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## Studies on the effect of $ZnCl_2$ on phosphatase enzyme activity and biomass of *Pseudomonas fluorescens* (ATCC<sup>®</sup> 13525<sup>TM</sup>)

R. Lalfakzuala<sup>1</sup>, H. Lalrinfeli<sup>1</sup>, and H. Lalruatsanga<sup>2\*</sup>

<sup>1</sup>Department of Botany, Mizoram University, Tanhril, Aizawl 796004, India

<sup>2</sup>Department of Botany, Pachhunga University College, Aizawl 796001, India

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### ABSTRACT

Living organism requires certain metals for their growth and metabolism and evolved an appropriate uptake mechanism for metals. However, it is particularly difficult to establish mutual relationship when soil is continuously contaminated with various heavy metals. Phosphatase enzyme significantly accelerates the release of inorganic P from organic compounds and returns it to the soil. Therefore, the study of Phosphatase enzyme that releases P from organic compounds by dephosphorylation of phosphor-ester or phosphoanhydride bond in an organic matter is an important tool in initiating biofertilizer for specific crops. The present study indicated that the availability of metal such as Zinc in its inorganic form can act as an inhibitory factor for the growth and activity of bacteria such as *Pseudomonas fluorescens* and the biomass of *P. fluorescens* decrease with increase  $ZnCl_2$  concentration. It can be concluded that  $ZnCl_2$  acts as an inhibitor to the growth of *P. fluorescens* and also the presence of  $ZnCl_2$  in the growth medium decreases the phosphatase enzyme activity of the bacteria, which reveal that metals can also affect the activity of the microbes.

**Key words:** Biofertilizers, enzymes, microbial ecosystems, phosphatase, *Pseudomonas fluorescens*,  $ZnCl_2$ .

### INTRODUCTION

The effects of metal on microbial ecosystems are primarily described in terms of the toxicity of heavy metals to microorganisms and their impact on microbial community, structure and function. Although a large amount of metals is

essential for growth, some can be harmful for living cells. This is because of the fact that heavy metals form complexes with protein molecules which render them inactive.<sup>1</sup>

The release of heavy metals and metalloids from various sources like metal industries, agrochemicals, waste disposals, refining, manufacturing, etc. is a major threat for the ecosystem. Pollution of the biosphere by heavy metals due to industrial, agricultural and domestic activities has created a serious problem for the safe and

Corresponding author: Lalruatsanga  
Phone: : +91-9436195356  
E-mail: [puiars@rediffmail.com](mailto:puiars@rediffmail.com)

rational utilization of soils.<sup>2-3</sup> Over the past century, there has been a progressive increase in the environmental load of toxic heavy metals. Their concentration varies considerably in polluted areas. When accumulated in the soil, the toxic metals inversely affect the composition of microbial population, including the plant growth-promoting rhizobacteria (PGPR) and their metabolic activities.<sup>4</sup>

The inhibition of soil enzymes activates depend on the nature and concentrations of heavy metals. Heavy metals usually inhibit enzymes activities by interacting with the enzymes substrate complexes, denaturing the enzyme protein and interacting with their active sites.<sup>5</sup>

Zinc, an essential micronutrient, plays a vital role in maintaining normal metabolism in higher plants. Zinc at high levels becomes strongly phytotoxic to cells and causes inhibition of plant growth or even death<sup>6</sup>. Photosynthesis is also sensitive to excessive Zinc, pigments and protein components of photosynthetic membranes are the targets.<sup>7</sup> In addition, Zinc toxicity is related to disturbances in the uptake of other essential elements.<sup>8</sup>

Phosphorous is an essential element that often limits the growth of plants mainly due to its low solubility and fixation in soil. A major part of phosphorous in soil is present in the form of insoluble phosphates which cannot be utilized by the plants. Therefore, phosphorous is added in the form of phosphatidic fertilizers, to be utilized by the plants but most of it is quickly transformed to insoluble form. Hence, only little percentage of the applied phosphorous is available to plants, making continuous application necessary.<sup>9</sup> The inability of the plants to absorb insoluble form of phosphorous means that it has to be converted into soluble form by phosphatase enzyme such as acidic and alkaline phosphatases.

