

## ASSESSING *IN VITRO* SOLUBILIZATION POTENTIAL OF DIFFERENT ZINC SOLUBILIZING BACTERIAL (ZSB) ISOLATES

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Submitted: March 12, 2003; Returned to authors: July 24, 2003; Approved: February 10, 2004.

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### ABSTRACT

Zinc solubilizing ability of *Bacillus* sp. and *Pseudomonas* sp. was assessed using zinc oxide, zinc sulphide (sphalerite) and zinc carbonate in both plate and broth assays. ZSB-O-1 (*Bacillus* sp.) showed highest dissolution in the zinc sulphide (Sphalerite ore), with 2.80 cm of dissolution zone and 14.50 cm<sup>2</sup> of area in the plate assay and 13.60 mg kg<sup>-1</sup> of zinc in the broth assay on the 15<sup>th</sup> day after inoculation. The ZSB-S-2 (*Pseudomonas* sp.) showed more solubilizing ability in the zinc oxide, with 3.30 cm clearing zone and 20.43 cm<sup>2</sup> area in the plate assay and 16.40 mg kg<sup>-1</sup> of zinc in the broth assay over the same inoculation period. The isolate ZSB-S-4 (*Pseudomonas* sp.) has highest solubilizing potential in zinc carbonate with 6.20 cm of dissolution zone and 13.40 cm<sup>2</sup> area in the plate assay and 13.40 mg kg<sup>-1</sup> of zinc in the broth assay. Thus, the solubilization potential varies among different cultures. The solubilization might be due to production of acids by the culture, since the pH of the culture broth has been shifted from 7.0-7.3 to 4.8-6.5 after 15 days of inoculation. The zinc tolerance limit for two cultures (ZSB-O-1 and ZSB-S-2) was studied and determined to be upto 100 mg kg<sup>-1</sup> of zinc in the *in vitro* broth assay.

**Key words:** *Bacillus* sp., *Pseudomonas* sp., zinc solubilizing bacteria

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### INTRODUCTION

Microorganisms require various nutrients for their growth and metabolism. Among the nutrients, zinc is an element present in the enzyme system as co factor and metal activator of many enzymes (9). The role of zinc in the nutrition and physiology of both eukaryotic and prokaryotic organisms is widely studied, especially its importance for activity of many enzymes (7). Zinc deficiency in fungi and bacteria is accompanied by impairment of the formation of pigments such as melanin, chrisogenin, prodigiosin, subtilin and others (4). 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<sup>-1</sup> available zinc) (2). Lichens and conifers are conspicuous for their high zinc content and the highest concentration of this element has been found in poisonous mushrooms (12). Zinc

solubilizing potential of few bacterial genera has been studied. Hutchins *et al.* (8), reported that *Thiobacillus thiooxidans*, *T. ferrooxidans* and facultative thermophilic iron oxidizers solubilized zinc from sulphide ore (sphalerite). Exogenous application of soluble zinc sources, similar to fertilizer application, has been advocated to various crops. This causes transformation of about 96-99 per cent of applied available zinc to various unavailable forms. The zinc thus made unavailable can be reverted back to available form by inoculating a bacterial strain capable of solubilizing it. Since zinc is a limiting factor in crop production, this study on zinc solubilization by bacteria has an immense importance in zinc nutrition to plants.

### MATERIALS AND METHODS

Seventeen bacterial isolates were obtained based on the solubilizing zone formation on the modified Bunt and Rovira

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media containing 0.1% insoluble ZnO (3). *Bacillus* sp. (ZSB-O-1) isolated from zinc ore sphalerite and *Pseudomonas* sp. (ZSB-S-2 and ZSB-S-4) isolated from paddy soil were screened as efficient zinc solubilizing isolates (ZSB) and tested on different insoluble zinc compounds. The solubilization potential was assessed both qualitatively and quantitatively under *in vitro* conditions.

#### Plate assay

The isolates were inoculated into agar medium containing 0.1% insoluble zinc compounds *viz* ZnO, ZnS and ZnCO<sub>3</sub>. The test organisms (ZSB-O-1, ZSB-S-2 and ZSB-S-4) were inoculated on these media and incubated at 30°C for 48 hours. The diameters of the clearing zones around the colonies were measured.

