کود زیستی چند منظوره از Pseudomonas putida PT: رویکردی برای تحریک همزمان رشد ذرت و زیست‌پالایی خاک‌های آلوده به کادمیوم

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چکیده

مقدمه: آلودگی خاک‌های کشاورزی با فلزات سنگین یک معضل جهانی است. هدف پژوهش تهیه کود زیستی چند منظوره با قابلیت حذف کادمیوم از خاک‌های آلوده و افزایش رشد گیاه است.

مواد و روش‌ها: جهت ارزیابی توانایی سویه Pseudomonas putida PT در برقراری رابطه‌ای موثر با گیاهان، تاثیر برخی ترکیبات موجود در ریشه گیاهان بر کموتاکسی، رشد و تشکیل بیوفیلم بررسی شد. آزمایش‌های گلدانی با کودهای زیستی تهیه شده از P. putida PT با دو روش متفاوت شامل غوطه‌وری بذر و تثبیت باکتری بر سبوس برنج به عنوان حامل، انجام شد. پس از محاسبه وزن تر و خشک گیاهان، غلظت‌های کادمیوم در بخش‌های هوایی گیاه و خاک در حضور و عدم حضور کودهای زیستی به وسیله طیف سنجی جذب اتمی اندازه‌گیری شد.

نتایج: پاسخ‌های کموتاکتیک مثبت P. putida PT به ساکارز، مانیتول، گلوکز، آلانین، هیستیدین، تریپتوفان، سوکسینیک اسید، مالیک اسید و سیتریک اسید مشاهده شد. مانیتول، ساکارز و سوکسینیک اسید رشد و تشکیل بیوفیلم را افزایش دادند. مالیک اسید تنها رشد باکتری و هیستیدین، تریپتوفان و گلوکز تشکیل بیوفیلم را بهبود بخشیدند. هر دو نوع کودهای زیستی وزن تر و خشک ذرت را حدود دو برابر افزایش دادند. حداکثر کاهش کادمیوم در خاک به ترتیب در حضور کودهای زیستی تهیه شده با روش‌های تثبیت سلولی (۹۷,۵۷٪) و غوطه‌وری بذر (۶۸,۶۷٪) مشاهده شد. غلظت کادمیوم در گیاهان از ۶۳۱۰ پی‌پی‌بی در آزمایش شاهد به ۸۸۴ و ۵. ۲۹۱۷ پی‌پی‌بی به ترتیب در حضور کودهای زیستی تهیه شده با تکنیک‌های تثبیت سلولی و غوطه‌وری بذر، کاهش یافت.

بحث و نتیجه‌گیری: استفاده از این کودها رویکردی جهت بهبود رشد ذرت در خاک‌های آلوده به کادمیوم توام با کاهش غلظت فلز در خاک و گیاه است.

واژه‌های کلیدی: بیوفیلم، تثبیت سلول، زیست پالایی، غوطه‌وری بذر، کادمیوم،کود زیستی، کموتاکسی، محرک رشد گیاه

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Multifunctional Biofertilizer from *Pseudomonas Putida* PT: A Potential Approach for Simultaneous Improving Maize Growth and Bioremediation of Cadmium-polluted Soils

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Abstract

Introduction: Pollution of agricultural soils with heavy metals is a serious problem in the world. The aim of this study was to produce a multipurpose biofertilizer which can remove cadmium from polluted soils as well as enhancing plant growth.

Materials and methods: To study the ability of *Pseudomonas putida* strain PT for establishing an effective relationship with plants, the effects of some compounds present in plant root exudates were examined on chemotaxis, growth, and biofilm formation of bacteria. Pot experiments were performed by using biofertilizers prepared from *P. putida* PT by two different methods including seed immersion and immobilization of the bacterial cells on rice bran as carrier. After measuring the fresh weight and dry weight of each plant, cadmium concentrations in plant aerial tissues and soil in the presence and absence of biofertilizers was measured by atomic absorption spectrometry.

Results: Results showed positive chemotactic responses of *P. putida* PT to sucrose, mannitol, glucose, alanine, histidine, tryptophan, succinic acid, malic acid, and citric acid. Succinic acid, mannitol, and sucrose promoted both biofilm formation and growth of *P. putida* PT. Malic acid enhanced only the bacterial growth, whereas histidine, tryptophan and glucose promoted biofilm formation. Both biofertilizers enhanced the fresh and dry weight of maize plants about 2-fold. The maximum reduction of cadmium concentration in the soil was observed in the presence of immobilized cells (97.57%), followed by the inoculation of bacterial cells by seed immersion method (68.67%). Cadmium concentration in plants was decreased from 6310 ppb in control experiment to 885 ppb and 2917.5 ppb in the presence of biofertilizers produced by cell immobilization and seed immersion techniques, respectively.

