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Effect of co-substrate on anaerobic slurry phase bioremediation of TNT-contaminated soil

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Abstract−Molasses and starch were used to estimate the effect of co-substrate on anaerobic slurry phase bioremediation of TNT (2,4,6-trinitrotoluene)-contaminated soil. Degradation efficiency of TNT in molasses and starch addition was approximately 97% and 87%, which is 50-60% higher than 38% without co-substrate addition. Molasses and starch addition enhanced TNT degradation. It was proved that the TNT degradation was a mainly biological process from the result of an abiotic control experiment. In case of molasses addition, overall first order degradation rate was 0.0161/day indicating the most active TNT degradation. The first order degradation rates (k_1=0.0056 for molasses, k_1=0.0033/day for starch) before 60 days were slower than those thereafter (k_2=0.0237 for molasses, k_2=0.0136/day for starch). Lower degradation rate in early stage might be due to adaptation of native soil microorganisms.

Key words: TNT, Anaerobic Slurry Phase Bioremediation, Co-substrate

INTRODUCTION

The worldwide annual production of TNT (2,4,6-trinitrotoluene), the primary explosive used in the manufacture of munitions, is estimated at two million lbs [1]. It is widely used in commercial explosives as a good sensitizer and is much safer in production and handling than nitroglycerine [2]. The molecular weight of TNT is 227.15 g/mole and it has a specific gravity of 1.654, a melting point of 80.65 ºC and a boiling point of 240 ºC at which point it explodes. The water solubility of TNT is 120 mg/L. TNT is introduced into soil and water ecosystems mainly by military and manufacturing activities.

TNT is toxic to a number of organisms including humans and may be carcinogenic. Because of its toxic and recalcitrant properties, the contamination of soil and groundwater by TNT represents a significant international environmental problem [3]. TNT contamination of soil and water occurs because of the manufacture, loading, assembly, packing and military related activities [4]. Due to the potential adverse effect of the compound on human health and the environment, contaminated environments must be remediated [5]. The Department of Defense has identified more than 1,200 explosive-contaminated sites in the United States [6]. In spite of unavailable information for TNT-contaminated soils in Korea, widespread use of TNT on military sites and explosives producing plants may result in numerous occurrences of TNT-contaminated sites.

Slurry phase bioremediation has been demonstrated to be an effective process for contaminated soils, sediments, and sludges [7]. Solid phase treatments such as composting or land farming cannot complete remediation of contaminated ones in short time-frames [8]. Slurry phase bioreactors are designed to optimize mass transfer of nutrients and electron acceptors by using mechanical mixing and aeration [9]. In general, contaminated soil is loaded into a reactor or tank to produce a water-based slurry (at least 30%, w/w).

Many workers have reviewed the recent literature concerning the biological degradation of TNT and other hazardous energetic nitroaromatic compounds. Under both aerobic and anaerobic culture conditions, the initial step in the metabolism of nitroaromatic compounds is typically a reduction of the nitro constituents to amino groups. McCormick et al. [10] hypothesized that aerobic DNT transformation by a *Microsporium* sp. proceeded through nitroso (-NO) and hydroxylamino (-NHOH) intermediates. However, these metabolites cannot be easily detected in contaminated soils. This reduction usually proceeds in steps with the para nitro group being the most susceptible to reduction [11,12]. The reduction of the para nitro group appears to be nonspecific and is performed by many bacterial cells and by some cell extracts, as long as some growth of the bacteria occurs or strong reducing conditions are used. The next reduction usually occurs at one of the ortho groups, producing DANT (diaminonitrotoluene) isomers [10,13]. The reduction of the third nitro group occurs only under strictly anaerobic conditions (Eh≤−200 mV) [14,15].

Cometabolism has recently been defined as the transformation of a non-growth substrate in the obligate presence of a growth substrate [16]. Osmon and Klasusmeier [17] reported that TNT degradation occurred only in the presence of supplemental organic material and TNT degradation was cometabolic. Various co-substrates such as glucose, succinate, acetate, malate, citrate, and molasses can be used.

