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(مقاله پژوهشی)

# تأثیر فرمولاسیون ارزان قیمت کود زیستی تهیه‌شده از *سودوموناس پوتیدا* سویه PT و مکمل‌های غذایی بر صفات رویشی ریحان

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## چکیده

**مقدمه:** در سال‌های اخیر تقاضای جهانی برای محصولات کشاورزی افزایش یافته است. یکی از روش‌های افزایش میزان محصولات همراه با کاهش مصرف کودهای شیمیایی، استفاده از کودهای زیستی است. هدف از مطالعه حاضر تولید یک فرمول کم‌هزینه از کود زیستی تهیه‌شده از *سودوموناس پوتیدا* سویه PT است.

**مواد و روش‌ها:** ویژگی‌های مختلف محرک رشد گیاه *سودوموناس پوتیدا* سویه PT مانند محلول‌سازی فسفر، تثبیت نیتروژن و تولید ایندول-۳-استیک‌اسید با استفاده از محیط Pikovskaya's (PVK)، معرف سالکوفسکی و محیط فاقد نیتروژن سنجش شدند. اثر سوبستراهای کم‌هزینه برای تهیه کود زیستی ارزان قیمت با اندازه‌گیری وزن خشک باکتری تجزیه و تحلیل شد. کود زیستی به روش غوطه‌وری بذر تهیه شد و آزمایش‌های گلدانی با استفاده از کود زیستی تهیه‌شده از *سودوموناس پوتیدا سویه* PT و مکمل‌های غذایی انجام شدند. اثر تیمارهای مختلف بر رشد و نمو گیاه *Ocimum basilicum* (ریحان) با اندازه‌گیری طول ریشه و اندام‌های هوایی، وزن تر اندام‌های هوایی و وزن خشک اندام‌های هوایی ارزیابی شد.

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**نتایج:** *سودوموناس پوتیدا* سویه PT قابلیت محلول‌سازی فسفات ($OD_{430nm}$ = 1.1)، تولید IAA ($OD_{530nm}$ = 0.86) و تثبیت نیتروژن را نشان داد. بالاترین زیست‌توده باکتریایی در حضور آب پنیر و به دنبال آن ملاس با درجه بریکس ۲/۵ همراه با آمونیوم سولفات مشاهده شد. کود زیستی تهیه‌شده از این باکتری به‌ویژه در صورت استفاده با فسفات نامحلول، رشد گیاه را به‌طور چشمگیری افزایش داد. افزودن کود زیستی و فسفات نامحلول به‌ترتیب باعث افزایش ۴۱/۶، ۸۳/۷ و ۲۵ درصدی طول اندام‌های هوایی، وزن تر اندام‌های هوایی و وزن خشک اندام‌های هوایی نسبت به گیاهان شاهد تیمارنشده شد.

**بحث و نتیجه‌گیری:** نتایج نشان می‌دهند *سودوموناس پوتیدا* سویه **PT** می‌تواند برای تهیه کود زیستی مقرون‌به‌صرفه به‌منظور افزایش رشد گیاه استفاده شود.

**واژه‌های کلیدی:** کود زیستی، ایندول-۳-اسیک‌اسید، تثبیت نیتروژن، انحلال فسفات، باکتری‌های محرک رشد گیاه

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Research Article

# The Effects of Low-cost Formulation of Biofertilizer Prepared from *Pseudomonas Putida* PT and Nutritional Supplements on Vegetative Characteristics of Basil

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## Abstract

**Introduction:** In recent years, the global demand for agricultural products has increased. One of the methods to increase crop yield, along with the decreased use of chemical fertilizers, is to apply biofertilizers. The purpose of the present study is to produce a low-cost formulation of biofertilizer synthesized from *Pseudomonas putida* PT.