Discussion and conclusion: Using these fertilizers is an approach to promote maize growth in cadmium contaminated soils along with decreasing metal concentrations in soil and plant.

Key words: Biofertilizer, Bioremediation, Biofilm, Cadmium, Cell Immobilization, Chemotaxis, Plant Growth Promoting, Seed Immersion.

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Introduction

Pollution of agricultural soils with heavy metals, due to the disposal of untreated toxic waste of industries such as mining, leatherworking, metallurgy and electric appliance manufacturing, has become a serious problem in the world (1-6). On the other hand, increasing world's population has dramatically increased the demand for high yield crops (7). This matter has caused to overuse chemical fertilizers, leading to a large-scale release of heavy metals into the environment, particularly Cd (8), and decline of soil quality and fertility (9). Soluble heavy metals in the soil solution or those that are easily solubilized by root exudates can be uptaken by plant (10). The reduction of plants growth and crops yield are the results of heavy metal accumulation in plant tissues (1, 11). On the other hand, plants contaminated with heavy metals (vegetables, fruits, and grains) are the most important metal entrance routes to the human food chain especially in the case of cadmium (12-15). Cadmium seriously threatens human health with its carcinogenic, mutagenic and teratogenic properties (16). Since heavy metals, unlike some other pollutants, cannot be decomposed to compounds with low toxicity, their toxic effects increase gradually in the environment (17). So, the remediation of metal-contaminated soils is necessary to keep sustainable agriculture and subsequently a healthy life. High costs, incomplete metal removal, forming secondary pollution, destruction of soil texture and decreasing soil fertility are the disadvantages of physicochemical methods used for remediating polluted soils. In recent years, biological methods (bioremediation) have gained more prominence as an alternative technology due to simplicity, efficiency and low costs (18). Plant growth promoting bacteria (PGPB) are a heterogeneous group of bacteria that can enhance plant growth by synthesizing or facilitating the uptake of nutrients from the soil. Also, they can protect plants against phytopathogenic microorganisms (19-26). Using heavy metal resistant microbes promoting plant growth is a low-cost and eco-friendly approach to remove metals from the soil and increase plant growth without effects on soil structure (27). The soil is an unpredictable environment, so finding a suitable niche for survival amongst the competitors, predators and harsh environmental conditions like toxic compounds for inoculated bacteria is often difficult. In general, after the inoculation of bacterial cells without a suitable formulation into the soil, the bacterial population decreases rapidly. A major function of inoculant formulation is to provide a proper microenvironment for improving the survival and retention of bacterial cells (28). Also, an appropriate formulation protects the stability of bacterial cells during storage and transportation (29). Formulation of inoculant is an industrial art which converts a laboratory proven bacteria to a commercial product. The aim of this study is to produce a multipurpose biofertilizer which can remove cadmium from polluted agricultural soils as well as enhancing plant growth. To achieve this objective: (1) the effects of some organic compounds present in plant root exudate on growth, chemotactic responses and biofilm formation of *P. putida* PT as a plant growth promoting bacteria were evaluated; (2) biofertilizers were produced by two different methods (seed immersion and bacterial cells immobilization on rice bran); and (3) the effect of each biofertilizer on growth of *Zea maize* and the amount of Cd^{2+} adsorption from the soil by this plant was compared.

Materials and Methods

Source of Bacterial Strain: *P. putida* PT was isolated previously from the rhizosphere of plants cultivated in agricultural soil polluted by heavy metals. The selected bacterial strain was characterized by using standard morphological, physiological and biochemical tests according to Bergey's Manual of Determinative Bacteriology, followed by molecular identification based on 16S-rRNA gene sequence analysis (GenBank accession NO. KX963368).

Chemicals and Media Preparation: A synthetic minimal salt medium (MSM) containing (g L^{-1}): KH_2PO_4, 2.25; K_2HPO_4, 2.25; $(NH_4)2SO_4$, 1.0; $MgCl_2.6H_2O$, 0.2; NaCl, 0.1; $FeCl_3.6H_2O$ was used as basal medium for chemotaxis, growth and biofilm formation tests. Some amino acids (i.e. alanine, histidine and tryptophan), sugars (i.e. sucrose, mannitol and glucose) and organic acids (i.e. succinic acids, malic acid and citric acid) which had been reported in previous studies as the most important compounds present in plant root exudate (30) were selected for further investigation in mentioned experiments. Stock solutions of these compounds were prepared in MSM. The metal stock solution was prepared in double-distilled water and sterilized by a syringe filter (0.45 µM). All chemicals were purchased from Sigma (Sigma Chemical Company, Germany) or Merck (Germany).