#### 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<sub>3</sub> and ZnS (Sphalerite ore material) by ZSB was assessed. Basal medium (glucose-10.0 g; ammonium sulphate-1.0 g; potassium chloride-0.2 g; dipotassium hydrogen phosphate-0.1 g; magnesium sulphate-0.2 g; distilled water -1000 ml, pH 7.0) was prepared, splitted in 50 ml aliquots in 100 ml Erlenmeyer flasks and 0.1% of these chemicals were added, steam sterilized for 30 minutes in an autoclave for 3 consecutive days. Then the flasks were inoculated with 1 ml suspension of the test culture with a cell load of 10<sup>7</sup> cells ml<sup>-1</sup>. Three flasks were maintained with an uninoculated control for each treatment. Experiments were done in triplicate. The samples were withdrawn at 0, 7, 14 and 20 days intervals, centrifuged to remove the debris and cells. Ten ml of this solution was fed to Atomic Absorption Spectrophotometry (AAS) to determination of the available zinc content.

#### Determination of pH

The pH of the ZSB culture filtrates and the uninoculated samples was determined at 0, 5, 10 and 15 days after inoculation. The culture was filtered using Whatman No.1 filter paper. The pH was estimated using Elico pH meter.

#### Zinc tolerance by ZSB isolates

Zinc is a nutrient at low concentration but toxic at higher concentration. The solubilization of zinc might limit the growth of the bacteria at higher level. Unless the cultures tolerate a higher level of zinc its solubilization may not continue. Therefore the ability of selected isolate to tolerate solubilized zinc was determined under *in vitro* condition in nutrient broth containing different concentrations of soluble zinc (ZnSO<sub>4</sub>). The nutrient broth was prepared and splitted in 10 ml aliquots in test tubes. ZnSO<sub>4</sub> was incorporated into the broth in such a way that the final concentration of zinc was 25, 50, 100, 200, 300, 400 and 500 mg kg<sup>-1</sup>. These solutions were divided in 10 ml quantities in test

tubes, sterilized at 15 psi for 15 min and inoculated with 0.1 ml of ZSB. An uninoculated control was also maintained. The total ZSB population was assessed by plating on nutrient agar media. The growth of bacteria in the zinc containing medium indicated their tolerance to zinc. The highest concentration at which poor growth was observed was taken as tolerance level. The experiments were carried out in a completely randomized design and the data obtained were subjected to statistical analysis of variance (10).

## RESULTS AND DISCUSSION

Zinc solubilizing potential varied with each isolate (Table 1). The ZSB-O-1 (*Bacillus* sp.) obtained from the zinc ore exhibited the highest potential in Sphalerite (ZnS) containing medium, producing a clearing zone of 2.80 cm with a zone area of 14.50 cm<sup>2</sup>. Its performance in zinc carbonate and zinc oxide was 1.50 cm of clearing zone and zone area of 8.50 cm<sup>2</sup> with zinc carbonate and 1.80 cm of clearing zone and zone area of 8.00 cm<sup>2</sup> with zinc oxide, respectively. On the other hand, the ZSB-S-2 (*Pseudomonas* sp.) produced maximum clearing zone of 3.30 cm and zone area of 20.43 cm<sup>2</sup> with zinc oxide and performed poorly in zinc carbonate, with a clearing zone of 2.00 cm and zone area of 7.00 cm<sup>2</sup>. The ZSB-S-4 (*Pseudomonas* sp.) showed the highest potential in zinc carbonate, with a clearing zone of 4.00 cm and 19.60 cm<sup>2</sup> zone area.