The reduction of the third nitro group occurs only under strictly anaerobic conditions and co-substrate addition is needed for biological treatment of TNT-contaminated soil. Therefore, this research

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was carried out to estimate the effect of addition of molasses and starch wastewaters as co-substrate on anaerobic slurry phase bioremediation of TNT-contaminated soil. If molasses and starch wastewaters could be effective as co-substrate, it could decrease the treatment cost of TNT-contaminated soil. Kinetics for anaerobic slurry phase bioreactor was also evaluated.

MATERIALS AND METHODS

1. Materials

The materials used for this research were soil, TNT, and co-substrate. The soil was collected from the top 15 cm of the surface soil on the campus of K University. The soil was sieved through a 2 mm sieve to remove large soil fraction such as stone and gravel. The soil was ground with mortar and pestle to obtain finer fraction. In general, contaminants will be associated with the finer fraction of the soil which has a larger surface to volume ratio, and besides, larger factions are difficult to keep in suspension and likely to settle in a slurry phase reactor. Table 1 shows physical and chemical characteristics of the soil for slurry phase treatment research. The texture of the soil was classified as a typical loam (the portions of sand, silt, and clay of soil were 36.1%, 37.3%, and 26.6%, respectively) according to the USDA definition [18]. The soil had a slight alkaline pH of 8.1 and contained organic matter of 4.5% by dry weight.

Military-grade TNT was used for this research and its purity was about 76% (wet weight basis). Also, it contained ADNT and DNT as the minor impurities.

Molasses and starch wastewaters were obtained from JENICO Corporation and Doosan Corporation, Korea, respectively. Molasses and starch wastewaters as co-substrates had an acidic pH of 5.1 and 4.1, respectively (Table 1). Molasses and starch wastewaters provide a variety of organic compounds for enhancing microbial metabolism, including sugar, amino acids, organic nitrogen, proteins, vitamins, and mineral. The co-substrates were added 0.3% (w/v) to the slurry phase reactor based on TS (total solids). A phosphate buffer solution was added to the reactor to control the pH to approximately 7. Molasses and starch had TOC of 23,976 mg/L and 4,410 mg/L, respectively.

2. Experimental Apparatus and Condition

Fig. 1 shows anaerobic bioreactor sets for this research. Anaerobic slurry phase bioreactors consisted of a series of five bottles with butyl rubber stopper and 10 mL capacity. To prevent photolysis of the added TNT, the reactors were incubated in the dark and operated at 30 °C. Park et al. [19] reported that the optimal conditions for the biodegradation of TNT were found to be 30 °C and pH 7.1. A phosphate buffer solution with KH_2PO_4 and K_2HPO_4 was added to the reactor to control the pH to approximately 7. The headspace of the reactor was purged with N_2 gas before sealing. The soil slurry was mixed with vortex mixer (Vision Scientific Co., KMC-1300V) for 5 minutes, twice a day.

Anaerobic slurry phase bioreactors were loaded with 30% (w/v) slurry of TNT-contaminated soil in water. Molasses and starch used as co-substrate were added 0.3% as dry weight basis to the volume of slurry phase bioreactors. Co-substrate control experiment was performed to find the effect of adding co-substrates. An abiotic control experiment with $HgCl_2$ of 2,000 mg/kg also was carried out to discriminate chemical and biological degradation. Anaerobic slurry phase bioreactors were operated for 200 days.

