

Characterization biodegradation of Benzene, Toluene, Ethylbenzene, and Xylenes by the Newly Isolated Bacterium *Pseudomonas putida* AY-10 in Rhizosphere of Wastewater Treatment Reed

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Abstract. The role of plant growth-promoting bacteria(PGPB) in the bioremediation of BTEX contaminated environment is important in overcoming its limitations for field application. In this study, by isolation BTEX-degradation bacteria were studied to assess the potential use of bioremediation. A plant growth-promoting bacterium, *Pseudomonas putida* AY-10, was isolated from the rhizosphere of wastewater treatment reed. This isolate has shown capacities for indole acetic acid production(10.72 mg/L of IAA accumulated during 5 days) and ACC deaminase(Absorbance value: 0.49 ± 0.01 72) by 72 hours incubation. In addition, Although *P. putida* AY-10 was incubated in BTEX-BH medium, it could degrade pure B, T, E, and X without any lag period. The *P. putida* AY-10 was able to completely degrade BTE after 15 h, almost 100% and X after 50 h under aerobic condition. These results indicate that *Pseudomonas putida* AY-10 can serve as a promising microbial inoculant for increased plant growth in BTEX contaminated environment to improve the bioremediation efficiency.

Keywords: Biodegradation, BTEX, Rhizosphere, *Pseudomonas putida*, Wastewater Treatment

1. Introduction

Benzene(B), Toluene(T), Ethylbenzene(E), and Xylenes(X) are components of gasoline. The gasoline used in industrial syntheses, and aviation fuels, and solvents of chemical(Smith, 1990). A considerable amount of gasoline enters the environment as leakage from underground storage tanks, accidental spills, and improper waste disposal practices(Bowlen and Kosson, 1995; Reinhard et al., 1984). When gasoline is in contact with water, benzene, toluene, ethylbenzene, and the xylene isomers(BTEX) account for as much as 90% of the gasoline components that are found in the water-soluble fraction(Saeed and Mutairi, 1999). Therefore, these chemicals are some of the most common contaminants found in drinking water(Prenafeta-Boldú et al., 2002). The BTEX compounds are carcinogenic and neurotoxic and are classified as priority pollutants regulated by the Environmental Protection Agency(Dean, 1985; EPA, 1977).

When organic pollutants like BTEX are released into the environment, the function and structure of the microbial communities are generally affected (Barker et al., 1987; Lovely, 2001). It is well known that microbial BTEX biodegradation occurs under both aerobic and anaerobic conditions(Edwards et al., 1992; Evans et al., 1991; Hutchins, 1991; Smith, 1990). The ability of natural microorganisms in the environment in BTEX degradation was first demonstrated by Gray and Thornton in 1928(Gray and Thornton, 1998). These researchers found 250 species of bacteria present in non-contaminated environment samples, so they could be used in environmental remediation for this mixture(Mazzeo et al., 2010). A variety of bacterium have been reported for the biodegradation of BTEX; *Phanerochaete chrysosporium*, *Rhodococcus* sp, *Pseudoxanthomonas spadix*, and *Pseudomonas putida*(Yadav and reddy, 1993; Lee and Cho, 2009; Kim et al., 2008; Mazzeo et al., 2010). In the current study, isolated and characterized was a new BTEX-degrading bacterium *Pseudomonas putida* AY-10 in rhizosphere of

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wastewater treatment reed. In addition, the effects of inoculation of *Pseudomonas putida* AY-10 remediation efficiency were evaluated in the contaminated system.

2. Materials and methods

2.1. Isolation, identification and characterization of a BTEX-degrading bacterium

Reed rhizosphere soil was used as a bacterial inoculum for an enrichment culture, as follows. Wet soil (10 g) was mixed with 90 mL of distilled water in a shaker at 180 rpm for 15 min. After settling, the supernatant was transferred to a 250 mL flask, with the addition of 50 mL of NFB medium (Malic acid, 5.0 g/L; K_2HPO_4 , 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; NaCl, 0.1 g/L; $CaCl_2$, 0.02 g/L; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g/L; $MnSO_4 \cdot H_2O$, 0.001 g/L; KOH, 4.5 g/L; biotin, 0.0001 g/L; Fe-EDTA (1.64% (w/v)), 4.0 mL/L; Agar, 15 g/L; Agar (case of semi), 1.75 g/L; yeast extract (case of agar), 0.02 g/L) or Burk's medium ($C_6H_{12}O_6$, 10 g/L; KH_2PO_4 , 0.41 g/L; K_2HPO_4 , 0.52 g/L; Na_2SO_4 , 0.05 g/L; $CaCl_2$, 0.2 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L; $FeSO_4 \cdot 7H_2O$, 0.005 g/L; $Na_2MoO_4 \cdot 2H_2O$, 0.0025 g/L; Agar, 15 g/L) or Congo medium (K_2HPO_4 , 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; NaCl, 0.1 g/L; yeast extract, 0.5 g/L; $FeCl_3 \cdot 6H_2O$, 0.015 g/L; DL-malic acid, 5 g/L; KOH, 4.8 g/L; Agar, 20 g/L). The flask was sealed with a stopper. The culture was incubated at 30°C for 7 days, with mixing at 180 rpm. The culture was enriched four times. And Nitrogen fixation ability of culture medium were evaluated and spread on Burk's agar, Congo Red agar and NFBA agar plate and allowed to grow at 30°C for 3-7 days. After cultivation, representative colonies were selected based upon morphology and color properties, and transferred to new plate. And each colony was to evaluate the ability of the nitrogen fixation. Nitrogen fixation ability was evaluated by referring to the Hong et al. (Hong et al., 2010).

