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# Cadmium Removal from Water and Soil by a Cadmium-Resistant Rhizobacterium and Its Effect on Plant Root Elongation

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**Abstract**

A cadmium resistant rhizobacterium, *Pseudomonas* sp. PM2, was isolated from plant roots. It is highly resistant to cadmium toxicity. The minimum inhibitory concentrations of cadmium for *Pseudomonas* sp. PM2 is 2100 mg/L. *Pseudomonas* sp. PM2 produced high levels of siderophore and rhamnolipid biosurfactant in 74.88 μM of deferoxamine mesylate equivalents and 329.54 mg/L, respectively. It was able to remove cadmium ion in an aqueous solution by 69.84% at the initial cadmium ion concentration of 25 mg/L. *Pseudomonas* sp. PM2 significantly increased cadmium bioavailability in contaminated soil by increasing DTPA-extractable cadmium concentration or a bioavailable form of cadmium. The increase in cadmium bioavailability in soil helps to promote cadmium uptake by plants for cadmium phytoextraction. In addition, *Pseudomonas* sp. PM2 had no positive or negative effects on seed germination and root elongation of *Glycine max* L. under the absence and presence of cadmium. Our findings suggest that *Pseudomonas* sp. PM2 could be useful in the further development of biological treatment of cadmium in contaminated water and soil.

***Keywords:*** cadmium; siderophore; biosurfactant; *Pseudomonas* sp.; *Glycine max* L.

## 1. Introduction

Cadmium (Cd) contamination in water and soil is one of an important environmental problem because it is not biodegradable and persists indefinitely in the environment. It causes adverse effects on human health and other living organisms by disintegrating cell organelles and disrupting the membranes and the physiological process (Khan *et al*., 2009). Several treatments have been applied to clean-up water and soil that are contaminated by cadmium. Biological treatment is the use of plants and microorganisms to remove cadmium, which is considered as the best-environmental friendly method for treatment of cadmium in contaminated water and soil. Phytoextraction is the use of living green plants to uptake and accumulate heavy metals from the soil or water into the roots and shoots of the plants (Kumar *et al*., 1995). However, cadmium phytoextraction has some limitations, in particular a low cadmium bioavailability in soil and a slow plant growth rate due to cadmium toxicity (Glick, 2010).

Rhizobacteria are root-colonizing bacteria which inhabit many plants. Rhizobacteria have facilitated plant growth and development via direct and indirect mechanisms which are called plant growth promoting rhizobacteria (PGPR) (Glick, 2010). They can promote plant growth in several ways e.g., fix nitrogen, solubilization of mineral phosphate and other nutrients, and production of siderophores, plant growth hormones and antimicrobial metabolites (Bloemberg and Lugtenberg, 2001). In particular, siderophores, iron-chelating agent, are secreted by microorganisms under iron-starved conditions and solubilize iron for increasing iron uptake for plant growth (Burd *et al*., 2000). They not only bind to iron, but they also bind to other divalent heavy metals e.g. zinc, copper, lead and cadmium and result in increasing metal solubilization or bioavailability in soil, thus enhancing heavy metal uptake by plants (Nair *et al*., 2007; Braud *et al*., 2009; Rajkumar *et al*., 2010). Apart from siderophores, biosurfactant can bind to metals and increase metal solubilization (Thavasi *et al*., 2008). Removal of zinc and cadmium from soil by immobilized biosurfactant-producing bacteria has been reported by Sarin and Sarin (2010).

*Pseudomonas* sp. PM2, a cadmium-resistant rhizobacterium, was isolated from the root of *Brussus flabellifer* Linn. by Duangboobpha *et al*. (2013). It can grow well in the culture medium containing cadmium. This research focuses on the production of siderophores and biosurfactant by *Pseudomonas* sp. PM2 and the correlation between the cell growth and siderophores and biosurfactant production. Other plant growth-promoting properties, including phosphorous solubilization and indole-3-acetic acid (IAA) production were

also assessed. The abilities of this strain on removal of cadmium from aqueous solution and increasing cadmium bioavailability in contaminated soil were evaluated. In addition, the effects of *Pseudomonas* sp. PM2 on the seed germination and root elongation of *Glycine max* L. under cadmium toxic conditions were also investigated.