Assimilation of phosphate from organic compounds by plants and micro-organisms take place through the enzyme "phosphatases" which is present in wide variety of soil microorganisms. The principle mechanism for solubilization is the production of organic acids and acid phos-

phatases which play a major role in the mineralization organic phosphorous in soil. Acid phosphatases are a group of enzymes that catalyze the hydrolysis of external phosphate esters.<sup>10</sup> They play an important role in mineralization of organic carbon, organic phosphate and low levels of free inorganic ions (Pi).<sup>11</sup> Extra cellular acid phosphatases are involved in routine utilization of Pi.

Keeping in view the importance of *P. fluorescens* as one of the major phosphate solubilizing bacteria and the adverse effect of zinc on the environment, the present experiment was performed to see the effect of different concentration of zinc on the phosphatase enzyme and biomass of *P. fluorescens*.

## MATERIALS AND METHODS

The experiment was designed to test the effect of heavy metal (zinc chloride) on phosphatase activity and biomass of bacteria. Since zinc is not readily available in its free form, zinc in the form of zinc chloride ( $ZnCl_2$ ) was used. *Pseudomonas fluorescens* (ATCC 13525) was selected as the test organism for this experiment and it was cultured in nutrient broth.<sup>12</sup>

### Nutrient broth composition:

Peptone	5.0g
Beef extract	3.0g
NaCl	8.0g
Distilled water	1000 ml

For the test culture, 1ml of the prepared stock culture was transferred to Pikovskaya's medium<sup>13</sup> which has been treated with 0.5 mM, 2.5 mM, 4.5 mM, 6.5 mM, 8.5 mM and 10.5 mM concentrations zinc chloride ( $ZnCl_2$ ).

Testing of different parameters of the experiment was done using this test culture (Pikovskaya's medium) that have been treated concentration of  $ZnCl_2$ . The bacterial test culture without any  $ZnCl_2$  treatment was taken as control.

### Bacterial Culture

*P. fluorescens* (ATCC 13525) was used for this experiment. Stock cultures were prepared in 250 ml conical flask containing 50 ml of nutrient broth.<sup>12</sup> The pH was adjusted to pH 7 before sterilization at 121°C and 15 psi. The stock cultures were then incubated at 30 ± 1°C for 5 days in a bacteriological incubator. The cultures were hand shaken several times daily. All the experiment was conducted in triplicates with cultures in the logarithmic phase of growth.

### Measurement of bacterial growth by bacterial biomass

The heavy metal used in this experiment was zinc chloride (ZnCl<sub>2</sub>). Test cultures for the bacterial growth were prepared by using Pikovskaya's medium<sup>13</sup> minus the agar. ZnCl<sub>2</sub> concentration ranging from 0.5 mM, 2.5 mM, 4.5 mM, 6.5 mM, 8.5mM and 10.5 mM was added to the medium and was autoclaved at 121°C for 30 minutes. Pure Pikovskaya's medium was used as control for the study. The test cultures were divided into triplicates each containing 50ml of the medium broth treated with different ZnCl<sub>2</sub> concentration in 250 ml conical flasks. To each flask 1ml of bacteria from the stock culture was added. After 48 hours of incubation at 30 ± 1°C in a bacteriological incubator the inoculated flasks were then it was washed to remove salts and nutrients. The whole portion of washed cells were dried at 190-220°C for 1 hour in an oven and weighed. The dry weight of solid matter of bacteria is a measure of their proportion. The dry weight was measured as

$$W_2 - W_1$$

Where, W<sub>1</sub> = initial weigh of filter paper  
W<sub>2</sub> = final weigh of filter paper

### Measurement of Phosphatase Enzyme Activity<sup>14</sup>

Phosphatase enzyme activity of *P. fluorescens* was assayed spectrophotometrically by p-

nitrophenyl phosphate (PNP) method. In this experiment 0.1ml of filtrate obtained from the bacterial biomass test was transferred into different test tubes maintaining three replicates for each treatment. To it 4 ml modified universal buffer (pH 6.5), 0.25 ml toluene and 1ml of 0.115 m p-nitrophenyl phosphate (PNP) were added and was incubated at 37°C for one hour. After incubation 1 ml of 0.5 m CaCl<sub>2</sub> and 4 ml of 0.5 m NaOH was added. The test tubes were swirled gently for few minutes and the absorbance was measured at 430 nm.