Chemotaxis Assay: Swim plates for the qualitative analysis of chemotaxis were prepared in MSM which was solidified by adding 0.2% agar (31). A colony of *P. putida* PT (grown on tryptic soy agar (TSA) medium (Typical composition (g/litre): pancreatic digest of casein 15.0; papaic digest of soya bean 5.0; sodium chloride 5.0; agar-agar 15.0.)) was inoculated into the agar at the center of each plate. Then, crystals of amino acids, organic acids and sugars were placed separately on the right side of each plate. After 24 h incubation at 30˚C, the halos of bacterial movement toward chemoattractants were investigated. The plate containing one drop of sterile distilled water instead of organic compounds was considered as negative control.

Evaluation of *P. Putida* PT Growth: *P. putid*a PT growth in the presence of some organic compounds present in plant root exudates was evaluated using 96-well microtiter plate based on the protocol described previously (32) with minor modifications. Briefly, each well of the microtiter plate was loaded with 150 µL of different concentrations of organic acids (25, 50 and 100 µM), sugars (10, 50 and 100 mM) and amino acids (5 and 10 mM). Then, 50 µL of diluted bacterial culture in MSM ($OD6_{00nm}$= 0.1) was added to each well. Control wells contained 150 µL of MSM and 50 µL of bacterial culture. All experiments were done in triplicate. After 24 h incubation at 30˚C, the OD (optical density) was measured by an ELISA reader at 630nm.

Biofilm Formation Assay: Biofilm formation was assayed by following the protocol described previously with some modifications (32). Flat-bottomed microtiter plates were loaded according to conditions mentioned in the previous section and incubated at 30˚C for 24h. The growth phase medium was removed carefully by pipetting, and each well was rinsed with 250µL of saline (0.09% NaCl). The remaining attached bacterial cells were fixed by adding 200 µL of ethanol to each well. After 15 min, the wells were emptied and left to dry at room temperature. Then, each well was stained with 200 µL of 0.1% (w/v) crystal violet solution for 10 min. Excess dye was rinsed off by normal saline slowly. Finally, 100 ml of 30% (v/v) acetic acid was added to each well, and the OD was read by ELISA reader at 630 nm after 15 min.

Biofertilizer Preparation: Biofertilizers were prepared in two different types of formulation: bacterial inoculation using the seed immersion method and immobilization on rice bran. *P. putida* PT was cultured in conical flasks containing 50 ml Tryptic Soy Broth (TSB) medium at 30°C on an orbital shaker at 120 rpm until reaching exponential growth phase. The bacterial suspension was adjusted to the approximate concentration of 2.5×10^9 CFU/ml.

In seed immersion method, maize (*Z. mays*) seeds provided by the Soil and Water Research Institute of Isfahan were transferred to the bacterial suspension (1 seed per 1 ml of bacterial inoculants) and allowed to incubate for 30 min at room temperature. To prepare immobilized cells on rice bran, the bacterial suspension was added to the sterilized rice bran (1 ml of bacterial suspension per 1 gram of rice bran) and incubated at room temperature for 5 days (33).

Pot Experiments: Pot experiments for studying the effect of the biofertilizers on the plant growth and heavy metal uptake in *Z. mays* were conducted in greenhouse condition. Physicochemical properties of the soil used in the experiments were as follows: 55% sand; 18.4% silt; 24% clay; cation exchange capacity (CEC) of 7.5 cmol/kg; 10000 mg/kg Fe; 330 mg/kg Mn; 83 mg/kg Zn; 25 mg/kg Cu and 30 mg/kg Pb. Soil samples were dried at room temperature and passed through a sieve with a diameter of 2 mm. The metal salt was dissolved in distilled water and mixed thoroughly with the soil (~ 40000 ppb Cd in each kg of soil). The experiment involved three separate treatments with three replications: (I) plants growing in the inoculated soil with the immobilized bacterial cells on rice bran; (II) inoculated plants by seed immersion method (III) control treatment (plants growing in soil in the absence of bacterial cells). After 70 days of incubation, plants were removed from the pots carefully and were rinsed with distilled water. After measuring the fresh weight, dry weight of each plant was determined after drying at 70 ˚C for 48h. The cadmium concentration in *Z. mays* aerial tissues was determined using Atomic Absorption Spectrometry (AAS). All experiments were performed triplicates.

Statistical Analysis: One-factor analysis of variance (ANOVA) followed by Scheffe's post hoc analysis were performed to assess the difference between different treatments. Level of significance unless specified is $P < 0.05$.