## 2. Materials and Methods

### *2.1. Cultivation of a cadmium-resistant rhizobacterium and its minimum inhibitory concentration of cadmium*

*Pseudomonas* sp. PM2 was cultured in Luria-Bertani (LB) agar (Criterion, USA) amended with 3 mM of cadmium nitrate. The long-term preservation of this strain was performed in LB broth supplemented with glycerol at -70°C (Watcharamusik *et al*., 2008). The minimum inhibitory concentration (MIC) of cadmium was determined according to the method of Raja *et al*. (2006) with some modification.

### *2.2. Quantitative determination of the levels of siderophores and biosurfactant production at different growth phases*

The levels of siderophores produced from *Pseudomonas* sp. PM2 which was cultivated in M9 miminal medium (Difco, USA) were determined in the form of deferoxamine mesylate (DFAM), one of the hydroxamate siderophores (Amico *et al*., 2008) according to the method of Schwyn and Neilands (1986). To determine the levels of biosurfactant production in form of rhamnolipid, *Pseudomonas* sp. PM2 was cultured in modified minimal medium (Pacheco *et al*., 2010) at 28°C with conditional shaking. The level of rhamnolipid biosurfactant was determined by orcinol assay (Chandrasekaran and Bemiller, 1980). In addition, bacterial growth was monitored by measuring cell density or optical density (OD) using spectrophotometer at wavelength 600 nm ($OD_{600}$).

### *2.3. Biosorption of cadmium ion from aqueous solution by a cadmium-resistant rhizobacterium*

The cadmium biosorption experiment was carried out by the method as described previously by Prapagdee *et al*. (2013). Briefly, *Pseudomonas* sp. PM2 was inoculated in LB broth and shook overnight. Bacterial cells were harvested by centrifugation and washed twice with a sterile 50 mM phosphate buffer. Cell pellets were re-suspended in different concentrations of cadmium ion, including 25, 50 and 75 mg/L, and shaken for 10 h at room temperature. Bacterial cells were separated from cadmium solution by centrifugation at 10,000 rpm for 20 min. The supernatant was digested by nitric acid using open tube digestion method. Cadmium concentration was analysed by Flame Atomic Absorption Spectrophotometer (FAAS) (Varian spectra model AA240FS, USA) and the percentage of cadmium removal efficiency was calculated.

### *2.4. Effects of a cadmium-resistant rhizobacterium on the increasing of cadmium bioavailability in contaminated soil*

Cadmium-contaminated soil was collected from an agricultural area at Mae Sot district, Tak province, northern Thailand. Soil was digested by hot plate method of 3050B US.EPA (1996). The digested sample was filtrated through Whatman filter paper No. 41 before analysis of total cadmium concentration using FAAS. The bioavailable or extractable form of cadmium in soil was extracted by diethylene triamine pentaacetic acid (DTPA)-triethanolamine (TEA) solution (Faust and Christians, 2000) and analysed by FAAS. The method for testing cadmium solubilization in contaminated soil was previously described by Kijawatworawet *et al*. (2014).

### *2.5. Study of plant growth-promoting properties in a cadmium-resistant rhizobacterium*

The ability on phosphorous solubilization in *Pseudomonas* sp. PM2 was assayed using the method of Picovskaya's (1948). Briefly, overnight cells of *Pseudomonas* sp. PM2 were dropped on Picovskaya's agar plate and incubated at 28°C for 7 days. The diameter of the halo zone which appeared on Picovskaya's agar was measured. The levels of indole-3-acetic acid (IAA) produced from *Pseudomonas* sp. PM2 at different growth phased were analyzed according to the method of Bric *et al*. (1991) with some modification. Briefly, a 1 mL of cell-free supernatant was mixed vigorously with 2 mL of Salkowski's reagent and allowed to stand for 20 min before measuring the absorbance at a wavelength of 530 nm. The levels of IAA were determined using a standard calibration curve generated from analytical grade IAA (Sigma, USA).