## RESULTS

### Bacterial biomass

The effect of ZnCl<sub>2</sub> on the growth of bacteria *P. fluorescens* was shown on the graph (Fig. 1), which shows that with the increase in ZnCl<sub>2</sub> concentration there is reduction in the growth of the bacteria. This is shown by the considerable decrease in the dry biomass with increase in the ZnCl<sub>2</sub> concentration. However, *P. fluorescens* growth was at its highest (0.32±0.01 µg) in control i.e. the culture with pure medium and lowest (0.07±0.02 µg) at 10.5 mM i.e. highest concentration.

### Phosphatase Enzyme Activity

The effect of ZnCl<sub>2</sub> treatment on phosphatase activity of *P. fluorescens* was shown on the graph (Fig. 2). The increase in concentration of ZnCl<sub>2</sub> decreases the enzyme activity. However, at certain concentration, there is no negligible decrease in the enzyme activity. *P. fluorescens* growth was at its highest (4.43±0.09 µg) in control i.e. the culture with pure medium and lowest (1.03±0.13 µg) at 10.5 mM i.e. highest concentration.

## DISCUSSION

From the result of this experiment, it can be found that the availability of metal such as Zinc

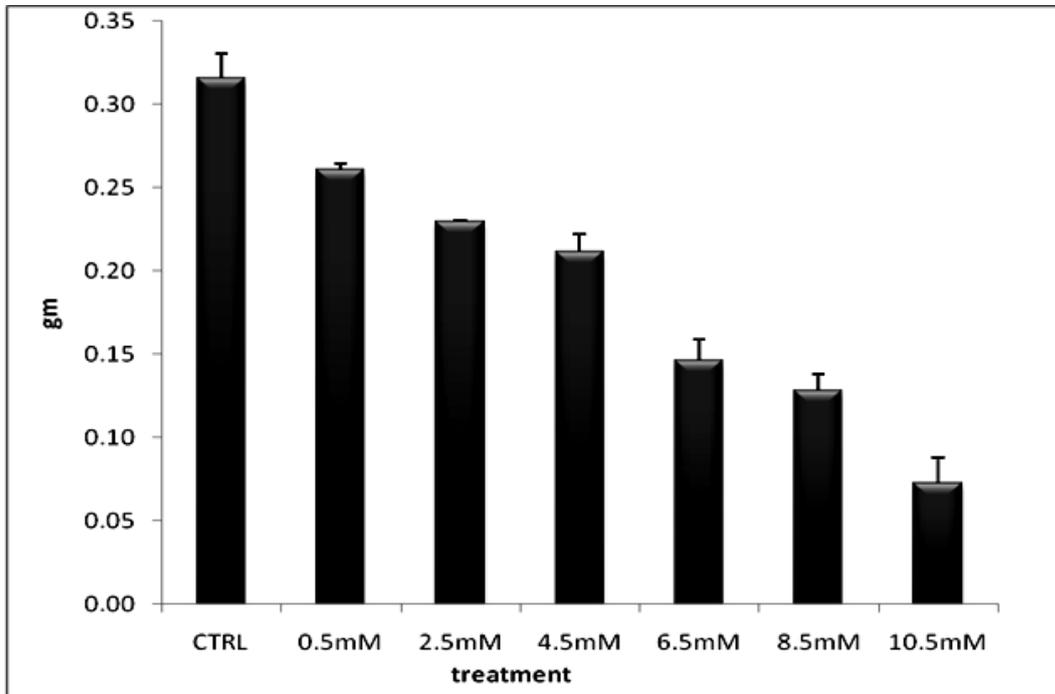


Figure 1. Bacterial biomass at different concentration of ZnCl<sub>2</sub>.