Results

Chemotaxis Assay: *P. putida* PT migration from the inoculation point to organic compounds added to swim plates exhibited positive chemotactic responses of this bacterium to all amino acids, sugars and organic acids tested in this study. No chemotactic response was detected in the negative control plate which was contained one drop of sterile distilled water instead of organic compounds (Figure 1).

Evaluation of *P. Putida* PT Growth: To evaluate the influence of plant root exudates on *P. putida* PT growth, different amino acids, sugars and organic acids at various concentrations were added to the microorganism culture medium. Citric acid at concentrations from 25 and 50 µM did not produce a significant effect on the microorganism growth. Malic acid at concentrations from 50 to 100 µM and succinic acid at the concentration of 100 µM significantly decreased *P. putida* PT growth. Succinic acid at concentrations from 25 to 100 µM and malic acid at the concentration of 25 µM significantly enhanced the growth compared to control (Fig. 2a). Alanine, tryptophan, and histidine revealed no significant effects on growth (Fig. 2b). Also, the results indicated that *P. putida* PT growth is independent regarding the variation in the concentration of

glucose. The growth of microorganism significantly decreased by mannitol and sucrose at the concentration of 10 mM but, these sugars at concentrations from 50 to 100 mM enhanced the growth significantly (Fig. 2c).

