Growth Response of *Avena sativa* in Amino-Acids-Rich Soils Converted from Phenol-Contaminated Soils by *Corynebacterium glutamicum*

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The biodegradation of phenol in laboratory-contaminated soil was investigated using the Gram-positive soil bacterium *Corynebacterium glutamicum*. This study showed that the phenol degradation caused by *C. glutamicum* was greatly enhanced by the addition of 1% yeast extract. From the toxicity test using *Daphnia magna*, the soil did not exhibit any hazardous effects after the phenol was removed using *C. glutamicum*. Additionally, the treatment of the phenol-contaminated soils with *C. glutamicum* increased various soil amino acid compositions, such as glycine, threonine, isoleucine, alanine, valine, leucine, tyrosine, and phenylalanine. This phenomenon induced an increase in the seed germination rate and the root elongation of *Avena sativa* (oat). This probably reflects that increased soil amino acid composition due to *C. glutamicum* treatment strengthens the plant roots. Therefore, the phenol-contaminated soil was effectively converted through increased soil amino acid composition, and additionally, the phenol in the soil environment was biodegraded by *C. glutamicum*.

**Keywords:** *Corynebacterium glutamicum*, bioavailability of phenol, *Daphnia magna*, *Avena sativa*, amino acids

Phenol and its derivatives are considered as priority pollutants in the US EPA list [23] because they have potentials as toxic, carcinogenic, mutagenic, and teratogenic agents [4]. Soil contamination with phenols is closely related to various industrial activities such as coal conversion processes, petroleum refineries, and manufacturing of phenolic petrochemicals [4, 19]. Hence, numerous studies on the biological treatment of phenol have been conducted over the last few decades [2], and they have been the focus of extensive research in environmental engineering, and bioremediation has been recognized as an economical approach [13].

Biological treatments are generally preferred owing to their lower operation costs and the possibility of complete mineralization than physical methods such as solvent extraction, adsorption, chemical oxidation, and incineration [1]. Specifically, soil microorganisms are widely used in studies on the degradation of phenol and its derivatives because they are able to metabolize that by common metabolic pathways [14, 22]. However, the major problem of phenol degradation by microorganisms is that some intermediates (*i.e.*, catechol) converted from phenol are observed to have a higher toxicity effect to the bacterial cells rather than phenol [20].

*Corynebacterium glutamicum*, a Gram-positive soil bacterium, is able to utilize phenol as carbon energy [15, 18]. Qi *et al.* [15] described the metabolic pathway for phenol degradation in *C. glutamicum* and its examination by proteomic and molecular methods. A previous study reported a novel process for the production of useful amino acids, such as glutamate and proline, coinciding with the degradation of phenol in *C. glutamicum* [10]. This integrated metabolic pathway was based on the concept that intermediates (*i.e.*, acetyl-CoA and succinyl-CoA) resulting from phenol degradation enhance the center pathway for energy metabolism, the tricarboxylic acid (TCA) cycle [15]. In particular, it was revealed that two regulatory proteins, ArgR and FarR, induce metabolic flux to amino acid biosynthesis derived from the TCA cycle in the presence of phenol in *C. glutamicum* [10, 12]. Therefore, the main

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advantages of using *C. glutamicum* include excretion of basic amino acids into the extracellular phase as well as reduction of phenol concentration by its own metabolic pathways [5, 10–12]. It is generally known that amino acids in soil affect to increase the chlorophyll content, antioxidative enzymes activities, and essential microelement uptake and transport in plants (e.g., tomato seedlings) [24]. Moreover, recent work has shown that efficient biodegradation of phenol in soil environments was achieved with *C. glutamicum* [10].

In the present study, the phenol biodegradation and soil amino acid composition were examined in *C. glutamicum*-treated soils. As mentioned before, toxicity from phenol and its intermediate should be removed to recover the contaminated soils. For this, the study was conducted on the growth variation in plants, such as lettuce and oat, both to evaluate the removal of hazardous phenol and to determine how the restoration of phenol-contaminated soil by *C. glutamicum* treatment increases various amino acids levels.

### MATERIALS AND METHODS

#### Microorganism

Strain *C. glutamicum* ATCC 13032 (America Type Culture Collection, Manassas, VA, USA) was used for phenol degradation in soil. It was cultured at 30°C and 150 rpm in Luria–Bertani medium [16].

#### Soil Remediation

The ability of *C. glutamicum* to remediate the phenol-contaminated soil sample was investigated by carrying out the biodegradation experiment in soil for 4 days under indoor laboratory conditions. A bulk soil outside of the rhizosphere was collected at the location of a field margin near Songcheon River in Jeonju, South Korea. The soil was completely sterilized in an autoclave at 121°C and 103.4 Pa for 30 min. Then 100 ml of 4.2 mM phenol solution (> 99.5% purity, Sigma-Aldrich) was mixed with the sterilized soil (100 g), and the mixed samples were layered in a glass column-type reactor (0.5 L; 9.5 cm diameter) that was capped with a stainless steel closure.