3. Analysis

Analytical mixing standards were purchased from AccuStandard

Table 1. Physical and chemical characteristics of soil and co-substrates

Classification		Soil	Classification		Co-substrates	
					Molasses	Starch
pH		8.1	pH		5.1	4.1
Volatile solids (%)		4.5	EC (mmhos/cm)		30.2	20.1
CEC (meq/100g dry matter)		21.3	TOC (mg/L)		23,976	4,410
Chemical composition	C	0.73	Solid contents (mg/L)	TS	71,013	11,050
(%, dry weight basis)	H	0.60		VS	44,847	6,110
	O[a]	2.81		SS	12,850	608
	N	0.12		VSS	9,500	528
	S	0.23				
	Ash	95.52				
Heavy metals	Pb	9.9	Heavy metals (mg/L)	Pb	10.3	1.9
(mg/kg, dry weight basis)	Zn	19.6		Zn	62.3	58.0
	Cu	30.8		Cu	1.9	1.4
	Cd	0.9		Cr	N.D[b]	0.5
Soil textures (%)	Sand	36.1		Cd	0.5	N.D
(Hydrometer)	Silt	37.3		Fe	29.0	89.9
	Clay	26.6		Mn	5.7	19.4
		Loam		As	0.5	0.2

[a]O=100−(C+H+N+S)−Ash

[b]N.D.: Not Detected

Fig. 1. Anaerobic slurry phase bioreactor for this research.

(Mix A (M8330A-R-10X) and Mix B (M8330B-R2-10X), New Haven, USA). The mixture contained the following compounds: Mix A (2-ADNT, 1,3-DNB, 2,4-DNT, HMX, NB, RDX, 1,3,5-TNB and 2,4,6-TNT) and Mix B (4-ADNT, Tetryl, 2,6-DNT, 2-NT, 3-NT and 4-NT). The mixture was dissolved in 1.0 mg/mL each in acetonitrile to MeOH (1 : 1). The standards were diluted in acetonitrile to achieve 10 ppm. Working standards were made by diluting the stock solution with HPLC-grade water. The HPLC was calibrated by using these standards. TNT and its metabolites have properties such as thermal lability and polarity. Many of these compounds thermally degrade or explode at temperature below 300 °C. Thus, methods based on gas chromatography are not recommended for routine use. Therefore, the sample extracts were analyzed with RP-HPLC (reversed-phase high performance liquid chromatography) method (SW-846 Method 8330A) [20].

A 2.00 g sample was placed in a 10 mL glass vial equipped with a Teflon-lined cap. Ten mL of acetonitrile with HPLC-grade (Fisher Scientific, A998-4) was added in the vial. The target compounds in sample were dispersed by vortex mixing for 1 min and extracted in an ultrasonic bath (Branson Ultrasonic Corp., 8210R-DTM) for 18 h. The bath was maintained at ambient temperature during extraction to minimize loss of materials which are thermally degradable. The vials were removed from the bath and allowed to settle for 30 min. A 5 mL aliquot was removed, placed in a glass vial and combined with 5 mL aqueous $CaCl_2$ (5 g/L). The vials were shaken and centrifuged at 2,000 g for 20 min to permit fine particles to flocculate. A 5 mL aliquot was filtered through a 0.45 μm PTFE filter (Millipore, JHWP02500) into a clean vial. The vials were sealed with butyl rubber stoppers and aluminum crimps. Extracts were stored at 4 °C in the dark until analysis.

RESULTS AND DISCUSSION

1. Effect of Co-substrate

Variation of TNT concentration for anaerobic slurry phase bioreactor with co-substrates is shown in Fig. 2. TNT concentration is expressed in the units of mg/kg (soil) as solid basis. TNT concentration was decreased in the anaerobic slurry phase bioreactors with molasses and starch as co-substrate. The TNT concentrations were little changed in the early stage of operation until 60 days, but decreased more rapidly thereafter. In case of the co-substrate control without co-substrate, TNT was slowly decreased relative to those with co-substrates during 200 days of operation period. This result indicates that the degradation of TNT in contaminated soil was enhanced by the addition of co-substrates such as molasses and starch. In the abiotic control with $HgCl_2$ of 2,000 mg/kg as biocide, there was little reduction in TNT, indicating that the degradation of TNT is a mainly biological process aided by soil microorganisms.