### *2.6. Plant root elongation assay on filter paper culture*

Preparation of bacterial cells and an *in vivo* root elongation assay were conducted according to the methods of Prapagdee *et al*. (2013). The seeds of *G. max* L. were surface sterilized with a mixture of an

equal volume of absolute ethanol and 30% hydrogen peroxide and washed twice with sterile distilled water. Various concentrations of cadmium ion, including 0, 25, 50 and 75 mg/L, were applied to sterile filter paper in a Petri dish. *G. max* L. seedlings were soaked with *Pseudomonas* sp. PM2 and aseptically placed on a sterile filter paper containing different concentrations of cadmium ion. *G. max* L. seedlings which were soaked in a sterile 50 mM phosphate buffer were carried out as a control experiment. Petri dishes were incubated at room temperature in dark condition for 10 days. The seed germination, root length and root fresh weight of these seedlings were observed and measured.

## 3. Results and Discussion

### *3.1. Growth and a MIC of cadmium*

*Pseudomonas* sp. PM2 grew well on LB agar amended with 3 mM cadmium nitrate. The number of viable cells in LB broth supplemented with 3 mM cadmium nitrate was $2.0\times10^8$ CFU/mL. The MIC value of cadmium for *Pseudomonas* sp. PM2 was 2100 mg/L. MIC represents the resistant ability of bacteria to a specific substance that causes inhibition of bacterial growth. Kijawatworawet *et al*., (2014) reported that MIC value of cadmium for *Pseudomonas* sp. TS32, a siderophores-producing bacterium, was 400 mg/L. *Pseudomonas aeruginosa* BS15 resisted to 500 mg/L of cadmium and it had resistant ability against other heavy metals e.g. chromium, lead, nickel (Raja *et al*., 2006). It indicates that *Pseudomonas* sp. PM2 has a high tolerance to cadmium toxicity.

### *3.2. Levels of siderophore and biosurfactant produced from a cadmium-resistant rhizobacterium*

The yield of siderophores in *Pseudomonas* sp. PM2 was slightly low in the beginning of a growth period (the lag phase) and sharply increased with an exponential phase of growth. The high levels of siderophores produced from *Pseudomonas* sp. PM2 were observed when cells entered the stationary phase of growth (24 h) and siderophore production stabilized throughout the incubation period (69.02-74.88 μM) (Fig. 1). The results found that the highest level of siderophores produced from *Pseudomonas* sp. PM2 was observed at the stationary phase of growth. Similar to the study of Kijawatworawet *et al*. (2014), the highest level of siderophores produced by *Pseudomonas* sp. TS32 (59.19 μM) was observed at the stationary phase. *Pseudomonas azotoformans* produced the highest siderophores after 48 h of incubation (Nair *et al*., 2007). The pH and the presence of iron and other heavy metals have an influence on siderophore production (Rajkumar *et al*., 2010). Siderophore biosynthesis in *Pseudomonas aeruginosa* strain KUCd1, a cadmium-resistant bacterium was induced by cadmium (Sinha and Mukherjee, 2008).

*Pseudomonas* sp. PM2 produced the highest level of rhamnolipid biosurfactant by 329.54 mg/L at 96 h of incubation period in modified minimal medium (Fig. 2).

