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Growth of Kerosene-Biodegrading Microorganisms in the Presence of α-Amino Acid

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Synthetic materials such as polypropylene have been widely used in oil spill cleanup because of their oleophilic and hydrophobic characteristics (Schatzberg 1971). These synthetic sorbents, however, are often non-biodegradable. Use of biodegradable sorbents can provide an alternative disposal method (biodegradation) of the oil-soaked sorbent over conventional landfilling or incineration (Choi and Cloud 1992). Our earlier studies indicated that natural fibers such as wool and cotton resulted in almost three times greater oil sorption than polypropylene mats or fibers (Choi 1996). Wool has great potential as natural oil sorbent due to its high oleophilic wax content, which is about 10 to 20% of fiber weight, and ready availability (Choi and Cloud 1992).

Composting has been used for bioremediation of soil and water contaminated with various toxic or hazardous chemicals (Beaudin et al. 1996, Boopathy et al., 1994). To utilize composting in biodegradation of the oil-soaked wool sorbents, it is necessary to know activity of various microorganisms in composting in the presence of wool. However, the laboratory-scale analysis in examining growth of microorganisms would be very difficult if wool is present during incubation. Our objective in this study was to examine the effects of three important a-amino acids in wool on growth of kerosene-biodegrading microorganisms.

MATERIALS AND METHODS

Soils obtained from near a cattle shed, Suwon, Korea, were air-dried in the shade after removing large plant roots and other organic debris and filtered through a standard sieve (No. 10, 2.0mm). Parameters of the soil were measured and included: 5.3, the pH of the soil, 3.3% organic carbon, and 19.7% moisture. 330,000 microorganisms per gram of soil were measured in nutrient agar by using bacterial counts. Five percent kerosene (Aldrich Chemical Co.) was mixed into the soil to prepare the kerosene-contaminated soil. Compost was prepared by mixing contaminated and uncontaminated soils (8:2 w/w soil), each with a mixture of cow manure, potato, and straw (20:40:40 v/v) and composted in a laboratory-scale composter constructed of a 10L acrylic cylinder insulated with 30cm thick Styrofoam for 13 days. Water-saturated air was supplied to the composting system at a constant flow rate (lm^3/kg per day) via a perforated aeration loop in the bottom of the reactor. Initial moisture content for composting was optimized to 50±5%, and the compost was completely mixed to eliminate any possible empty space around the composter wall.

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A 10g aliquot of the compost sample was obtained at 36°C and appropriate dilutions were made with sterilized distilled water. One milliliter of the appropriate diluted medium was placed into a tube containing M56 medium [10.6g KH_2PO_4, 43.98 $Na_2HPO_4 \cdot 12H_2O$, 4mL 10% $MgSO_4 \cdot 7H_2O$, 40mL 10% $(NH_4)_2SO_4$, 2mL 1% $Ca(NO_3)_2$, 2mL 0.05% $FeSO_4 \cdot 7H_2O$, and 15g agar] and 1% kerosene, and incubated for seven days at 36°C. After incubation, characteristics of colonies of microorganisms such as shape, colony color, and biochemical reactivity were examined. Colonies were then inoculated into new M56 medium in the absence of kerosene, incubated, and stored at 4°C. Kerosene-degrading activity of microorganisms was determined by examining growth of colonies after inoculation into M56 medium containing 1% kerosene. If microorganism formed the colony in M56 medium containing kerosene, it was considered as kerosene-degrading bacteria and its characteristics were determined.

The kerosene-degraders from compost were inoculated in 10mL of P-Y (Peptone-yeast extract, pH 7) agar slants containing 5g peptone, 3g yeast extracts, and 15g agar, and 10mM $CaCl_2$. The test tubes were then inoculated at 36°C for 24 hrs. Each loopful of kerosene-degrader was re-inoculated in 10mL P-Y broth and incubated at 36°C in a shaking incubator (Jeio Tech, Model SI-900R). When optical density (OD) at 600 nm reached a range of 0.4 and 0.6, an aliquot was transferred to a sterilized micro propylene tube containing 10% sterilized glycerol and then stored at -20°C. Stored tubes containing kerosene-degraders were thawed and diluted OD 0.1 at 600 nm with sterilized distilled water. 1mL of an aliquot of diluted microorganisms was mixed in a conical tube, centrifuged at 4°C for 10min (9000 g), rinsed twice with sterilized saline solution, re-centrifuged, and mixed with sterilized distilled water which was used as a mixed culture for further study with a-amino acid and kerosene.