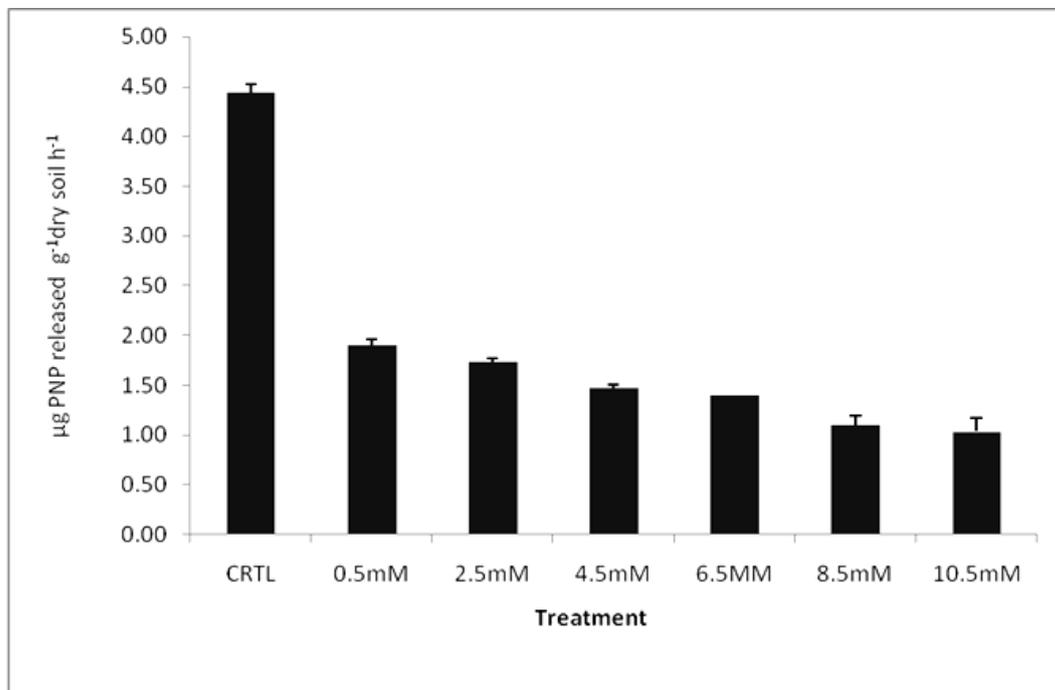


Figure 2. Phosphatase enzyme activity of *P. fluorescens* at different concentration of ZnCl<sub>2</sub>.

in its inorganic form can act as an inhibitory factor for the growth and activity of bacteria such as *P. fluorescens*. Since the biomass of *P. fluorescens* decrease with increase ZnCl<sub>2</sub> concentration, it can be concluded that ZnCl<sub>2</sub> acts as an inhibitor to the growth of *P. fluorescens*, and further, the presence of ZnCl<sub>2</sub> in the growth medium decreases the phosphatase enzyme activity of the bacteria. It thus clearly shows that metals can also affect the activity of the microbes. The overall decrease in the growth and enzymatic activity of *P. fluorescens* observed due to increase concentration of heavy metal contamination is in agreement with the findings of previous workers.<sup>15,16</sup> The overall profile of the bacteria remained the same with that of control.

The experiments result showed that even at low concentration the presence of heavy metal such as zinc in its available form is crucial to cause heavy metal toxicity. Even though the average concentration of ZnCl<sub>2</sub> in the experimental setup was very low but it caused a very high effect on bacterial growth and activity. This shows that the presence of heavy metal in the natural environment could have an adverse affect on the microbial community and activity as a whole. The activity and community composition of microorganism are closely related to soil fertility and environment quality. It has also provided evidence that heavy metals decrease the proportion of microbial biomass in the total soil matter.<sup>17</sup>

Lowest increase in biomass was observed at 10.5 mM concentration of ZnCl<sub>2</sub> which is the highest heavy metal treatment in the experimental setup. While the bacteria in control condition i.e. without ZnCl<sub>2</sub> treatment shows luxuriant growth. Thus, it clearly shows that the concentration of ZnCl<sub>2</sub> in the growth medium is inversely proportional to the increase in biomass of bacteria i.e. with increase in ZnCl<sub>2</sub> concentration there is decrease in the growth of bacterial biomass

With increase in ZnCl<sub>2</sub> concentration in growth medium, there is considerable decrease in the phosphatase enzyme activity of the bacteria. In control condition, the bacteria i.e. *P.*

*fluorescens* is found to have 4.4 phosphatase activity (µg PNP released g<sup>-1</sup> dry soil h<sup>-1</sup>). This shows that the presence of heavy metal in the growth medium greatly inhibit the enzymatic activity of the bacteria. However, at certain concentration of ZnCl<sub>2</sub> in the growth medium does not further reduce the phosphatase enzyme activity of the bacteria. 8.5 mM and 10.5 mM concentration of ZnCl<sub>2</sub> in the medium, the phosphatase enzyme activity remains relatively constant. This may be due to that ZnCl<sub>2</sub> has reached its saturation point so that it cannot further affect the enzymatic activity of the bacteria.

The result of this study is significant for the fundamental study of toxicity and inhibition effect of various heavy metals on soil microbial population. Also this study can help in estimation of the effect of heavy metals on enzyme activity of certain microbes.

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