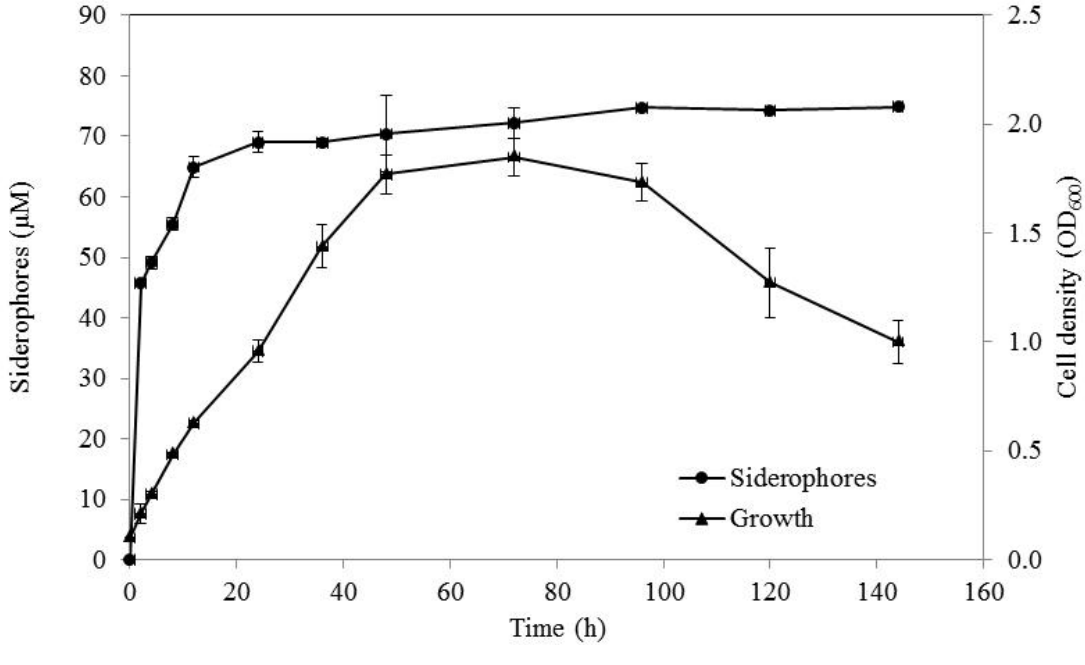


Figure 1. Time-course experiments of siderophores production and growth of *Pseudomonas* sp. PM2 cultured in a M9 minimal medium

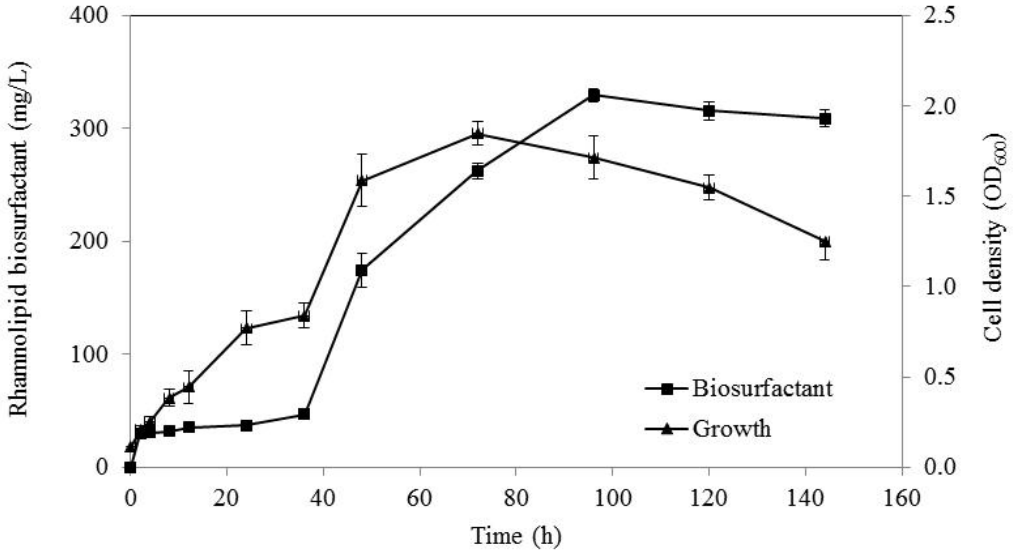


Figure 2. Time-course experiments of rhamnolipid biosurfactant production and growth of *Pseudomonas* sp. PM2 cultured in a modified minimal medium