Three abundant a-amino acids in wool (arginine, cysteine, and glutamic acid) were used as nitrogen sources instead of using NH_4NO_3 to determine their effects on growth of kerosene-degrading microorganisms in duplicate. Cysteine was used in lieu of cystine to enhance aqueous solubility. This nitrogen-limiting medium was composed of lg KH_2PO_4, 7g $Na_2HPO_4 \cdot 12H_2O$, 20mg 10% $MgSO_4 \cdot 7H_2O$, 2mg ferric citrate, 10mg $CaCl_2 \cdot 2H_2O$, and 20mM yeast extract as a carbon source, and an appropriate amount of a-amino acid. The pH of the medium was adjusted to 7 to eliminate any pH effects. All chemicals used in this study were reagent grade from Aldrich Chemical Co.

RESULTS AND DISCUSSION

We identified a total of 13 different microorganisms from our compost pile as shown in Table 1. Petroleum-contaminated environments resulted in a change in the balance of the microbial community structure. It has been demonstrated that the number of hydrocarbon-oxidizing bacteria increases in areas that suffer from oil pollution (Leahy and Colwell 1990, Song et al. 1986). Also the ratio of hydrocarbon-utilizing bacteria to total heterotrophic bacteria has been reported to be an indicator of petroleum contamination (Song et al. 1986). Under kerosene composting, various bacteria were isolated at temperatures ranging from 17 to 55°C. Isolates included heterotrophic, gram negative, rod or *cocobacillus*-shaped, non-spore forming, and facultative anaerobic bacteria, and also had the capability of degrading kerosene. Most strains were isolated at 36°C with the *Corynebacteria* genera being predominant. These results were accordant with the observation on that the highest rate of kerosene degradation occurred at 36°C during 13 days of cornposting.

Table 1. Characteristics of 13 kerosene-biodegrading strains of microorganism isolated from compost incubated at 36°C.

Group	Gram's stain	Shape	Colony color	Motility	Oxygen demand[a]	Indol	Kerosene degrading activity
KR-1	+	Rod	Orange	-	FA	-	+
KR-2	-	*Coccobacillus*	Orange	-	FA	-	++
KR-3	+	Rod	Orange	-	Ae	-	+
KR-4	+	Rod	White	-	Ae	-	++
KR-5	+	*Coccobacillus*	Orange	-	FA	-	++
KR-6	-	Rod	Yellow	-	FA	-	++
KR-7	+	*Coccobacillus*	Orange	-	FA	-	++
KR-8	+	Rod	Orange	-	FA	-	++
KR-9	+	Rod	White	+	FA	+	+
KR-10	+	*Coccobacillus*	White	+	FA	+	+
KR-11	+	Rod	White	+	FA	-	++
KR-12	+	Rod	Orange	-	FA	-	++
KR-13	+	Rod	White	-	FA	-	+

[a]FA: facultative anaerobic; Ae: aerobic

Figure 1 shows effects of three amino acids as a nitrogen source on growth of kerosene-biodegrading microorganisms in the absence of kerosene. As a control, no amino acid was added. At relatively low concentrations (0.01% and 0.1%) growth of microorganisms in the presence of arginine and glutamic acid was much higher than that of control. Contrarily, at high concentration (0.5%) of both amino acids, growth was inhibited. It should be noted that since the initial pH of the incubation medium was adjusted to pH 7 after addition of a-amino acid, the pH was not expected to influence growth. In addition, no significant pH change occurred during the incubation.

On the other hand, final growth of microorganisms at three different concentrations of cysteine was greater than that of control in the absence of α–amino acid. Nevertheless, initial growth of microorganisms was inhibited at high concentration of cysteine. Growth of microorganisms was generally lower than that of arginine- and glutamic acid-containing systems at 0.01% and 0.1% cysteine, but higher at 0.5%.

Results in Table 2 show that Carbon/Nitrogen (C/N) ratios decreased with increase in concentration of a-amino acid. It was known that an initial C/N ratio should be adjusted to a maximum of 40: 1 to provide sufficient nitrogen nutrients for effective composting (Rosenberg 1991). However, in this study no direct relationship was found between C/N ratio and growth of microorganisms in the absence of kerosene.