**Materials and Methods:** Various plant growth-promoting characteristics of *P. putida* PT such as P-solubilization, N. fixation, and Indole-3-acetic acid (IAA) production were assayed using Pikovskaya's (PVK) medium, Salkowski reagent, and N-free medium, respectively. The effects of low-cost substrates to prepare inexpensive biofertilizers were analyzed by measuring the bacterial dry weight. The biofertilizer was prepared by the seed immersion method, and pot experiments were performed by using prepared biofertilizer from *P. putida* PT and nutritional supplements. The effect of various treatments on the growth and development of *Ocimum basilicum* (basil) was evaluated by measuring the root and shoot lengths, fresh weight of aerial parts, and dry weight of aerial parts.

**Results:** *P. putida* PT exhibited capability of P-solubilization ($OD_{430nm}$=1.1), IAA production ($OD_{530nm}$=0.86), and N-fixation. The highest bacterial biomass was observed in

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the presence of cheese whey, followed by 2.5 °Bx molasses plus $(NH_4)_2SO_4$. The prepared biofertilizer from this bacterium increased significantly plant growth, especially when it was used along with insoluble phosphate. The addition of biofertilizer and insoluble phosphate increased shoot length, fresh weight of aerial parts, and dry weight of aerial parts by 41.6%, 83.7%, and 25% compared to the untreated control plants, respectively.

**Discussion and Conclusion:** The results of the study signify that *P. putida* PT can be used to prepare a cost-effective biofertilizer for the enhancement of plant growth.

**Key words:** Biofertilizer, Indole Acetic Acid, N-Fixation, P-Solubilization, Plant Growth-Promoting Bacteria

## Introduction

In recent years, the global demand for agricultural products has increased due to the increasing world population (1). The use of chemical fertilizers, therefore, has raised to enhance crop yields. The excessive use of chemical fertilizers in agriculture has caused the large-scale release of heavy metals into the environment because some fertilizers contain high concentrations of these elements (2). Heavy metals can arrive into the human food chain through contaminated crops as a result of their uptake by plants (3). Some heavy metals like lead and cadmium have no role in biological reactions. They are toxic and threaten serious human health, even at low concentrations (4). Continuous application of chemical fertilizers gradually increases heavy metal content in the soil, because heavy metals are non-biodegradable (5). Soil degradation, salt burns, excessive growth, and high expenses are other disadvantages of chemical fertilizers (6).

Basil (*Ocimum basilicum*) is an aromatic plant that is commercially cultivated worldwide. This plant is frequently consumed in the food, pharmaceutical, and cosmetic industries. Basil is generally cultivated in urban and peri-urban fields, thereby it is subjected to numerous pollutants related to the development of urbanization and industrialization processes. The consumption of contaminated vegetables causes a risk to human health. Thus, the employing an easy and effective approach is vital for rising crop yields and safety (7). Plant growth-promoting bacteria (PGPBs) are a beneficial group of microorganisms that exist in the rhizosphere, on the surface of roots, or associated with plant roots. They can stimulate plant growth by increasing the availability of nutrients (phosphate solubilization, nitrogen fixation, potassium solubilization, and siderophore production). Also, these bacteria can protect plants against phytopathogens through the production of antibiotics and lytic enzymes (8, 9). In other words, PGPBs can be used as biofertilizers to reduce the need for chemical fertilizers (10). Plant roots release a wide range of compounds as root exudates, including carbohydrates, organic acids, amino acids, fatty acids, and vitamins.