Plant growth-promoting activities (PGPA) of the screened colonies were investigated using DF salts minimal medium supplemented with 3 mM 1-aminocyclopropane-1-carboxylic acid (ACC) for ACC deaminase activity test (Dell'Amico et al., 2005), IAA Production ability test (Koo and Cho, 2007) and chrome azurol S blue agar plate for siderophore synthesis ability test (Schwyn and Neilands, 1987). In order to evaluate the BTEX biodegradation ability of the screened colonies. The degrading ability for BTEX of the isolated strain was performed. For the identification of the isolate, its 16S rDNA was partially sequenced in the same manners as described previously (Koo and Cho, 2007).

2.2. BTEX biodegradation assay of *Pseudomonas putida* AY-10

To examine the ability of isolated microorganisms to degrade Benzene, Toluene, Ethylbenzene, and xylene (B.T.E.X). The degrading ability for BTEX of the isolated strain was performed. At first, to assess each substrate for use, cells were collected from the precultured BH medium ($MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2$, 0.02 g/L; KH_2PO_4 , 1 g/L; K_2HPO_4 , 1 g/L; NH_4NO_3 , 1 g/L; $FeCl_3$, 0.05 g/L), washed with distilled water, and resuspended in 20 mL of new BH medium. After 20 mL of the resuspended cells had been transferred to each 120 mL vial, each substrate was added (Benzene: 120 mg/L, Toluene: 90 mg/L, Ethylbenzene: 80 mg/L, m-p Xylene: 80 mg/L, o-Xylene: 60 mg/L), cultivated at 30°C and 180 rpm. Samples of the air in the headspace were withdrawn from the vials every 3 hours with a 500 μ L gas-tight syringe, and residual BTEX were analyzed using Gas chromatography (HP 5890 series II Gas chromatograph, Hewlett Packard, USA), with a DB-WAX capillary column (0.25 mm \times 30 m, 0.25 μ m; Agilent, USA) and FID. The oven temperature of injector and detector were increased from 60°C for 2°C/min to 80°C with a heating, 10°C/min to 200°C and remains, 250°C and 250°C, respectively. Carrier gas was using N_2 .

3. Results and discussion

AY-10, isolated from the rhizosphere of wastewater treatment reed, was identified by comparison of the 16S rDNA gene sequences in the NCBI database using a BLAST analysis. The closest relatives of strain AY-10 was found to be *Pseudomonas putida*, with 99% similarity. AY-10 was 99% similar to *Pseudomonas putida*, which is capable of degrading BTEX (Hojae Shim et al., 2002; Morlett-Chávez et al., 2010). A BTEX-degrading bacteria was successfully obtained from an enrichment culture using B, T, E, and X as the sole carbon source. The results of BTEX degradation are shown in Fig. 1. Although AY-10

was incubated in BTEX-BH medium, it could degrade pure B, T, E, and X without any lag period. The AY-10 was able to degrade completely BTE after 15 h and of X after 50 h under aerobic condition.

