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Zinc solubilizing plant growth promoting microbes produce zinc nanoparticles

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Abstract

Strains of *Pseudomonas*, *Bacillus*, and *Azospirillum* with plant growth promoting ability were checked for their zinc solubilizing ability at ICAR-CRIDA, Hyderabad, India. Efficient zinc solubilizing microorganisms were evaluated for their ability to produce nano-scale zinc particles. The nanoparticles from the cell-free culture filtrates obtained from these strains were characterized for particle size, Zeta potential and functional groups. Presence of Zn nanoparticles in the bacterial culture filtrate was confirmed by particle distribution and Scanning electron microscope (SEM) analysis. Most properties of nanoparticles are size dependent. Zinc nanoparticles were observed to be spherical in shape and size ranged from 52.0 to 106.0nm. Zeta potential of the Zn nanoparticles was estimated to understand the stability of the particles. The measured zeta potentials varied from -14.5mV to +179.10 mV indicating high stability and dispersion of the zinc nanoparticles. FTIR peaks at different wave numbers depicted the role of functional groups of proteins in the biosynthesis of Zn nanoparticles. These results demonstrate the green synthesis of zinc nanoparticles by the plant growth promoting and zinc solubilizing strains of *Azospirillum*, *Pseudomonas* and *Bacillus*.

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Introduction

Zinc (Zn) is one of the vital minerals required for optimum plant growth as well as animals, including human beings such as mental retardations, impairments of the

immune system and overall poor health. In recent years, the Zn deficiency problem has received increasing attention and appears to be the most serious micronutrient deficiency together with vitamin A deficiency especially

among children. It plays a vital role in biochemical reactions in plants and its deficiency is displayed as a remarkable reduction in plant height and develops whitish-brown patches that turn necrotic subsequently (Singh 1999). In India, >50% of agro-ecosystems are Zn deficient, especially the Indo-Gangetic plains spanning across the States of Punjab and Uttar Pradesh. Overall, zinc deficiency in the country has increased from 42% to 49% in the past four decades and it is expected to increase up to 63% by 2025 (Singh 2009). Plant response to Zn deficiency occurs in terms of membrane integrity loss, reduced synthesis of metabolites and susceptibility to heat stress (Bhupinder et al., 2005). Zn is supplemented exogenously to plants in the form of chemical fertilizers like zinc sulphate that subsequently transforms (96-99%) into different insoluble forms depending upon the soil types, physico-chemical reactions. However, external supplementation of micronutrients is not only expensive, but also leads to unwanted accumulation of them in the soil in fixed forms. Bacteria-mediated solubilization of zinc has been widely studied in fungi and bacteria (Suseelendra Desai et al. 2012).

Decreased particle size resulted in increased number of particles per unit weight of applied Zn and increase in the specific surface area of a fertilizer (Mortvedt 1992). Nanoparticles of Mg, Zn, Fe and P are the structural component of enzymes (phosphatases and phytase), polysaccharides and chlorophyll. These nanoparticles can be tagged to agrochemicals or other substances as delivery agent to plant system and tissues for controlled release of chemicals. Nanoparticles

with small size and large surface area are expected to be the ideal candidates for use as a Zn fertilizer in plants (Prasad et al. 2012). First evidence of biosynthesis of nanoparticles was reported using *Pseudomonas stutzeri* where the nanoparticles were deposited on the cell membrane (Klaus et al. 1999). Subsequently, *Lactobacillus* strains (Ahmad et al. 2003); *Fusarium oxysporum* (Minaeian et al. 2008); *Bacillus subtilis* (Nair et al. 2002); *Cornybacterium* sp. (Saifuddin et al. 2009); *Klebsiella pneumonia* (Zhang et al. 2005), and *Escherichia coli* (Gurunathan et al. 2009) have been found to produce nanoparticles. On the other hand, *E. coli* was shown to be involved in extra- and intra-cellular synthesis of nanoparticles (Gurunathan et al. 2009). However, very few reports suggest synthesis of Zn nanoparticles by plant growth promoting microorganisms. In the present study, we report the ability of Zn solubilizing rhizobacteria to produce Zn nanoparticles.

