7.0-7.2 to 4.5-6.5. The MPFM culture showed the lowest pH value (3.5) on 5th day after inoculation (Fig 3).

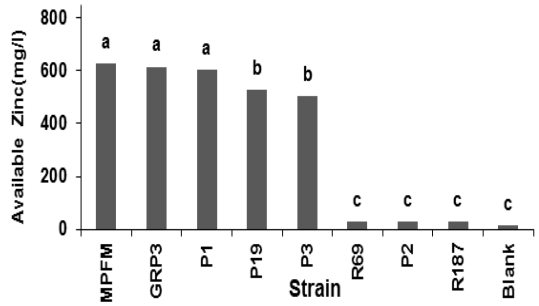

Fig 1. Available Zinc (mg/l) in broth containing zinc oxide

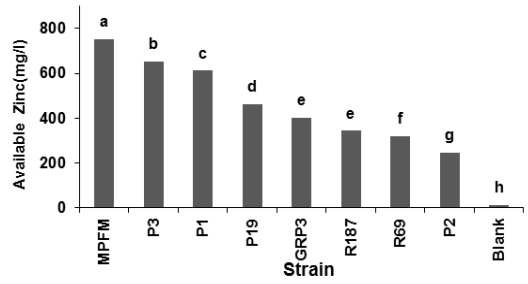

Fig 2. Available Zinc (mg/l) in broth containing zinc carbonate

The results showed a varied solubilization potential that were found among the bacterial isolates in ZnO and $ZnCO_3$ containing media. This might be related to differences in genomics and plasmid properties of strain that affected by the location from which they were isolated. All strains showed higher solubilizing ability in the $ZnCO_3$ containing medium which may be attributed to the fact that these strains were isolated from calcareous soils, presenting a higher potential than other

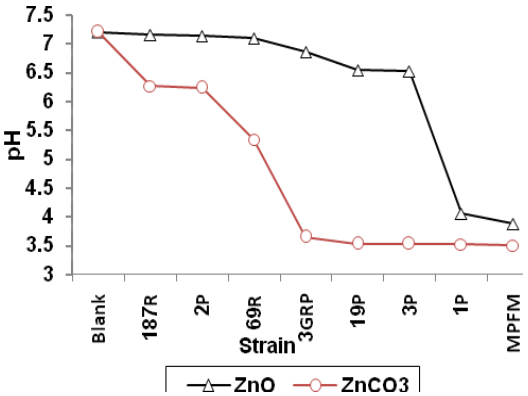

Fig 3. Changes in pH with zinc oxide and zinc carbonate as zinc source in broth media

Zn containing chemical substrates, making their adherence with the carbonate particles capable of solubilizing zinc carbonate and also it is depend the chemical properties of $ZnCO_3$ that easier than others affected by acidic exudates of bacteria. Electron microscopy studies showed evidence of direct attack from microorganisms to the surface of minerals, and have shown that bacteria attach themselves to surfaces, causing deep pitting and eventually dissolution of the crystal (Bennett and Tributsch, 1978). This may be the case also in the present study. From the results, it is clear that available zinc levels increased with the decrease pH after 5 days of inoculation. Dissolution of the zinc carbonate and zinc oxide may be due to production of organic acids, like gluconic acids. Gluconic acid, and its 2- and 2,5-keto-derivatives, are produced by fungi, such as *Penicillium luteum* and *Aspergillus niger,* and bacteria belonging to *Pseudomonas* or related genera as a result of an external oxidative pathway effective on glucose and other aldose sugars (Whiting et al., 1976; Babu-Khan et al., 1995; Williams et al., 1996). The gluconic acid is subsequently taken up by transport systems of the cell and utilized by cellular metabolic pathways therefore the external oxidation of glucose usually produces only transient increases in the concentration of gluconic acid (Drosinos and Board, 1994). The zinc phosphate solubilization by *Pseudomonas fluorescens* was investigated by Di Simine et al., (1998). They found that gluconic acid and 2 keto gluconic acids produced in the culture broth helped in the solubilization of the zinc salts. Acidic environments, such as those of the present investigation, revealed that the expected mechanism by which gluconic acid is able to dissolve insoluble metal compounds is mainly by acidification. gluconic acid which is known as a metal-chelating agent

(Mattey, 1992). In acidic environments, such as all the cultures showed a shift in pH towards acidic range, it gives a clue that organic acid might be involved. Thus, the obtainment of an elite culture or a consortium of strains capable of utilizing different unavailable insoluble forms of zinc and tolerant to higher zinc levels may be useful to make zinc available in the soil system (Saravanan et al., 2003). Soils are naturally rich in total zinc but poor in available forms. Application of soluble zinc component to alleviate zinc deficiency in certain soils is a costly practice. Zinc in soil system is constantly changing forms and being converted to unavailable forms. This particular study gains momentum for zinc nutrition using microbes.

Conclusion: We isolate 8 strains that formed clearing zone in plate assay and available zinc in broth assay. Selection and inoculation of zinc solubilizing bacteria either alone in soils inherently rich in native zinc or along with cheaper insoluble zinc compounds, like ZnO or $ZnCO_3$, will lead to lot of saving in crop husbandry, besides curtailing the expenditure on agro input.

Acknowledgements: This work was supported by the Department of Soil Microbiology, Soil and Water Research Institute in Tehran, Iran. We thank all the technical staff in our laboratory for assisting with the exposure system.

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