Plant root exudates can provide carbon and energy sources for PGPBs. Therefore, plant root exudates can serve as a suitable source for the isolation of these bacteria. Designing effective formulation plays a key role in the development of PGPB as a biofertilizer. Two main strategies are presently being used for biofertilizer preparation: (1) seed inoculation (by coating the seed with inoculant) and (2) soil inoculation (by delivering the inoculant directly into the soil) (11). The use of agro-industrial wastes as renewable and inexpensive sources of nutrients for microbial growth not only has environmental benefits but also reduces the costs of microbial biomass production. Cheese whey is a by-product of the dairy

industry and because it is generated in large quantities, its disposal causes serious environmental pollution. Another by-product used for microbial growth is sugar beet molasses (12, 13). The purpose of the present study is to produce a low-cost formulation of biofertilizer synthesized from *Pseudomonas putida* PT. Therefore, the objectives of this study are (1) to evaluate the ability of this strain to enhance plant growth, (2) to examine the effect of low-cost carbon sources on the production of bacterial biomass, and (3) to clarify differences between synthetic compounds and the bacterial strain on the growth of *O . basilicum*.

## Materials and Methods

**Source of Bacterial Strain:** The bacterial strain was previously isolated from agricultural soils, sited in Talkhooncheh village (32.2307° N, 51.5360° E), and identified as *P. putida* PT based on 16S rRNA gene sequence analysis (GenBank accession number: KX963368) (14).

**Evaluation of Plant Growth Promoting Traits**

**Screening and Quantification of P-solubilization:** Screening of P-solubilization ability was done on Pikovskaya's (PVK) agar medium containing: 10 g/l glucose, 5 g/l $Ca_3(PO_4)_2$, 0.5 g/l $(NH_4)_2SO_4$, 0.2 g/l NaCl, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l KCl, 0.5 g/l yeast extract, 0.002 g/l $MnSO_4 \cdot 2H_2O$, 0.002 g/l $FeSO_4 \cdot 7H_2O$, and 15g/l agar. Briefly, 0.1ml of overnight bacterial culture was spread on PVK agar medium and incubated at 30 °C for 5 days leading to the formation of a clear zone around the bacterial colony. Quantitative analysis of phosphate solubilizing activity was carried out in the PVK liquid medium. Next, 0.1ml of fresh bacterial culture was inoculated to 10ml of PVK liquid medium and incubated at 30 °C on a rotatory shaker at 180 rpm for 4 days. The supernatant was obtained by centrifugation at 8000 rpm for 10. Then, 0.5 ml of the supernatant was added to 2.5 ml of Barton's reagent and the final volume was adjusted to 50 ml with deionized water. After 10 min, the intensity of the yellow color (due to the formation of phosphomolybdate complex) was measured by the microtiter plate reader at 430 nm (15).

**Indole-3-acetic acid (IAA) production:** An overnight bacterial culture in a liquid Luria Bertani (LB) medium was inoculated to LB medium supplemented with 0.1% L-tryptophan. After 3 days of incubation at 30 °C in darkness on a rotary shaker, the pellet was discarded by centrifugation (5000 rpm for 20 min). One ml of supernatant was mixed with 2ml of Salkowski reagent and OD was read at 530nm after 30min. The development of the pink color indicated the production of IAA (16, 17).

**Nitrogen Fixation:** Bacterial culture was streaked on N-free medium comprising 20 g/l glucose, 3 g $CaCO_3$, 0.4 g/l $FeSO_4$, 0.25 g/l $KH_2PO_4$, 0.75 g/l $K_2HPO_4$, 0.5 g/l $MgSO_4$, 0.005 g/l $FeSO_4$, 0.02 g/l $Na_2MoO_4$, and 15 g/l agar (18) and incubated at 30 °C for 2 weeks. The growth of bacterial colonies on the surface of the N-free medium revealed the ability of the bacterial cells for nitrogen fixation.

**Detection of Bacterial Growth in the Presence of Cost-effective Substrates:** Cheese whey (6%) and sugar beet molasses (2.5, 5, and 10 °Bx) with or without supplementation of ammonium sulfate (0.2%) were selected as low-cost media. A volume of 1ml overnight bacterial culture was inoculated into Erlenmeyer flasks which contained 100 ml of selected media. The culture media containing sugar beet molasses were incubated on a rotary shaker at 180 rpm at temperatures of 30, 37, and 42 °C. The flasks containing cheese whey medium were incubated under the same conditions described previously, but at a temperature of 30 °C. The pellets were obtained after 15, 24, and 48 h of cultivation by centrifugation at 6000rpm for 15 min, washed three times with normal saline and dried. Finally, the amount of bacterial biomass was determined by dry

weight measurement. Experiments were carried out in duplicates (19-21).