The yields of rhamnolipid biosurfactant production sharply increased after 36 h of growth period (the exponential phase). Rhamnolipid biosurfactant which produced mainly by *P. aeruginosa* and *Burkholderia* spp. consists of one or two rhamnoses linked to one or two chains of β-hydroxy fatty acid (Perfumo *et al*., 2006). Rosa *et al*. (2010) reported that *P. aeruginosa* LBM10 produced rhamnolipid biosurfactant by 4500 mg/L. Modified minimal medium contains glycerol, a simple fatty acid precursor, which served as a carbon source for bacterial growth (Silva *et al*., 2010). The suitable sources of carbon for rhamnolipid production in *P. aeruginosa* are glycerol, glucose, n-alkanes and triglycerides (Lang and Wullbrandt, 1999).

*3.3. Ability of a cadmium-resistant rhizobacterium on cadmium removal in aqueous solution*

The percentages of cadmium removal in aqueous solution by *Pseudomonas* sp. PM2 at cadmium ion concentrations of 25, 75 and 100 mg/L were 69.84, 47.86 and 41.68%, respectively. It indicates that *Pseudomonas* sp. PM2 acts as cadmium biosorbent to remove the cadmium ion from an aqueous solution. Heavy metals can bind to bacterial cell walls and extracellular polymeric substances and precipitated in aqueous solution (Ahalya *et al*., 2003). The percentages of arsenic removal using *P. azotoformans*, a siderophore-producing bacterium, diaminetetra acetic acid (EDTA) and citric acid were 92.8, 77.3 and 70.0%, respectively (Nair *et al*., 2007). In addition, Huang and Liu (2013) reported that *Pseudomonas* sp. LKS06, a biosurfactant-producing bacterium was able to remove cadmium and lead ions from aqueous solution. Our findings indicate that the efficiency of cadmium ion removal in aqueous solution by *Pseudomonas* sp. PM2 involved the actions of siderophore and biosurfactant.

*3.4. Increasing of cadmium bioavailability in contaminated soil by a cadmium-resistant rhizobacterium*

*Pseudomonas* sp. PM2 was added in a sterilized cadmium contaminated soil at the initial soil cadmium concentration of 46.37 mg/kg for 7 days compared to the uninoculated control. The results of concentrations of soil cadmium, water soluble cadmium and DTPA-extractable cadmium are presented in Table 1. Concentrations of soil cadmium and water-soluble cadmium in soil with *Pseudomonas* sp. PM2 inoculation and the uninoculated control were not significant difference ($p<0.05$). Interestingly, concentration of DTPA-extractable cadmium in soil with *Pseudomonas* sp. PM2 inoculation was higher than that of the uninoculated control soil. DTPA-extractable fraction is suitable for assessing the bioavailability of cadmium in soil (Prokop *et al*., 2003). DTPA, a chelating agent, has the potential to increase the bioavailability of unavailable heavy metal fractions (Karami and Shamsuddin, 2010).Our result indicates that *Pseudomonas* sp. PM2 was able to increase cadmium bioavailability in contaminated soil. The bioavailable form of cadmium is easily uptaken by plants (Sheng and Xia, 2006). Several investigations have reported the application of siderophore or biosurfactant-producing bacteria for improving heavy metal bioavailability and heavy metal phytoextraction in contaminated soil (Braud *et al*., 2009; Rajkumar *et al*., 2010; Sarin and Sarin, 2010; Rufino *et al*., 2012).