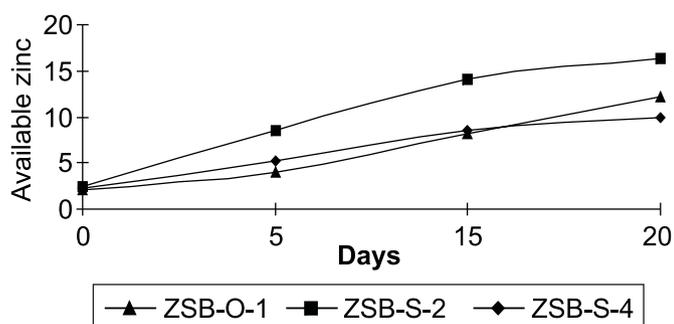
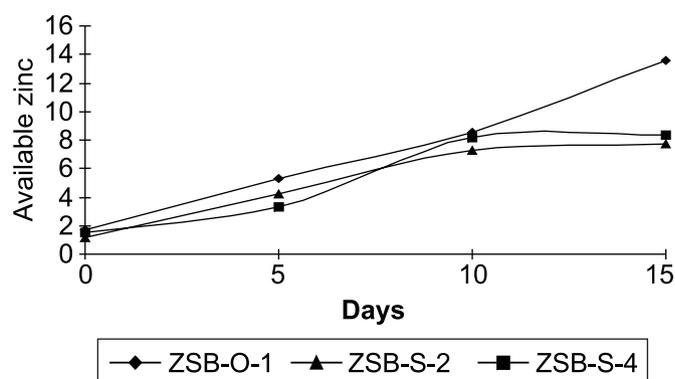
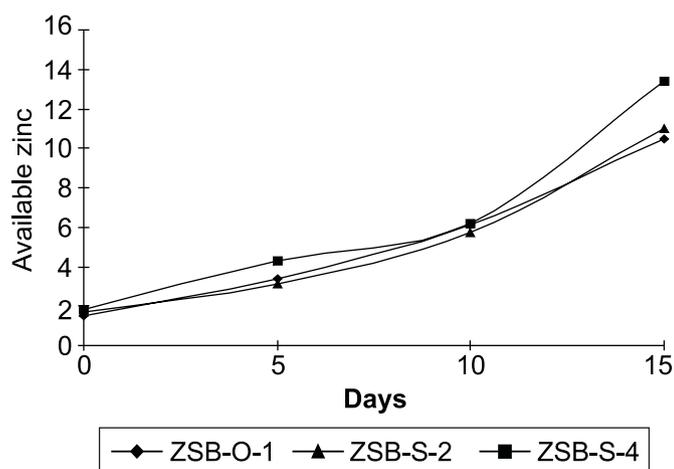
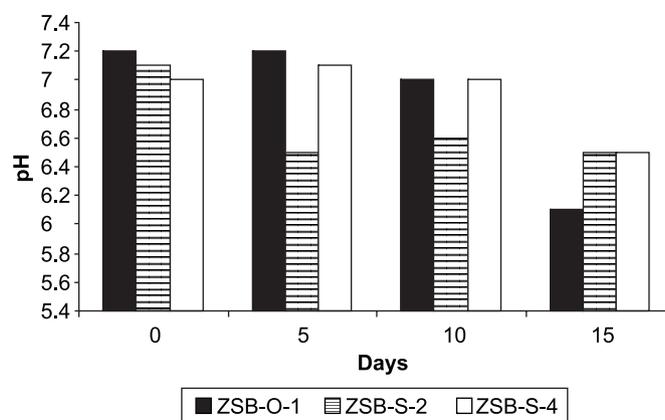
The findings were confirmed in the broth assay using the same chemicals (Figs. 1, 2 and 3). The results revealed higher Zn solubilization on 15<sup>th</sup> day by the isolates ZSB-O-1, ZSB-S-2 and ZSB-S-4 with 13.6 mg kg<sup>-1</sup> of zinc in sphalerite, 16.4 mg kg<sup>-1</sup> in the ZnO, and 13.4 mg kg<sup>-1</sup> ZnCO<sub>3</sub> respectively.

All cultures showed a shift in pH after growth in the broth (Figs. 4, 5 and 6). After 15 days, the pH of the broth was acidic in all cultures. The pH shifted from 7-7.3 to 4.8-6.5. The ZSB-S-4 culture showed the lowest pH value (4.8) on 15<sup>th</sup> day after inoculation, indicating a higher acidity due to growth.

Even at 25 mg kg<sup>-1</sup> concentration, there was reduction in population within 24 hours and afterwards population remained stable up to 8 days (Table 2). At zinc concentration above 100 mg kg<sup>-1</sup>, a further reduction in population was observed, which was more pronounced at 200 mg kg<sup>-1</sup>. The results showed the inherent capacity of the isolates to tolerate various levels of zinc. At 500 mg kg<sup>-1</sup> level, ZSB-S-2 was completely inhibited at the 8<sup>th</sup> day, while ZSB-O-1 recorded 2 x 10<sup>4</sup> cells ml<sup>-1</sup> at the 8<sup>th</sup> day after inoculation compared to 180 x 10<sup>6</sup> cells ml<sup>-1</sup> observed just after inoculation. For both isolates, there were no significant differences on the 24<sup>th</sup> h of inoculation, but on 8<sup>th</sup> day after inoculation in the ZSB-O-1, the population (2 x 10<sup>4</sup> cells / ml<sup>-1</sup>) recorded at 25 mg kg<sup>-1</sup> concentration was found to be superior than in any other concentration of zinc. All other treatments are statistically on par. In ZSB-S-2 isolate, the population recorded in 25, 50, 100, 200 and 300 mg kg<sup>-1</sup> of zinc were on par with each

**Table 1.** Zinc solubilizing potential of zinc solubilizing bacterial (ZSB) isolates with different insoluble zinc compounds.