**Biofertilizer Preparation:** Biofertilizer was prepared by the seed immersion method. For this purpose, an overnight bacterial culture was diluted 1:100 (v/v) into fresh tryptic soy broth (TSB) medium and incubated at 30° C for 24 h on a rotary shaker at 100 rpm. The pellet was obtained by centrifugation at 3000 rpm, washed three times, and re-suspended in normal saline (~1 $0^{11}$ CFU/ml). Then, bacterial suspension (3 ml of bacterial inoculants per 1 seed) was added to the surface of basil seeds (22).

**Pot Experiments:** Pot experiments were conducted to investigate the effects of the biofertilizer and chemical supplements on the growth of *O. basilicum* under greenhouse conditions (air temperatures ranged between 45°C (day) and 30°C (night)). The various treatments and combinations in each pot are listed in Table 1 (Appendix). Fifteen seeds treated with bacterial inoculum were planted in each pot. After 30 days of germination, the plants were harvested and different growth parameters including root and shoot lengths, fresh weight of aerial parts, and dry weight of aerial parts were recorded. All experiments were performed in triplicates (23).

**Statistical Analysis:** The obtained data were subjected to the one-way analysis of variance (ANOVA) followed by Duncan's tests. The results were considered statistically significant when $P < 0.05$. All statistics were performed using the Statistical Package for the Social Sciences (SPSS) program (Version 16.0) (11).

Table 1- Combinations and amounts of Compounds used in the Pot Experiments (values are grams per pot)

| Soil mix 1 | Soil mix 2 | Soil mix 3 | Soil mix 4 | Soil mix 5 (control) |
|---|---|---|---|---|
| $K_2HPO_4$ (1)+$KH_2PO_4$ (1)+Soil (1000) | Inoculant+$Ca_3PO_4$ (2) + Soil (1000) | NH3 (0.5)+Soil Soil (1000) | Inoculant + Soil (1000) | Soil (1000) |

## Results

**Evaluation of Plant Growth Promoting Traits:** After 5 days of incubation, *P. putida* PT showed a considerable transparent zone on PVK agar medium. To further analyze the quantitative P-solubilization activity, the soluble P content was evaluated in the PVK liquid medium .Under the conditions tested, the strain was able to release soluble phosphate to the culture medium ($OD_{430nm}$=1.1). IAA production was also evaluated in the present study with the use of the Salkowski reagent. Color development was first visible within minutes and continued to increase in intensity for 30 min ($OD_{530nm}$=0.86). Then, nitrogen fixation ability was detected in the N-free medium. After 2 weeks of incubation at 30 °C, the growth of *P. putida* PT on this medium indicated its ability to fix nitrogen.

**Detection of Bacterial Growth in the Presence of Cost-effective Substrates:** The growth of *P. putida* PT in the presence of sugar beet molasses (2.5, 5, and 10 °Bx) with or without supplementation of ammonium sulfate (0.2%) was investigated at different temperatures (30, 37, and 42 °C) within three-time points (15, 24, and 48 h). The highest dry biomass (6.9 g/l) was observed in the presence of 2.5 °Bx molasses and ammonium sulfate at 30 °C after 48 h. The lowest dry biomass (0.01 g/ml) was obtained in the presence of 10 °Bx molasses (with or without $(NH_4)_2SO_ε$) at temperatures of 37 °C and 42 °C after 15 h (Figure 1). Also, cheese whey (6%) was used as another low-cost substrate for bacterial growth. In this case, bacterial dry weight was increased up to 51 g/l.