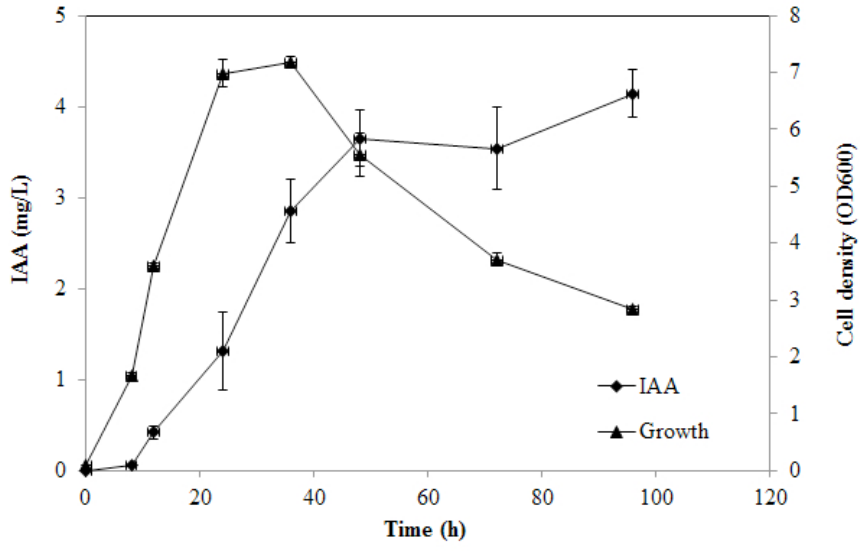


Figure 3. Time-course experiments of IAA production and growth of *Pseudomonas* sp. PM2 cultured in LB broth

*3.5. Other plant growth promoting properties of by a cadmium-resistant rhizobacterium and its effects on seed germination and root elongation of G. max L. under cadmium toxic condition*

*Pseudomonas* sp. PM2 was not able to solubilize phosphate and produced very low levels of IAA (Fig. 3). The ability of rhizobacteria on phosphate solubilization and IAA production are important properties of plant-growth promoting rhizobacteria. It suggests that *Pseudomonas* sp. PM2 did not have these plant-growth promoting properties. In order to apply *Pseudomonas* sp. PM2 for cadmium phytoextraction, the effects of this cadmium-resistant rhizobacterium on seed germination and plant root elongation were assessed. The results found that *Pseudomonas* sp. PM2 had no effect on seed germination of *G. max* L. compared to the uninoculated control. The root length and root fresh weight of *G. max* L. seedlings decreased with increasing cadmium concentrations. Root length and root fresh weight of *G. max* L. seedlings at toxic concentrations of cadmium were not significantly increased by *Pseudomonas* sp. PM2 (Fig. 4). It was due to the fact that, *Pseudomonas* sp. PM2 did not produce high amounts of IAA. IAA promotes root elongation and cell division in plants that enhances nutrient uptake. It has been reported that IAA-producing rhizobacteria promoted root elongation in *Helianthus annuus* L. seedlings under cadmium toxic conditions (Prapagdee *et al*., 2013). Neither positive nor negative effects on root elongation of *G. max* L. seedlings by *Pseudomonas* sp. PM2 were observed under the absence or presence of cadmium. Our findings suggest that *Pseudomonas* sp. PM2 can be safely used for improving cadmium bioavailability in contaminated soil for cadmium phytoextraction.

## 4. Conclusion

*Pseudomonas* sp. PM2, a cadmium-resistant rhizobacteria, produced siderophore and rhamnolipid biosurfactant. It was able to remove cadmium ion in aqueous solution. In addition, this strain effectively increased cadmium bioavailability in cadmium contaminated soil resulting in increasing cadmium uptake by plants. *Pseudomonas* sp. PM2 neither inhibited nor promoted the seed germination and root elongation of *G. max* L. under the absence and presence of cadmium. Our findings clearly demonstrate that *Pseudomonas* sp. PM2 could be useful for further development of biological treatment of cadmium in polluted water and soil.