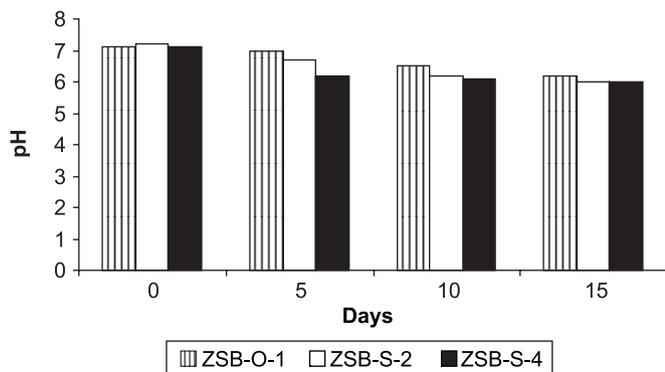
Isolates	Zinc oxide			Zinc carbonate			Sphalerite		
	Colony diameter (cm)	Clearing zone (cm)	Area (cm <sup>2</sup> )	Colony diameter (cm)	Clearing zone (cm)	Area (cm <sup>2</sup> )	Colony diameter (cm)	Clearing zone (cm)	Area (cm <sup>2</sup> )
ZSB-O-1	1.4	1.8	8.0	1.8	1.5	8.5	1.5	2.8	14.5
ZSB-S-2	1.8	3.3	20.43	1.0	2.0	7.0	1.8	2.5	14.5
ZSB-S-4	1.2	2.4	10.18	1.0	4.0	19.6	1.0	1.2	3.8

**Figure 1.** Available zinc (mg kg<sup>-1</sup>) in broth containing zinc oxide.**Figure 3.** Available zinc (ppm) in broth containing Sphalerite.**Figure 2.** Available zinc (mg kg<sup>-1</sup>) in broth containing zinc carbonate.**Figure 4.** Changes in pH with zinc oxide as zinc source.

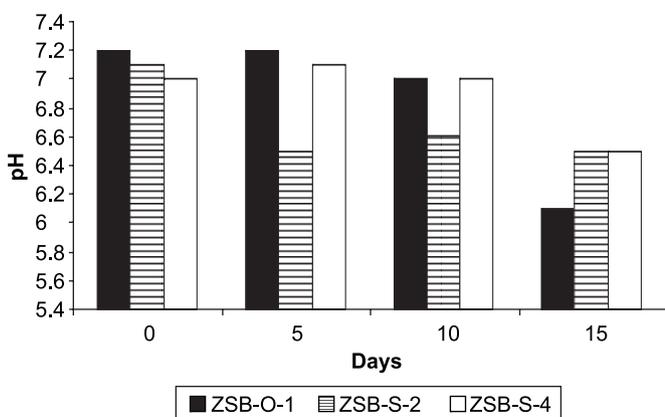
other. But, statistical difference existed in populations at 400 and 500 mg kg<sup>-1</sup> of zinc.

From the results, it is clear that available zinc levels increased with the increase in incubation period. This might be due to the presence of excess zinc even after the absorption by the microbial cells. The bioaccumulation of Cu, Zn Cd and Pb in the

cells of *Bacillus* sp. *Bacillus cereus*, *Bacillus sphaericus* and *Bacillus subtilis* were well studied (6). After enough absorption, the excess remains in the solution. This is in agreement with the findings of Cozzi *et al.* (5), who stated that the complexing agents produced by microbes stabilized the inorganic ions in the solution and kept them from precipitation or in some cases,



**Figure 5.** Changes in pH with zinc carbonate as zinc source.



**Figure 6.** Changes in pH with Sphalerite as zinc source.

prevented from being oxidized or reduced and converted to insoluble forms.