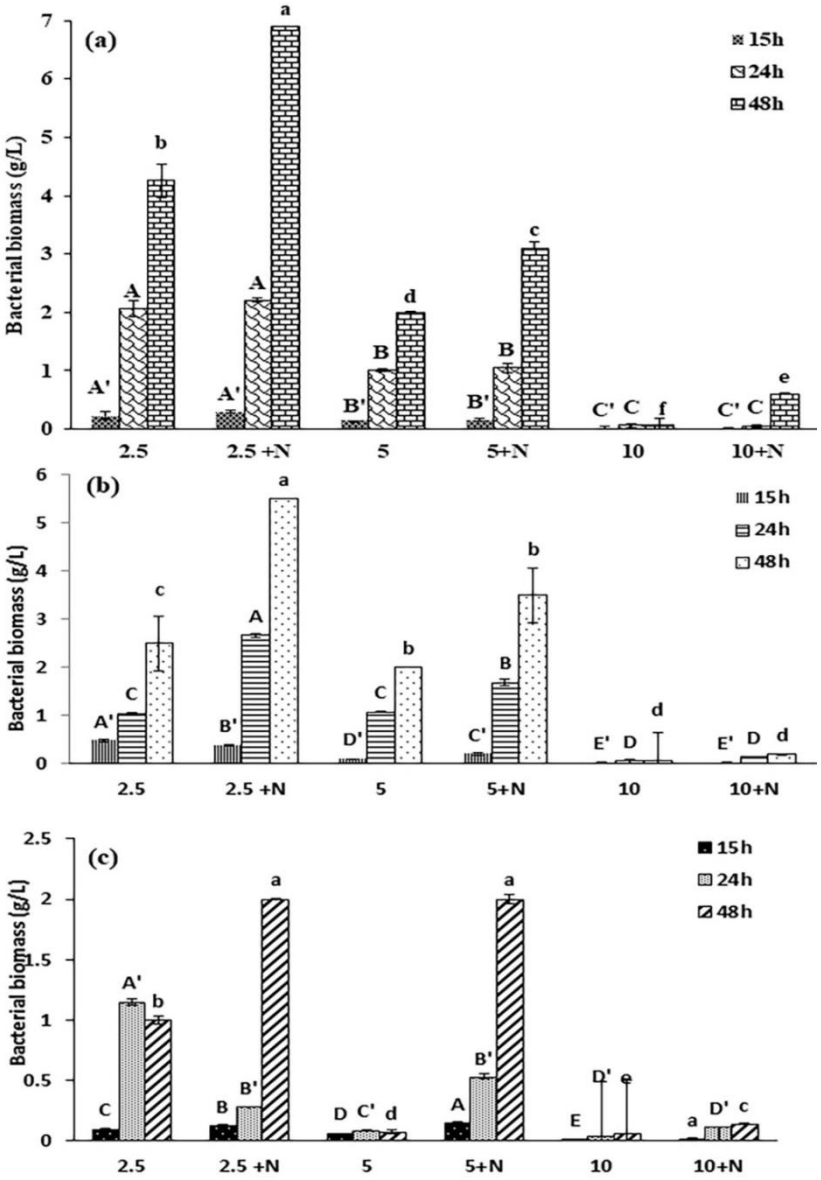


Fig. 1- Effects of time and different concentrations of sugar beet molasses in the absences or presence of 0.2% ammonium sulfate (N) on *P. putida* PT biomass at (a) 30°C, (b) 37°C, and (c) 42°C

**Pot experiments:** Root and shoot lengths, fresh weight of aerial parts, and dry weight of aerial parts of *O. basilicum* were measured to compare the effects of each treatment on plant growth. The addition of the biofertilizer increased significantly root length ($P<0.05$) and the highest root length (15 cm, 53.3% increase compared to the control) was observed in the pots, which contained bacterial inoculation (Figure 2A). The highest shoot length (12 cm), fresh weight of aerial parts (1.6 g), and dry weight of aerial parts (0.2 g) were observed in the pots which were treated with bacterial inoculant and insoluble phosphate, due to the ability of *P. putida* PT to release phosphate from insoluble phosphate compounds (data is shown in Figure 2). According to the results, the addition of the biofertilizer and insoluble phosphate increased shoot length, fresh weight of aerial parts, and dry weight of aerial parts by 41.6%, 83.7%, and 25% compared to the untreated control plants, respectively.