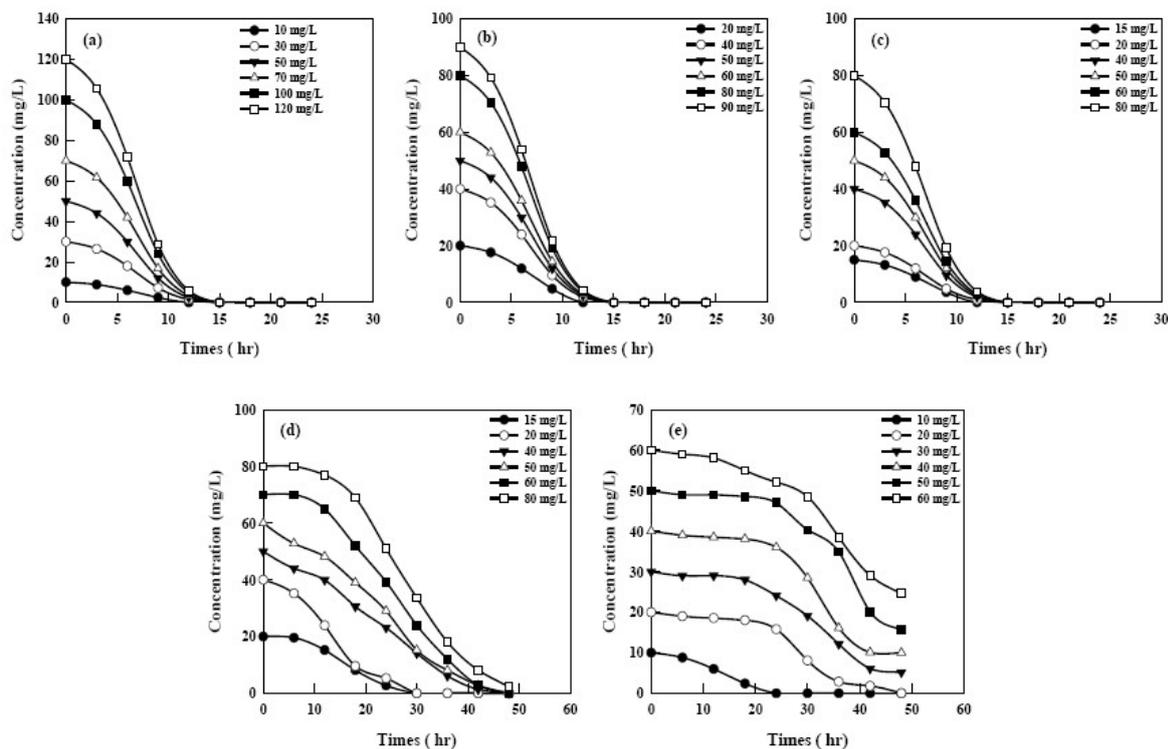


Fig. 1. Biodegradation of BTEX by *Pseudomonas putida* AY-10(a, Benzene; b, Toluene; c, Ethylbenzene; d, m,p-xylene; e, o-xylene).

The ACC deaminase can degrade ACC, a precursor of ethylene produced by plant stress(Glick, 2003), and IAA was phytohormones. AY-10 possessed the ability for both IAA production, ACC deaminase and synthesize siderophores(Fig. 2). The IAA concentration in the culture broth increased according to the growth of AY-10, with 10.72 mg/l of IAA accumulated during 5 days(Fig. 2(a)). The area of the orange halo around the AY-10 colony in the CAS-agar plate increased linearly during the incubation, indicating that SY5 could synthesize siderophores(data not shown). The case of ACC deaminase activity, Absorbance value by 72 hours incubation were measured. As a result, it was 0.49 ± 0.01 .

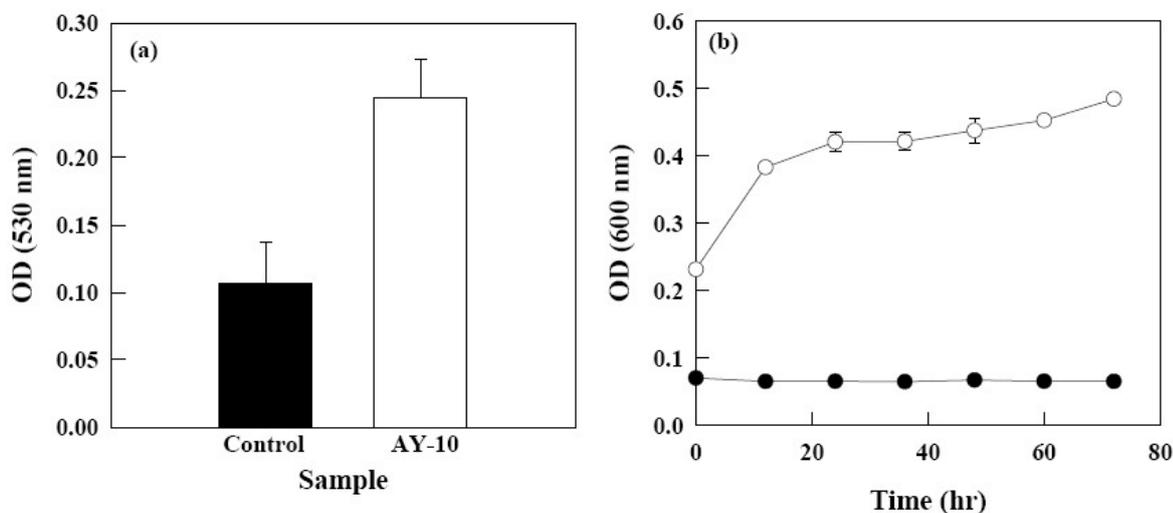


Fig. 3. IAA productivity(a) and ACC deaminase activity(b). Symbols: closed circle, control; open circle, *Pseudomonas putida* AY-10.

Plant growth-promoting bacteria (PGPB) colonizing the surface or inner part of roots play an important positive role that directly or indirectly influences plant growth and development (Gerhardt, 2009; Glick, 1999). In this study, a new PGPB, *Pseudomonas putida* AY-10 was isolated from the rhizosphere of reed; this bacterium also showed both the ability for IAA productivity (Fig. 2(a)) and ACC deaminase activity (Fig. 2(b)). In addition, it could degrade pure B, T, E, and X without any lag period. Our study has demonstrated a PGPB, *Pseudomonas putida* AY-10, which could be applied as a promising microbial inoculant for the direct stimulation of plant biomass production, and to degrade BTEX by plants and bacterium in the bioremediation of BTEX-contaminated environments.

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