A varied solubilization potential was found among the bacterial isolates. This might be related to differences in the location from which they were isolated. The ZSB-O-1 showed higher solubilizing ability in the ZnS (sphalerite) ore containing medium and this may be attributed to the fact that this strain was isolated from the same ore material, presenting a higher potential than other cultures, making their adherence with the ore particles capable of solubilizing ore. Electron microscopy studies evidenced direct attack of microorganisms to the surface of minerals, and has shown that bacteria attach themselves to surfaces, causing deep pitting and eventually dissolution of the crystal (1). This may be the case also in the present study. Dissolution of the ore and other material may be due to production of organic acids, like gluconic acids (especially 2 keto gluconic acids). The zinc phosphate solubilization by *Pseudomonas fluorescens* was investigated by Simine *et al.* (11). They found that gluconic acid and 2 keto

**Table 2.** Extent of zinc tolerance of zinc solubilizing bacterial (ZSB) isolates.

Strain N <sup>o</sup>	Zinc concentration (mg kg <sup>-1</sup> )	Population (1X10 <sup>6</sup> ml <sup>-1</sup> )					
		ZSB-O-1			ZSB-S-2		
		0h	24h	8DAI	0h	24h	8DAI
1.	0	180	250	250	200	270	200
2.	25	180	28	25	200	16	20
3.	50	180	21	21	200	16	20
4.	100	180	20	20	200	18	18
5.	200	180	16*	14*	200	14*	12*
6.	300	180	11*	12*	200	14*	13*
7.	400	180	6*	8*	200	8*	7*
8.	500	180	4*	2*	200	5*	*
	SEd		3.22	1.50		1.61	1.21
	CD		7.14	3.22		3.47	2.60

\* Population 10<sup>4</sup> cells ml<sup>-1</sup>; h – hours; DAI – Days After Inoculation; SEd - Standard Error Deviation; CD – Critical Difference.

gluconic acids produced in the culture broth helped in the solubilization of the zinc salts. Since all the cultures showed a shift in pH towards acidic range, it gives a clue that organic acid might be involved.

The zinc tolerance potential also varied with the organisms. The ZSB-O-1 cultures is a *Bacillus* sp., and had shown a cell count of 2 x 10<sup>4</sup> ml<sup>-1</sup> on the 8<sup>th</sup> day after inoculation in a 500 mg kg<sup>-1</sup> concentration of zinc. This might be due to production of spore cells by the *Bacillus* sp. which when transferred to a rich media like nutrient agar were able to retain their growth, whereas the ZSB-S-2 was completely inhibited at 500 mg kg<sup>-1</sup> concentration after 8 days of inoculation since it is a *Pseudomonas* sp. It may not be able to survive the adverse condition of higher level of Zn and so a complete inhibition was observed.

Thus, the obtention 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. Soils are naturally rich in total zinc but lack available zinc. Application of soluble zinc sulphate to alleviate zinc deficiency in certain soils is a costly practice. 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<sub>3</sub>, will lead to lot of saving in crop husbandry, besides curtailing the expenditure on agro input. Since zinc in soil system is constantly changing forms and being converted to unavailable forms, this particular study gains momentum for zinc nutrition using microbes.

## RESUMO

**Avaliação *In vitro* do potencial de solubilização de diferentes bactérias solubilizadoras de zinco (ZSB)**

A capacidade de *Bacillus* sp. e *Pseudomonas* sp. solubilizar zinco foi avaliada usando óxido de zinco, sulfeto de zinco e carbonato de zinco, em ensaios em placas e em caldo. A cultura ZSB-O-1 (*Bacillus* sp.) apresentou maior dissolução no sulfeto de zinco, com 2,80 cm de zona de dissolução e 14,50 cm<sup>2</sup> de área no ensaio em placa e 13,60 mg kg<sup>-1</sup> de zinco no ensaio em caldo, no 15º dia de incubação. A cultura ZSB-S-2 (*Pseudomonas* sp.) apresentou maior capacidade de dissolução no óxido de zinco, com 3,30 cm de zona de dissolução e 20,43 cm<sup>2</sup> de área no ensaio em placa e 16,40 mg kg<sup>-1</sup> de zinco no ensaio em caldo no mesmo período de inoculação. A cultura ZSB-S-4 (*Pseudomonas* sp.) apresentou maior potencial de solubilização em carbonato de zinco, com 6,20 cm de zona de dissolução e 13,40 cm<sup>2</sup> de área no ensaio em placa e 13,40 mg kg<sup>-1</sup> de zinco no ensaio em caldo. Assim, o potencial de solubilização variou de acordo com a cultura. A solubilização pode ser devida à produção de ácido, uma vez que o pH do caldo abaixou de 7,0-7,3 para 4,8-6,5 após 15 dias de incubação. O limite de tolerância ao zinco para as duas culturas (ZSB-O-1 and ZSB-S-2) foi mensurada, verificando-se ser 100 mg kg<sup>-1</sup> no ensaio *in vitro* em caldo.

**Palavras-chave:** *Bacillus* sp., *Pseudomonas* sp., bactérias solubilizadoras de zinco

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