Materials and Methods

Bacterial strains used in this study were obtained from the culture bank of ICAR-Central Research Institute for Dryland Agriculture, Hyderabad. Based on previous qualitative and quantitative screening, five efficient zinc solubilizing strains viz. *Bacillus* B116, *Pseudomonas* P29, *Pseudomonas* P33, and *Azospirillum* 20 were selected for nanoparticle analysis [6]. All the isolates were grown in Minimal salt medium amended with 0.1% ZnO with an initial population containing 2×10^8 CFU/ml and incubated at room temperature in an orbital shaking incubator with 180 rpm speed. The culture medium was centrifuged after 72h of inoculation and the supernatant was collected.

Supernatant was analysed for the presence of nanoparticles in nanoparticle analyzer.

Ten ml of the supernatant was transferred to a cuvette and placed into the nanoparticle analyzer (Horiba Nanoparticula 100). Three replicas for each sample were maintained respectively. Each sample was read three times and the mean was considered as particle size. The zeta potentials were recorded using Particle size analyzer (Horiba Nanoparticula 100, Japan). This apparatus uses Dynamic Light scattering (DLS) phenomenon. The sample holder temperature was maintained at 25⁰C, a technique used to determine the stability profile of small particles in solution. Nano scale Zinc particles were observed under SEM. To observe nanoparticles of Zinc, 10 µl of the suspension was spotted on to the metal slide. Metal slide was placed inside a desiccator over night to remove moisture completely. After removing the slide from the desiccator, a gold sputtering of approximately 1nm was done over the suspension.

For Fourier Transform Infra Red (FTIR) spectroscopy measurement, the bio-transformed product present in cell-free filtrate was analyzed. FTIR spectrum (Bruker Tensor 27, USA) was recorded in mid IR region in the range of 400–4000 wavenumber cm⁻¹. A drop of the sample solution was carefully added to the potassium bromide crystals using a micropipette and the spectrum was recorded in transmittance (%) mode.

Results and Discussion

Plant growth promoting strains of *Pseudomonas*, *Bacillus* and *Azospirillum*

found to solubilize ZnO and ZnCO₃ were formulated and field-evaluated. While trying to understand their mode of action of Zn solubilization, it was found that these strains synthesized Zn nanoparticles *in vitro* when cultured in MS broth supplemented with ZnO or ZnCO₃. The results of the particle size distribution (PSD) dynamic light scattering method conforms the presence of Zinc nanoparticles. In comparison to control, in all treatments, the Z-Average particle size was 10.5 to 14.6 times smaller than control. Smallest particle size was observed in P29 treatment. However, the treatments did not differ significantly (Table 1).

Table1. Particle Z-Average size and Poly dispersity index of Zn nanoparticles produced by different bacterial strains in ZnO incorporated broth

Strains	Z-Average particle size (nm)	Poly dispersity Index
B116	593.0	0.60
P 29	428.8	0.51
P 33	482.0	0.37
Asp 20	539.0	0.48
Control	6279	1.00

Electron Microscopy (SEM) analysis image provided further insight into the shape size, morphology and distribution of nanoparticles. The SEM micrographs showed uniformly distributed spherical or roughly spherical nanoparticles. The size of the Zn nanoparticles ranged from 106 to 52nm. In control the size was 5200nm (Fig 1).

Fig 1. SEM image of Nanoparticles of zn produced by a) P 29 (52nm) b) P 33 (74nm) c) Asp 20 (53nm) d) B 116 (106nm) in media amended with 1ppm ZnO.

There were differences among the strains in synthesizing different sizes of zinc nanoparticles. According to particle size distribution and SEM analysis, *Pseudomonas putida* P29 produced smallest zinc nanoparticles of the size 52 nm followed by Asp20 and B116 produced large particles (106 nm) as against control where the particle size was 5200 nm. All treatments did not differ significantly as compared to control indicating that nanoparticles produced by all organisms

were within a range. Using the nano-particle analyzer, the Zeta potential was measured as the magnitude of Zeta Potential (-200mV to +200mV) gives an indication of the potential stability of the colloidal system. The Zeta potentials of Zn nanoparticles produced by B116, P33, P29 and Asp20 were +179.1 mV, -14.5mV, +0.5mV and 0.5 mV, respectively, clearly indicating the stability of the nanoparticles (Fig 2).