Once kerosene was added during incubation, trends in growth of microorganisms were completely changed. As shown in Figure 2, growth of microorganisms was the greatest at 0.5% arginine. By addition of kerosene during incubation, there was no change in C/N ratio compared with that of N-limiting medium without kerosene (Table 2). This result suggested that nitrogen supplementation would be necessary in the presence of arginine and kerosene. However, addition of kerosene during incubation inhibited growth of microorganisms in the presence of 0.1% and 0.5% cysteine. Only at low concentration (0.01%) of cysteine was growth similar to controls without amino acid. Glutamic acid tended to inhibit

growth at high concentration (0.5%), but at other concentrations growth rates were much higher than controls without amino acid. Growth was slightly higher than the control at 0.5% glutamic acid. Order of microorganism growth at different concentrations of glutamic acid was the same as that in the absence of kerosene.

Table 2. Carbon/nitrogen ratios of the incubation baths.

α-Amino acid	% Concentration	N-limiting medium (no kerosene)	With kerosene
Arginine	0.01	116.3	119.0
	0.1	11.6	11.9
	0.5	2.3	2.4
Glutamic acid	0.01	391.7	400.6
	0.1	39.2	40.1
	0.5	7.8	8.0
Cysteine	0.01	309.1	316.2
	0.1	30.9	31.6
	0.5	6.2	6.3

Results also showed that general growth of microorganisms in the presence of cysteine was much lower than that with arginine and glutamic acid as demonstrated by low OD in the cysteine-containing system. Low growth rates with cysteine occurred in both the absence and presence of kerosene during incubation.

Initial study showed that in both the absence and presence of kerosene general growth of microorganisms with three amino acids was amino acid-specific and concentration-specific, indicating the presence of other governing factors beside C/N ratios. Further study is needed to examine combined effects of different α–amino acid in the presence of kerosene.

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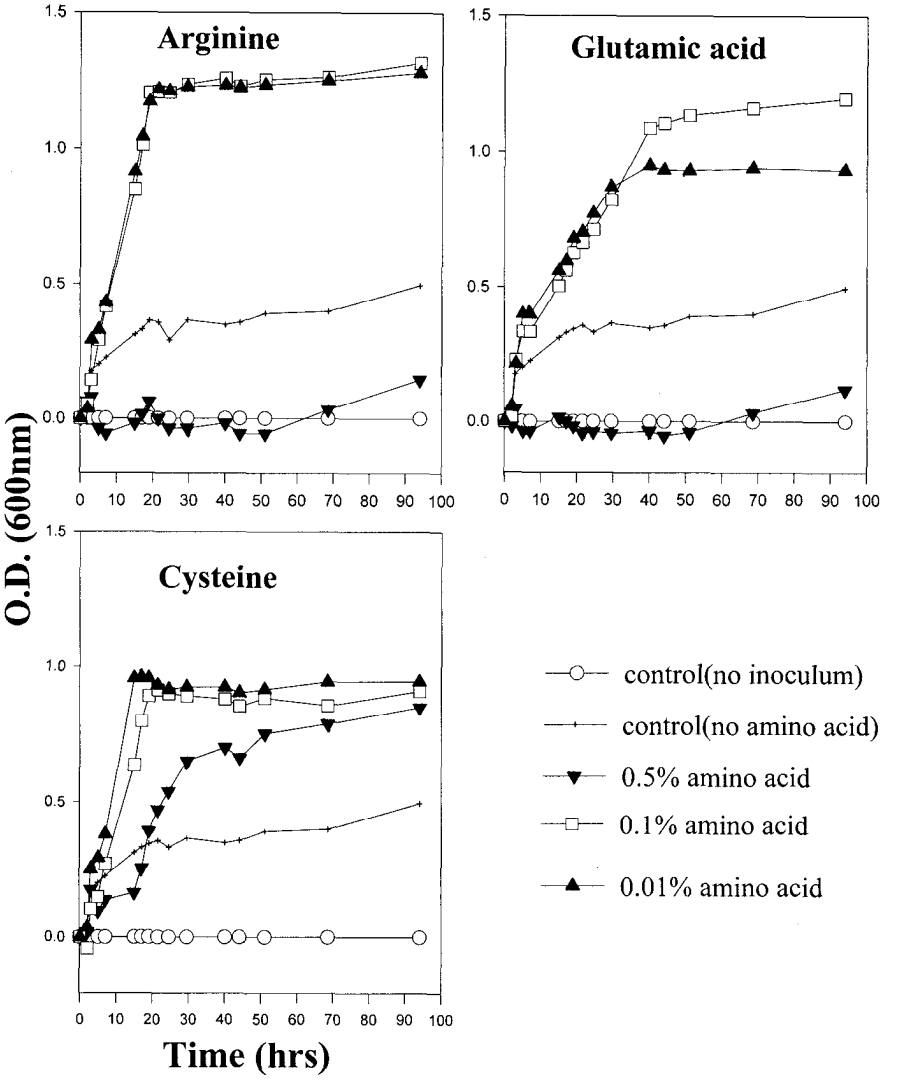