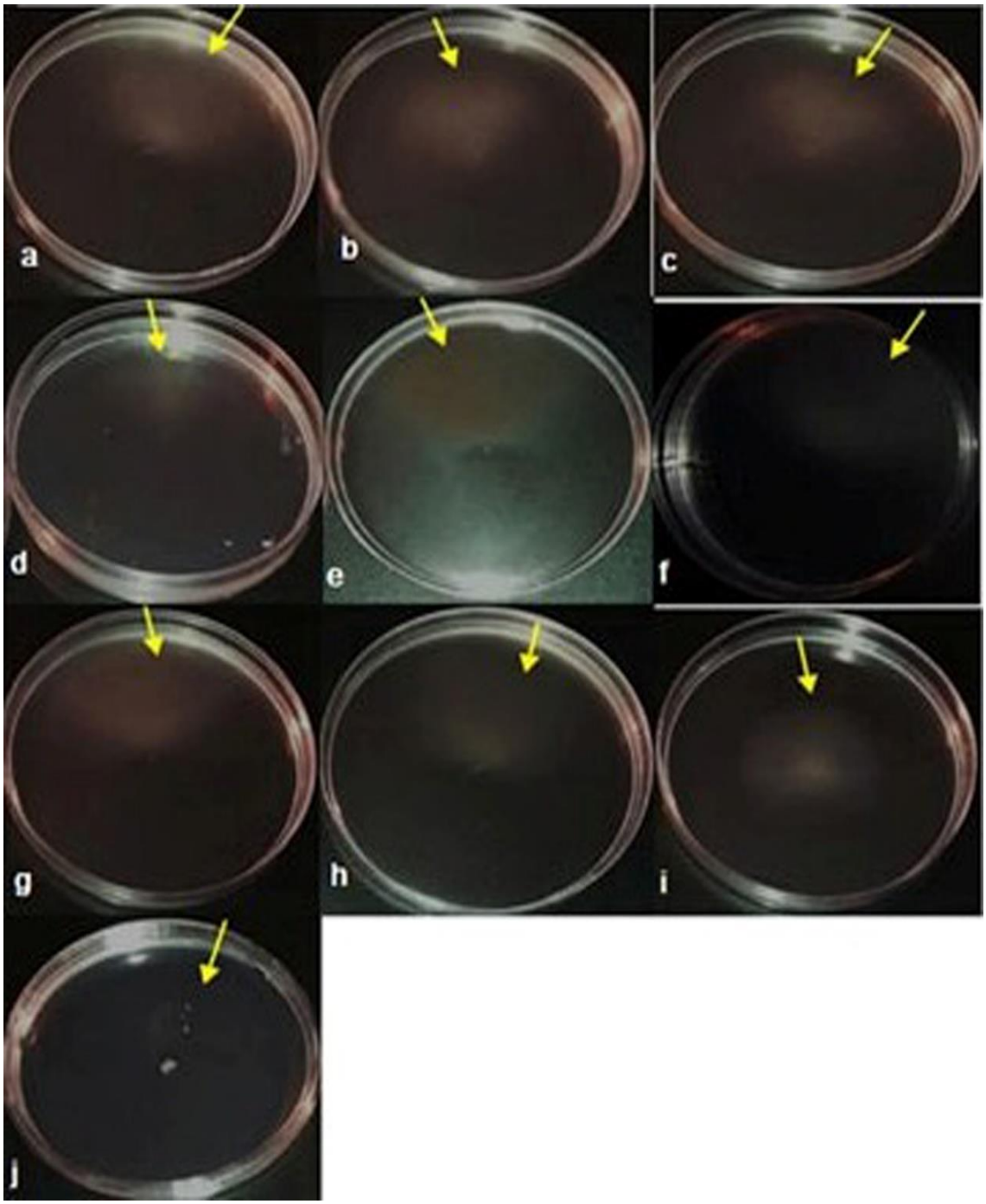

Fig. 1- *P. putida* PT chemotactic response to (a) tryptophan, (b) alanine, (c) histidine, (d) malic acid, (e) citric acid, (f) succinic acid, (g) mannitol, (h) glucose and (i) sucrose in qualitative swim plate assays. A positive chemotactic response is indicated by the formation of a swimming ring toward the chemoattractant (arrows represent *P. putida* PT movement in the direction of the chemoattractants). No response was detected when only a drop of sterile distilled water was added (plate (j) as negative control).

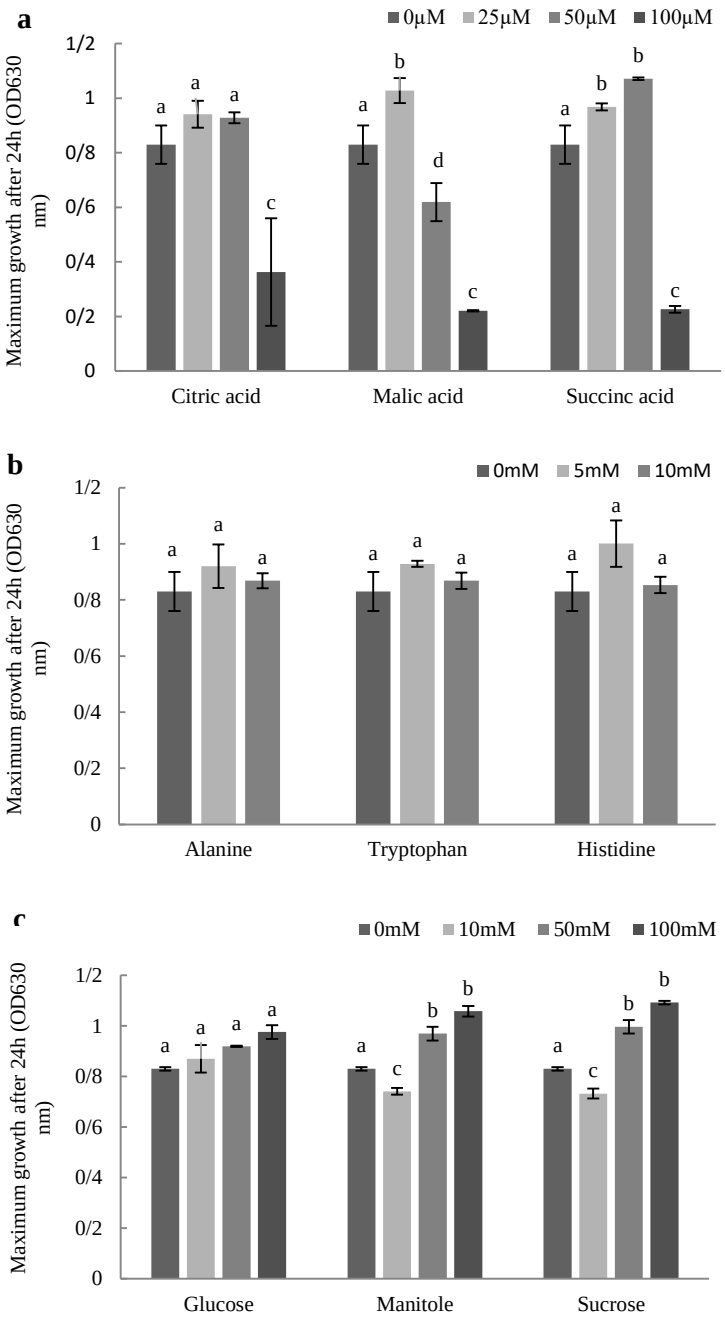

Fig. 2- Effects of different types and concentrations of (a) organic acids, (b) amino acids and (c) sugars which are present in root exudates on *P. putida* PT growth. Each value is the mean of triplicates. Bars represent standard error. Data of columns indexed by the same letter are not significantly different ($P < 0.05$) according to the Scheffe test ($p < 0.05$).