To the mixture, 1 ml of 10% yeast extract solution was added and then 1 ml of *C. glutamicum* solution (7.4 log10 CFU/ml) was inoculated and incubated for 4 days at 30°C, 150 rpm in a rotary shaker [10]. Each experiment was performed in triplicate. To determine biodegradation, the soil-phase phenol concentrations were analyzed at 0, 2, and 4 days.

#### Extraction and Determination of Residual Phenol in Soil

Triplicate soil samples (~1 g wet soil) from each container were mixed thoroughly with 1 ml of ethyl acetate, and the residual phenol was extracted. The organic phase extractions were combined and dried with anhydrous sodium sulfate. To evaluate the residual toxicity, we recovered the aqueous phase from the soil after 4 days of treatment with *C. glutamicum*.

The amount of residual phenol in the soil samples was determined by gas chromatography (GC) using an Agcme 6000 GC (Young Lin Instrument Co., Ltd., Korea) equipped with an HP-5ms capillary column (Agilent Technologies Inc., USA) and a flame ionization detector. The injector and detector were maintained at 200°C, and the column temperature was set to 70°C.

#### Determination of Amino Acids Contents in Soil

High-performance liquid chromatography (HPLC, Waters Alliance 2690 Analytical HPLC system) was used to determine the amino acid content of the soil. The system was equipped with a Nova-Pak C18 column and a Waters 747 scanning fluorescence detector (Waters Co., USA). All the results represent the data from at least three independent experiments and include a mean value.

#### Acute Toxicity Test Using *Daphnia magna*

The 24 h acute toxicity tests using *D. magna* were performed according to US EPA protocols [10]. All tests were conducted at 20 ± 1°C, in a 16:8 h (light:dark) photoperiod cycle. Test vessels consisted of a 60 ml glass beaker with 50 ml of test solutions eluted from phenol-contaminated soils and 5 to 8 *D. magna* neonates. All experiments were performed in triplicate. Control solutions were composed of reconstituted water equal to the volume used in the highest test concentration plus distilled water, summing to a total volume of 50 ml. Neonates, from females aged 2- to 5-weeks-old, were removed from the *Daphnia* culture vessels less than 24 h after culturing and were randomly transferred to test vessels containing of 1 ml *Daphnia* reconstituted water [10] using a clean glass pipet. Following 24 h exposure, the neonates were examined and determined as alive or dead (determined by lack of movement of the body, appendages, and heart through a microscope) [10]. The concentration lethal to 50% of the *D. magna* (LC50) was computed by evaluating the mortality percentage as a ratio of aqueous soil extract concentration.

#### Seed Germination/Root Elongation Test

The seed germination and root elongation toxicity test was performed according to US EPA protocols [18], as modified by Chang et al. [3]. The silica sand (20 meshes) was used as a diluent in the dose-response test. In this test, the phenol-contaminated soil samples were mixed with silica sand at dilutions of 0, 10%, 25%, or 50%. For example, 50% soil means that 1 g of phenol-contaminated soil is mixed with 1 g of silica sand. Each dilution was tested in triplicate in glass Petri dishes. One species of dicotyledon, lettuce (*Lactuca sativa*), and one species of monocotyledon, oats (*Avena sativa*), were studied using seeds purchased from a local supplier. For each dish, 10 seeds were placed on top of the test soil mixture. All dishes were incubated at room temperature with a 16:8 h light/dark cycle. At the end of 3 days, the number of germinated seeds was determined, and the total root length was measured.

#### RESULTS AND DISCUSSION

#### Biodegradation of Phenol-Contaminated Soil

After 4 days of incubation, the remaining phenol amounts were measured in 4.2 mM phenol-spiked soil samples. The recovery rate of phenol was determined initially by GC analysis; the average rate of phenol recovery was 83.6 ± 5.1%. In this study, four individual experiments were performed to degrade phenol in soil samples, as shown in Fig. 1. In the two control samples (i.e., no microorganism or supplements), phenol degradation was not observed.
within 4 days of incubation. Although *C. glutamicum* was inoculated in the soil, phenol was not degraded without the supplement of 1% yeast extract. Phenol degradation was only observed in soil in the presence of *C. glutamicum* with 1% yeast extract. At the fourth day, over 95% of the phenol in the soil sample was degraded by *C. glutamicum*. Low levels of yeast extract (below 1%) did not promote phenol degradation in soil samples by *C. glutamicum* (data not shown). Hatzinger *et al.* [7] described that the biodegradation of methyl tert-butyl ether (MTBE) [7], a pollutant of groundwater, was greatly enhanced by the addition of a small amount of yeast extract in the culture medium of *Hydrogenophaga flava*. Yeast extract can serve as a nitrogen source for microorganisms [9]. This suggests that *C. glutamicum* needs 1% yeast extract for effective phenol degradation in soil environments.