Figure 1. Turbidity of cultures growing in the presence of a-amino acids.

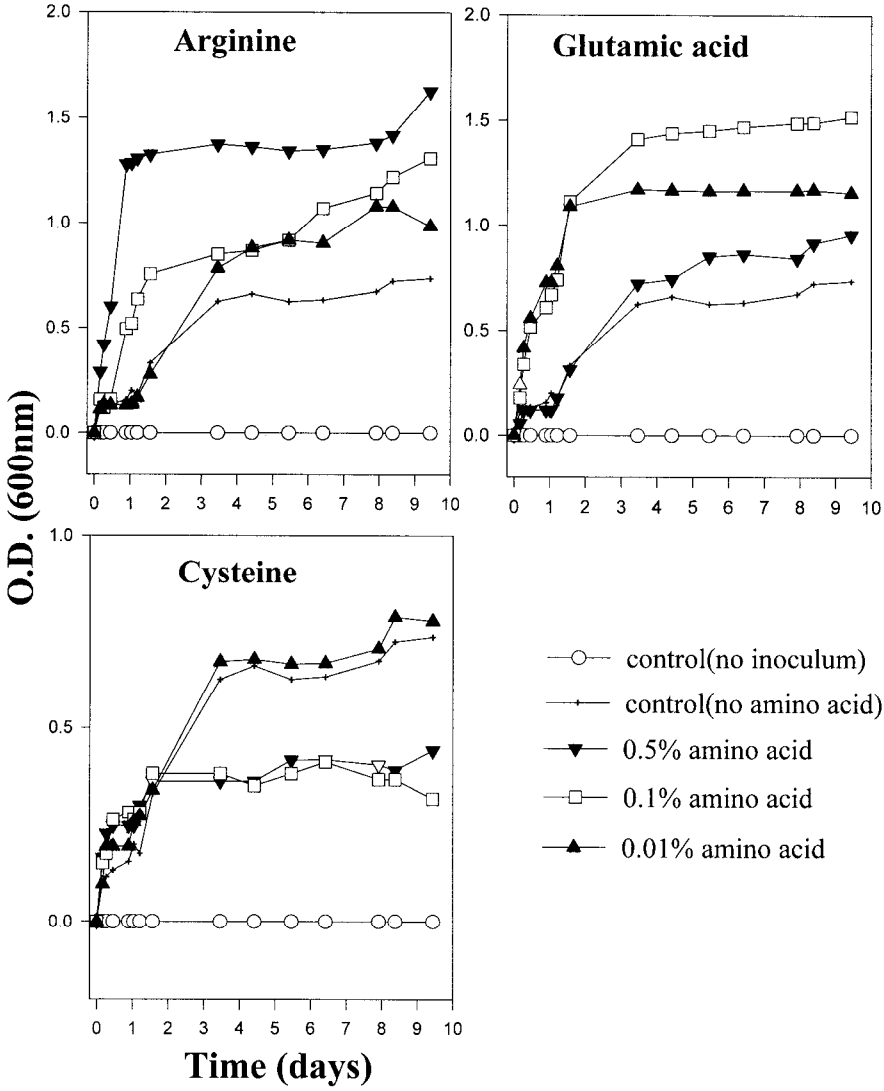

Figure 2. Turbidity of cultures growing in the presence of a-amino acid and kerosene.

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