Biofilm Formation Assay: Biofilm formation in *P. putida* PT was measured following incubation in the presence of different types of amino acids, sugars, and organic acids which are present in plant root exudate. Twenty four hours after incubation, succinic acid at the concentration of 50 µM could significantly enhance the biofilm formation of *P. putida* PT. Malic acid and citric acid revealed no effects on the biofilm formation (Fig. 3a). Histidine at concentrations from 5 to 10

mM and tryptophan at the concentration of 10 mM significantly stimulated the *P. putida* PT biofilm formation compared to the control. Alanine represented no significant effect on the biofilm formation (Fig. 3b). All of the sugars tested in this study induced biofilm formation and this stimulatory effect was concentration-dependent. Glucose and sucrose at concentrations from 50 to 100 mM and mannitol at the concentration of 10 mM significantly increased biofilm formation (Fig. 3c).

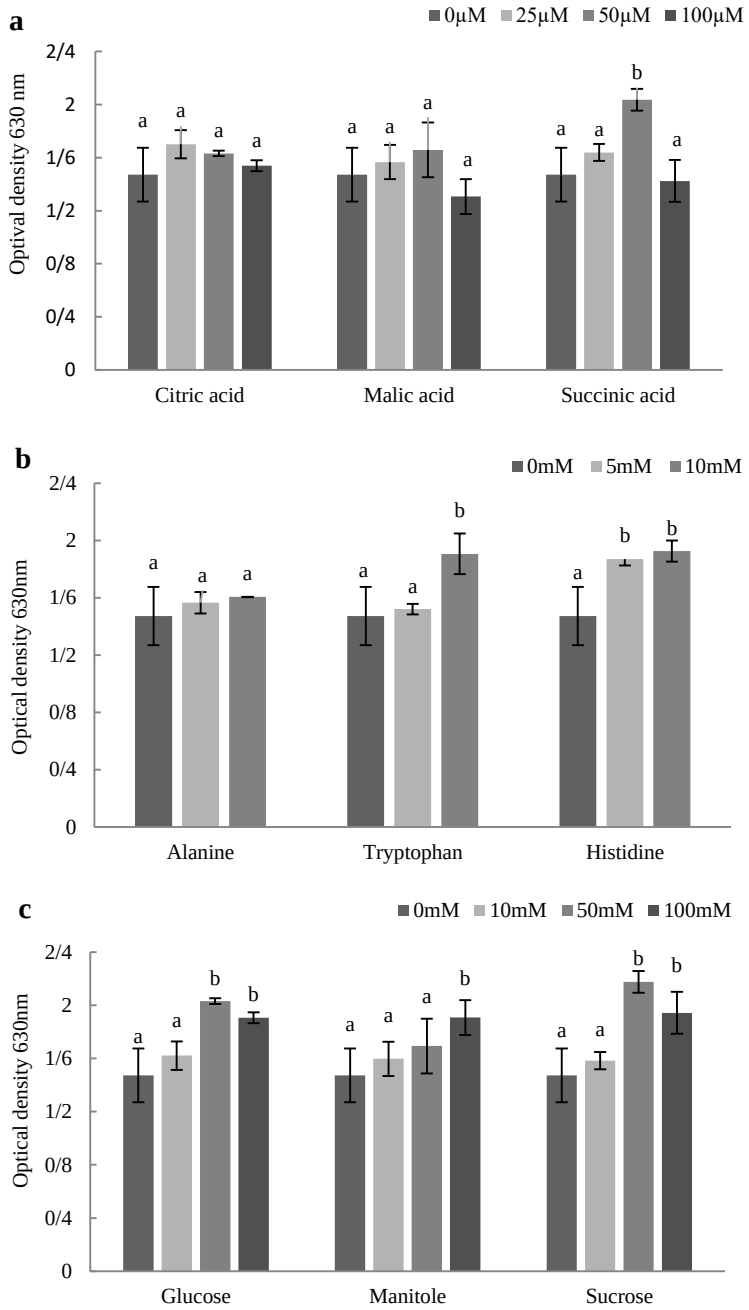

Fig. 3- Effects of different types and concentrations of (a) organic acids, (b) amino acids and (c) sugars which are present in root exudates on biofilm formation in *P. putida* PT. Each value is the mean of triplicates. Bars represent standard error. Data of columns indexed by the same letter are not significantly different according to the Scheffe test ($p < 0.05$).

Plant Growth-Promoting Effects of the Biofertilizers on *Z. mays* Biomass: The fresh and dry weights of *Z. mays* were measured to determine the effects of biofertilizers on plant growth. Both types of biofertilizers prepared in the present study significantly enhanced the fresh and dry weights of *Z. mays* at the harvest time (70 days after sowing). After inoculation of immobilized bacterial cells on rice bran, a 2-fold increase of fresh weight and a 2.15-fold increase of dry weight were observed. After bacterial inoculation using the seed immersion method, fresh and dry weights were respectively 1.7-fold and 1.65-fold higher than the control treatment, (Fig. 4).