**Toxicity Removal Evaluation Using D. magna**

The toxicity of phenolic compounds in aquatic environments has been investigated primarily through acute toxicity tests on freshwater organisms. A 24 h acute test with *D. magna* was used to evaluate the toxicity removal of phenol in aqueous soil extracts (Fig. 2). After 4 days of *C. glutamicum* inoculation, we collected the aqueous phase from phenol-contaminated soils by centrifugation. To estimate the toxicity of phenol, 50% lethal concentrations (LC$_{50}$) were calculated using each soil extract. The lethality of the test organisms contained a higher phenol concentration, which increased with increasing concentrations of the aqueous soil extract. In addition, in this case, a lower of LC$_{50}$ value means higher toxicity for organisms. In three samples from *C. glutamicum* untreated and/or soil without supplementation of 1% yeast extract, the LC$_{50}$ were 18.3%, 25.1%, and 18.2% aqueous soil extract, respectively. In contrast, the *C. glutamicum*-treated sample with 1% yeast extract showed a higher LC$_{50}$ (74.8% aqueous soil extract). As mentioned previously, phenol degradation in soils occurs when *C. glutamicum* is supplemented with 1% yeast extract (Fig. 1). Although the toxicity associated with phenol was not completely removed, our approach would decrease the toxicity of phenol-contaminated soils.

**Change of Amino Acid Compositions in Phenol-Contaminated Soils**

Phenol in the soil samples was effectively degraded by *C. glutamicum* supplemented with 1% yeast extract (Fig. 1). In a previous study, we verified that intracellular production of some amino acids, such as glutamate, proline, threonine, and histidine, was enhanced by phenol utilization in *C. glutamicum* [11, 12]. This study investigated the amino acid compositions of phenol-contaminated soils to confirm whether amino acid levels were increased after phenol degradation by *C. glutamicum* treatment after 4 days of incubation. As shown in Fig. 3, almost all the amino acid levels in phenol-contaminated soils were elevated under *C. glutamicum* treatment conditions with or without 1% yeast extract. Overall, the concentrations of eight amino acids (glycine, threonine, isoleucine, alanine, valine, leucine, tyrosine, and phenylalanine) in soil samples treated by *C. glutamicum* were greater than those of untreated samples. However, proline and glutamate concentrations decreased or did not change with *C. glutamicum* treatment compared with the two untreated conditions. Comparing these results with intracellular levels [11] suggests that glutamate and proline in soil samples may be imported into the bacterial environments.
cell by phenol stimulation [8]. This result suggests that *C. glutamicum* can fertilize phenol-contaminated soil via the increase of essential amino acids in soil. It might be progressed by integrated metabolic pathways such as the TCA cycle and amino acid biosynthesis pathway in *C. glutamicum* [12]. Moreover, phenol removal and soil fertilization were accomplished simultaneously by *C. glutamicum* supplemented with 1% yeast extract.

**Plant Growth Promotion After Treatment of Phenol-Contaminated Soil by *C. glutamicum***

To validate the applicability of the fertilized and/or biodegraded phenol-contaminated soils, we conducted seed germination and root elongation tests.

The seed germination of lettuce was extremely sensitive to phenol-contaminated soils after 4 days of incubation (Fig. 4), even though phenol was degraded completely by *C. glutamicum* (Fig. 1). It was found that the germination percentage of lettuce varied from very low to approximately 10–40% in soil containing 1% yeast extract without phenol. The germination of lettuce was improved in soil treated with *C. glutamicum* and 1% yeast extract compared with the other conditions. Yeast extract can be used as a nitrogen source for lettuce, but excess causes nitrogen stress for lettuce growth [17, 21]. The results from the oat germination test helped to characterize the soil fertilized by *C. glutamicum*. In the test, oat showed a statistically significant reduction in germination relative to control soil (zero soil percentage): 51.9 ± 11.5% (not treated with *C. glutamicum*), 41.7 ± 26.0% (treated with *C. glutamicum*), and 46.2 ± 16.3% (supplemented with 1% yeast extract only). Soil treated by *C. glutamicum* with 1% yeast extract was an exception.

Root elongation values of lettuce and oat were measured 3 days after seeding. Lettuce root length showed low values (below 17%) compared with that of the control at all soil conditions. Clearly, only the soil treated with *C. glutamicum* and 1% yeast extract did not affect the oat root length (Fig. 5). In addition, oat root elongation results showed lower toxicity in the *C. glutamicum*-treated sample than in the untreated sample. This probably reflects that increased soil amino acid composition due to *C. glutamicum* treatment strengthens the plant root against toxic molecules (phenol) by giving tolerance.

This first-attempt study demonstrated an approach to the bioremediation of soil contaminated by *C. glutamicum* for phenol degradation and the development of environmentally friendly fertilization technology. According to HPLC analysis, various amino acids were greatly increased in *C. glutamicum*-treated soil. However, it was demonstrated that only supplementation with 1% yeast extract could help *C. glutamicum* to use phenol as a carbon energy source in phenol-contaminated soil. Therefore, treatment of *C. glutamicum* with 1% yeast extract is necessary to achieve
Effective fertilization of phenol-contaminated soil. Furthermore, based on plant tests, oat growth rate on the phenol-contaminated soils was restored by the metabolic activity of \textit{C. glutamicum}.

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References