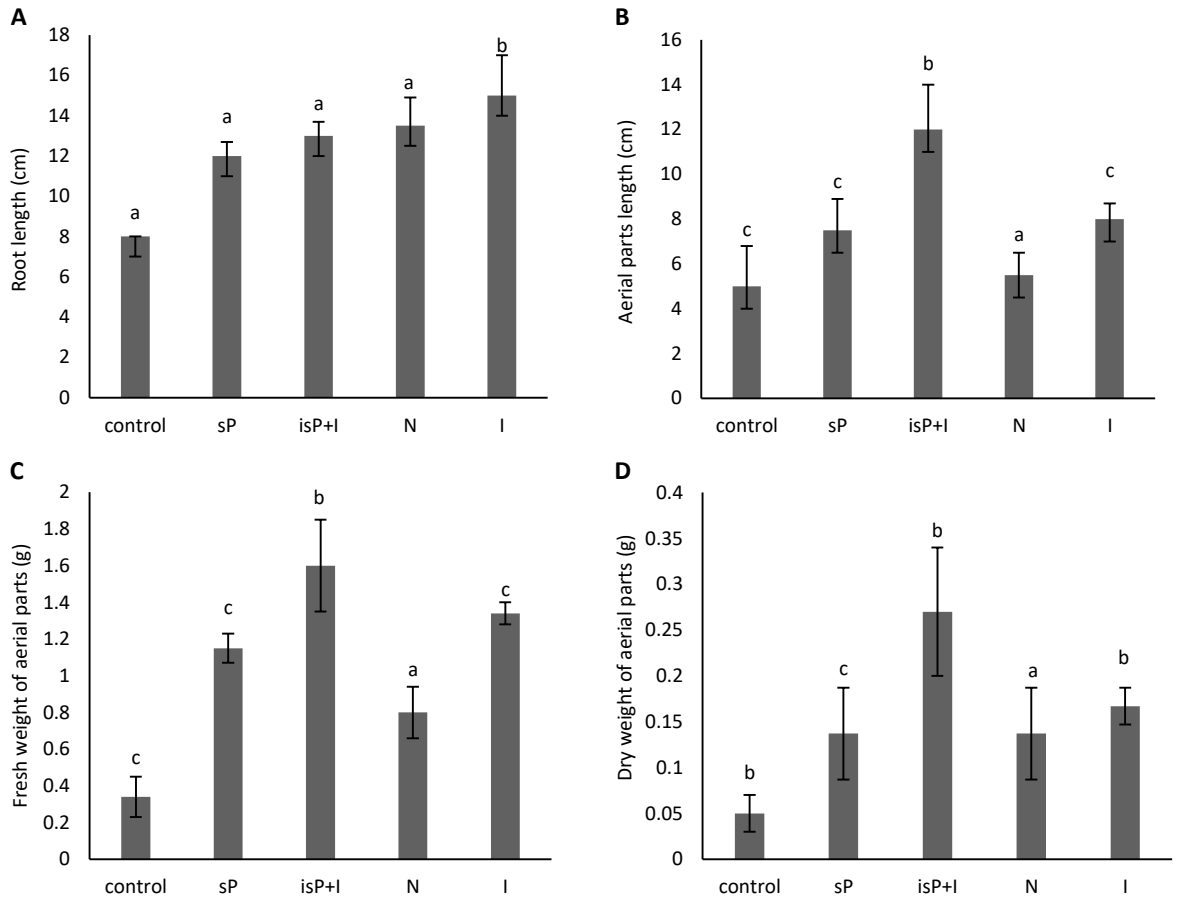


Fig. 2- Effects of different treatments on (A) root length, (B) shoot length, (C) fresh weight of aerial arts and (D) dry weight of aerial parts of *Ocimum basilicum*. Similar letters on each bar denote no significant difference between matrices within the same temperature ($P \leq 0.05$). (I: Inoculant, N: NH3, O: Soil without ant treatment, sP: soluble Phosphate, isP: insoluble Phosphate)

## Discussion and Conclusion

Chemical fertilizers are commercial products composed of known amounts of nutrients such as nitrogen, phosphorus, and potassium. In the last decades, the overuse of chemical fertilizers, due to the growing demand for food, has led to environmental problems like air, soil, and groundwater pollution (6). Therefore, recent efforts have focused on the production of biological-based fertilizers (24). The present study focused on the preparation of an efficient biofertilizer, which can improve plant growth. In the first step, we endeavored to identify plant growth-promoting traits of *P. putida* PT as a promising agent for biofertilizer preparation by evaluating IAA production, P-solubilization, and N-fixation abilities.

Nitrogen is the most essential nutrient for plant life. It is a major component of chlorophyll, amino acids, nucleic acids, and energy-transfer compounds, such as ATP (25). Also, phosphorus plays a significant role as the second most important macronutrient in vital plant reactions such as photosynthesis, development of roots, stems, flowers, and seeds, crop quality, and resistance to plant diseases (26). Indole-3-acetic acid is the most common plant growth hormone of the auxin category which is produced naturally by plants, fungi, and bacteria. IAA plays a key role in regulating many important physiological processes in plant growth and development, such as cell enlargement and division,

tissue differentiation, and responses to light and gravity (25-27). It enhances plant growth by creating a mutual communication between plants and microorganisms as a chemical signal (26).