Fig 2. Zeta potential of Zn nanoparticles produced by B116 (+179.1 mV), P33 (-14.5 mV), P29 (+0.5 mV), Asp 20 (+0.8 mV), and in ZnO amended MS medium

FTIR analysis of the cell-free culture filtrates of ZnO amended MS medium was done to identify the interactions between Zn and

bioactive molecules enabling synthesis and stabilization of Zn nanoparticles (Fig 3).

Fig 3. IR spectrum of cell-free culture filtrates of ZnO amended MS medium inoculated with different strains of bacteria

The FTIR spectra of cell-free extracts of ZnO amended MS medium showed prominent peaks at 3346, 2923, 1636, 1459 and 538 wave numbers cm^{-1} . The strong broad wave at 3346 cm^{-1} is attributed to NH stretch of amides. The wave observed at 2923 cm^{-1} is stretch vibration of primary and secondary amides of protein. The band observed at 1636 cm^{-1} is assigned to the bending vibrational mode of amides. The peaks at 1459 refer to amino and amino-methyl stretching groups of protein. Peak observed at 529 cm^{-1} corresponds to the stretching vibrations of Zn nanoparticles.

The exact mechanism for the synthesis of nanoparticles using biological agents has not yet been elucidated but it has been suggested that various biomolecules are responsible for the synthesis of nanoparticles. The mechanism of extracellular biosynthesis of nanoparticles is proposed as a reductase-mediated reaction, which brings about bio-reduction of metal ions and synthesizes nanoparticles (Honary et al. 2012). In the present study, FTIR spectrum of the nanoparticles indicated the presence of various chemical groups, one of which is an amide. An amide I band was observed at 1636 wavecm^{-1} , which is the bending vibrational mode of amides. The stretching band of amide at 3346 wavecm^{-1} further confirmed this result. The 1636 wave cm^{-1} corresponds to amide I due to carbonyl stretch in proteins. FTIR spectrum showed the presence of functional groups such as amide linkages and $-\text{COO}-$, possibly between amino acid residues in protein and the synthesized Zn nanoparticles. Similar results supporting the interactions between the proteins and

nanoparticles during the biosynthesis were reported (Honary et al. 2012). Surface properties of the nanoparticles are known to be one of the most important factors that govern their stability and mobility as colloidal suspensions, or their adsorption or aggregation and deposition. Zeta potential can be related to the stability of colloidal dispersions as it indicates the degree of repulsion between adjacent and similarly charged particles in dispersion and high zeta potential values indicate high stability and with decrease in the particle size, the stability also decreases (Vielkind et al. 2013). Highest zeta potential of +179.10 mV was observed for the particle size 106.0 nm produced by B116 indicating best stability. However, nanoparticles with zeta potential values greater than +25mV and -25mV typically are stable (Sonika et al., 2015). Hence, it could be inferred that the nanoparticles produced with lower zeta potentials such as +0.5mV may not be stable even though they are only 52 nm. Further, low stability nanoparticles tend to form aggregates in the medium, which is influenced by various factors. Aggregation of metal oxide nanoparticles in aqueous solutions depends on ionic strength, pH, surface charge and charge density (Domingos et al., 2009; Guzman et al. 2006). In the present study also, such aggregation was noticed in cases where the zeta potential was low. Poly dispersity index was lowest in P33 treatment whereas it was highest in B116 (Table 1). However, all the treatments did not differ significantly as against control. Results of particle size distribution clearly indicated that the obtained Zinc nanoparticles are both mono- and poly-dispersed in nature towards as the values

ranged from 0.37 to 0.60 across treatments. Generally samples with very broad distribution size have >0.70 poly-dispersity index and thus while studying extracellular synthesis of ZnO nanoparticles, an average particle Z-Average size of 46.61 nm and Poly dispersity index of 0.552 was also considered as mono-dispersed in nature (Nagarajan & Kuppusamy 2011).

Conclusion

The present study clearly shows that Zn nanoparticles are produced by Zn solubilizing strains of *Pseudomonas*, *Bacillus* and *Azospirillum* spp. These results open a new field for exploration as to mode of uptake of nutrients by the plants vis-à-vis role of plant growth promoting rhizomicroorganisms. Similar experiments also could be planned for other microbe-mediated plant nutrient supplementation studies to understand the dynamics of crop nutrition.

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