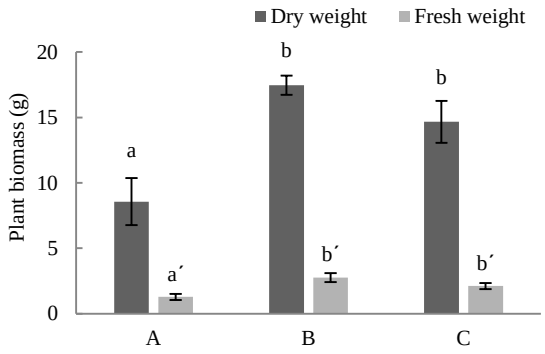

Fig. 4- Fresh and dry weight of *Z. mays* (A) without inoculation, (B) inoculation with the immobilized bacterial cells on rice bran and (C) inoculation with bacterial cells by seed immersion method. Each value is the mean of triplicates. Bars represent standard error. Data of columns indexed by the same letter are not significantly different according to the Scheffe test ($p < 0.05$).

Effects of the Biofertilizers on Cadmium Accumulation in *Z. mays*: At the end of the experiment, cadmium concentration in the aerial parts of *Z. mays* in the presence and absence of biofertilizers was measured by AAS. The maximum accumulation of cadmium (6310 ppb) was observed in plants grown in the absence of bacterial cells. Inoculation of plants with biofertilizers significantly decreased the quantity of cadmium accumulation in the maize tissues compared to the control treatments (Fig. 5). The minimum cadmium concentration was observed in plants grown in the presence of immobilized bacterial cells on rice bran (885 ppb) followed by the plants treated with the immersion method (2917.5 ppb), (Fig. 5).

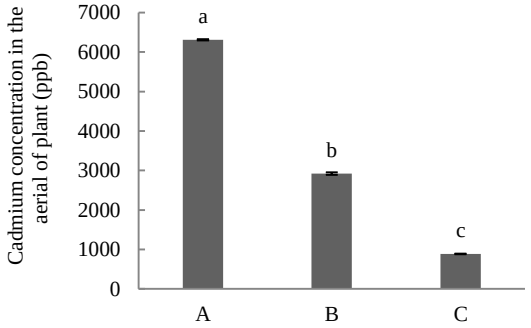

Fig. 5- Cadmium concentration in the aerial tissues of *Z. mays* grown in the (A) absence of bacterial cells, (B) presence of bacterial cells by seed immersion method and (C) presence of immobilized bacterial cells on rice bran. Each value is the mean of triplicates. Error bars represent SD. Statistical differences between means are indicated by different letters according to the Scheffe test ($p < 0.05$).

Effects of Biofertilizers on Soil Cadmium Content: The inoculation of soil with the biofertilizers resulted in a significant decrease in the soil cadmium concentration compared to the control plants since 49.5% of soil cadmium concentration decreased in the control treatments. Inoculation of bacterial cells by seed immersion method further augmented the cadmium decrease to 68.67%. The maximum decrease in soil cadmium concentration was observed in the presence of immobilized bacterial cells on rice bran (95.78%), (Fig. 6).

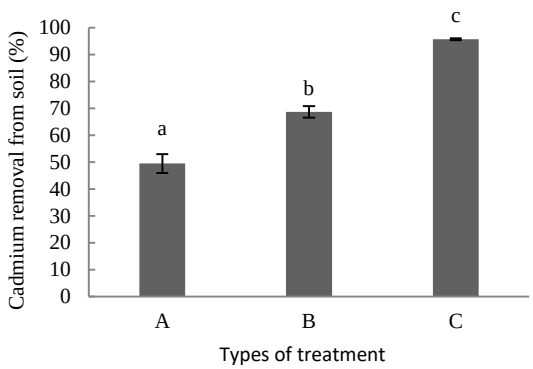

Fig. 6- Cadmium removal (%) in soil after harvesting of *Z. mays* grown in the (A) absence of bacterial cells, (B) presence of bacterial cells by seed immersion method and (C) presence of immobilized bacterial cells on rice bran. Each value is the mean of triplicates. Error bars represent SD. Statistical differences between means are indicated by different letters according to the Scheffe test ($p < 0.05$).