According to the results of the study, *P. putida* PT can be considered a plant growth-promoting bacterial strain because it exhibited the capability of P-solubilization, IAA production, and N-fixation. Similarly, some bacterial species that have the potential for IAA production, P-solubilization, and N-fixation, such as *Rhizobium daejeonense*, *Pseudomonas brassicacearum*, and *Enterobacter ludwigii* have been described as PGPBs in previous studies (14, 27, 28). The effects of sugar beet molasses and cheese whey in bacterial biomass production were compared to prepare the biofertilizer at a large scale with low production cost. Using low-cost substrates such as industrial wastes in biotechnological processes plays a key role to convert a product from lab scale to industrial scale.

Cheese whey and sugar beet molasses are byproducts of the dairy industry and sucrose production, respectively, which are produced in large amounts as industrial wastes (11). Therefore, we used these substrates as low-cost carbon sources to grow *P. putida* PT. The highest bacterial biomass was observed in the presence of cheese whey (6%) followed by 2.5 °Bx molasses plus ammonium sulfate (0.2%). These differences in the effects of each substrate on the bacterial growth may be due to the inhibitory effects of high sugar concentration in sugar beet molasses. *O. basilicum* (basil) is a commercial plant that is used extensively as an important food additive and a natural source for the production of phenylpropanoids and terpenoids in traditional medicine (29, 30). Therefore, we investigated the effects of different treatments on basil growth. Biofertilizer was prepared by the seed immersion method in this study. Seed immersion is one of the most frequently applied methods in the production of biofertilizers because it is easy to use and requires a relatively small volume of inoculant (31). This strain had positive effects on the growth parameters of *O. basilicum* such as root and shoot lengths, and fresh and dry weights of aerial parts especially when it was used along with insoluble phosphate. Thus, *P. putida* PT can be used to prepare a cost-effective biofertilizer for the enhancement of plant growth.

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