Table 1. Cadmium concentrations in contaminated soil

| Treatment | Cadmium concentration (mg/kg) | | |
|---|---|---|---|
| | Soil cadmium | Water-soluble cadmium | DTPA-extractable cadmium |
| No bacteria added (Control) | 42.43 ± 1.97[a] | 0.77 ± 0.06[a] | 26.13 ± 0.45[a] |
| *Pseudomonas* sp. TS32 | 40.37 ± 2.13[a] | 0.75 ± 0.00[a] | 28.73 ± 0.15[b] |

Means and the S.E. (n = 5) followed by the different letter within columns were significantly different ($p < 0.05$) according to Duncan's multiple range test.

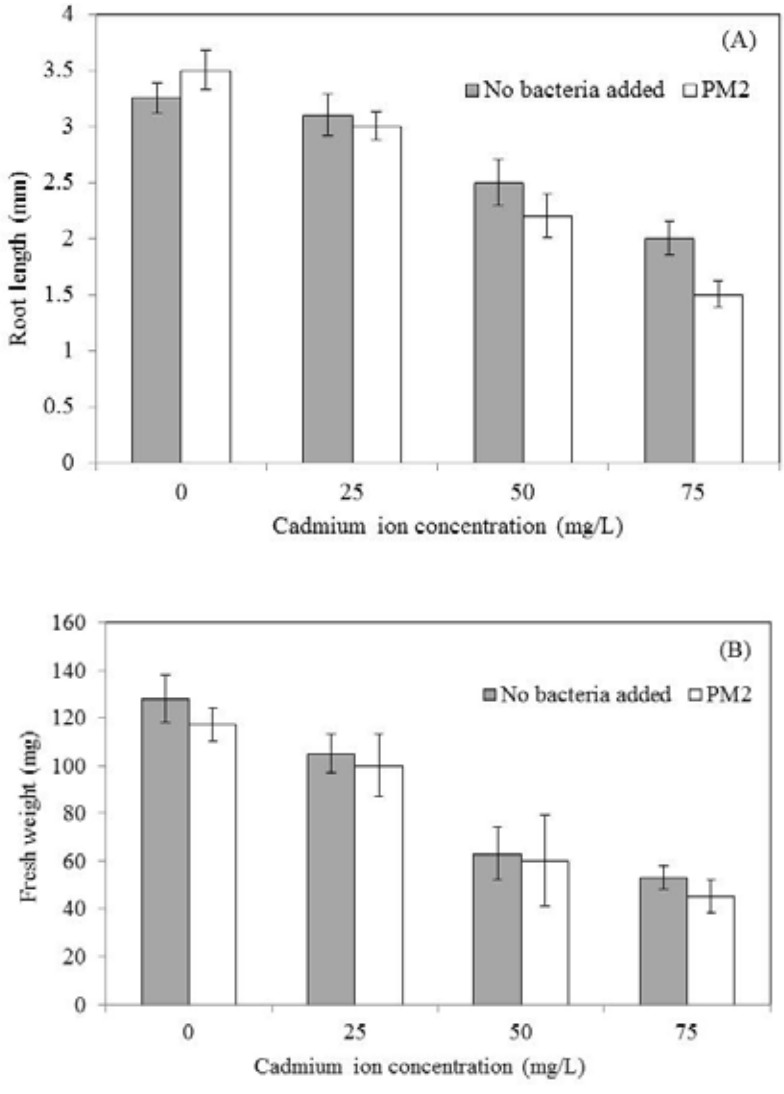


Figure 4. Effects of *Pseudomonas* sp. PM2 on the (A) root length and (B) fresh weight of *G. max* L. seedlings under cadmium toxic condition after incubation for 10 days. Means and the S.E. ($n = 10$) were not significantly different ($p < 0.05$) according to the analysis of variance.

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