Discussion and Conclusion

Root exudates play important roles in initiating and modulating plant and microbes interactions in the rhizosphere. Root exudates act as a chemical signal to attract beneficial microorganisms (such as PGPB) or repel phytopathogenic microbes present in the rhizosphere. PGPB receive this signal and move towards plant roots (positive chemotaxis) to colonize them and utilize root exudates as carbon and energy sources. So, chemotaxis and colonization are the two primary and critical steps in the establishment of plant-microbe interactions. Biofilm formation on plant roots represents the effective colonization by bacteria because plant-associated biofilms can decrease microbial competition in the soil and protect PGPG from external stress. Enhancing plant growth and yield, crop quality and biocontrol of soil-borne disease are the results of the root system effective colonization by PGPR (34, 35). Organic acids, sugars and amino acids used in the present study have been reported as the main components of the root exudates in previous studies (30). So, positive effects of these compounds on chemotaxis, growth and biofilm formation in *P. putida* PT represent the ability of this bacterial strain to establish an effective relation with plants. Positive chemotactic responses of *P. putida* PT toward all of the selected organic compounds show that root exudate components can serve as chemical signals to attract *P. putida* PT towards plant roots. Also, the stimulation of growth and biofilm formation in the presence of most of the amino acids, sugars and organic acids tested in this work, indicate that *P. putida* PT is able to participate in the cross-talk with plants. Many potentially useful strains in bioremediation and plant growth promotion reported in previous studies did not appear on the commercial marketplace, maybe due to inappropriate formulation (36). The heterogeneity and complexity of the soil and the presence of the better-adapted native competitors are the key obstacles which often don't allow the inoculated bacteria with an inappropriate formulation to find a suitable microenvironment in the soil. Designing effective formulation is a key factor in the development of PGPB as a commercial product (28). Thus, the second focus of this study was on the preparation of different biofertilizer formulations by using *P. putida* PT considering the limitations of the farmers and the manufacturer' interests. Farmers are seeking easy ways to handle and reasonably price products. Therefore, in the present study, biofertilizers were prepared by seed immersion method and bacterial immobilization on rice bran. Seed immersion is the most common technique for the placement of bacteria into the soil because it is easy to use and requires a relatively small amount of inoculant (36). Providing a high bacterial biomass, easy transposition, regeneration of bacteria, reusability, providing a suitable microenvironment for protecting cells against harsh conditions and toxic

compounds are the advantages of immobilized cells in a suitable carrier (29). Rice bran, which is produced in large scale as the by-product of the rice milling industry, has an insoluble structure in water with high stability and mechanical strength (37, 38). So, rice bran was used as a natural, available and economical carrier for *P. putida* PT immobilization. Also, in our previous study, rice bran was reported as a suitable carrier for immobilization of *P. putida* PT which could provide appropriate conditions for cells proliferation and protection of bacterial cells from undesirable conditions like high or low temperatures and acidic or alkaline pH (33). Maize is one of the most cultivated grain in the world with low production costs and high consumption for animal and human, which can be processed into many kinds of food and industrial products, including starch, sweeteners, oil, glue, beverages and fuel alcohol (39). High amount of toxic metals can be absorbed by maize grown in the contaminated soils and then arrive into the human food chain (40). Length reduction of roots and shoots, leaf bleaching, lowering of photosynthetic activity and reduction of growth and grain yield have been reported as the negative effects of cadmium accumulation in maize in previous studies (41, 42). Therefore, the final aim of this study was to examine the influences of biofertilizers on maize growth and cadmium uptake in the Cd-contaminated soil. Both biofertilizers had positive effects on the growth of *Z. mays*. According to our previous work, *P. putida* PT can uptake cadmium from the environment by bioaccumulation and biosorption mechanisms (33). Our finding in the present study showed that the plant growth promotion caused by biofertilizers was also accompanied by a decrease in the cadmium concentration in soil and *Z. mays* tissues. These results indicate that bacterial cells by adsorption of cadmium from the soil before entering the plant, reduce the metal concentration in both soil and plant. The comparison of the performance of two different formulations in the adsorption of cadmium showed that the inoculation of the immobilized bacterial cells on rice bran was better than the inoculation of bacterial cells by seed immersion method. Angular surfaces and numerous functional groups (such as carboxyl and silanol), due to the presence of cellulose, hemicellulose, lignin and protein in the rice bran structure, could explain the positive effect of this carrier on metal adsorption capacity of immobilized bacterial cells (38). In addition, based on previous finding, rice bran can play an important role in metal bioremediation process by providing high population of viable bacterial cells during plant growth period (33).

In conclusion, for the first time, we have designed multifunctional biofertilizers using *P. putida* PT by two different techniques: immobilization of bacterial cells on rice bran as an economical and available carrier and seed immersion method. They can be useful to promote *Z. mays* growth in cadmium contaminated soils with the adsorption of cadmium. Also, due to the influence of these biofertilizers in the decrement of soil cadmium concentration, using them can be an eco-friendly approach for cadmium removal from contaminated areas. In other words, inoculation of *Z. mays* with biofertilizers can be used as an alternative approach to chemical treatments to facilitate plants' growth without increasing metal content in the plant aerial tissues and secondary pollutions formed by chemical treatments.

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