Fig. 3 shows the final degradation efficiencies of TNT in contaminated soil after 200 days of operation. The highest degradation efficiency (about 97%) of TNT was observed in molasses addition during 200 days of operation. In case of starch addition, the degra-

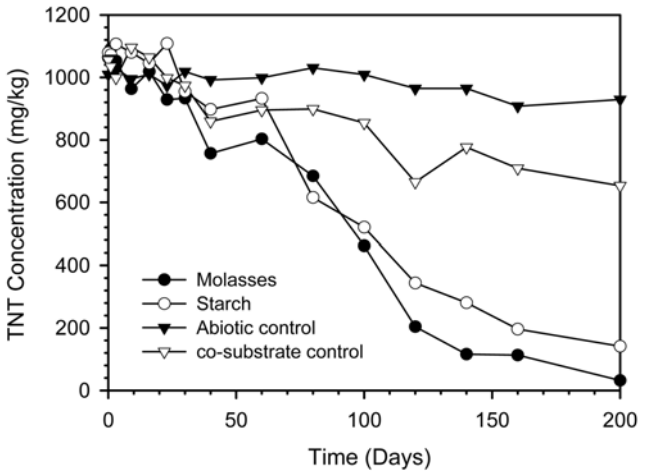

Fig. 2. Variation of TNT concentration with co-substrate addition.

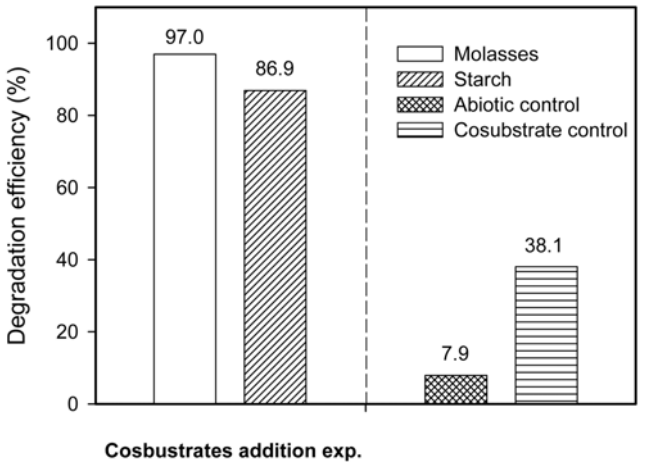

Fig. 3. Final degradation efficiency with co-substrate addition.

dation efficiency was approximately 87%. When no co-substrate was present in the anaerobic slurry phase bioreactor, the degradation efficiency was low as 38%, which is 50-60% lower than that of co-substrate addition. This means that molasses or starch addition enables transforming of TNT in contaminated soil into its metabolites.

USEPA [21] reported anaerobic slurry phase treatment (known as SABRE^{TM} process) of TNT-contaminated soil. TNT-contaminated soil was mixed with water to form a slurry in a 1 : 1 ratio (by weight). The degradation efficiency based on the TNT pre-treatment slurry concentration of 1,500 mg/kg and the TNT post-treatment slurry concentration of 8.7 mg/kg was 99.4% for 270 days. Relative toxicity testing of the slurry was performed before treatment and on day 150. This testing included early seeding growth, root elongation, and earthworm survival and reproduction tests. The results showed that the toxicity of soil was reduced greatly.

Anaerobic condition might be established by providing molasses and starch to indigenous aerobes, which then utilize oxygen, creating anaerobic conditions. Fig. 4 shows variation of redox potential in this experiment. Redox potential dropped from initial values of +270 mV to approximately −160 mV within 9 days and then slightly increased up to −120 mV until 23 days, and thereafter maintained a range from approximately −200 to −250 mV throughout the experiment period. In case of co-substrate control without co-substrate, redox potential slowly decreased and maintained high value compared to co-substrate addition. This means that co-substrates such as molasses or starch wastewaters used in this research helped lower redox potential and created anaerobic conditions.

The number of anaerobic bacteria was enumerated by the anaerobic roll tube method [22]. A method was developed in which agar medium was distributed as a thin layer over the internal surface of test tubes charged with an anaerobic atmosphere for the isolation of obligately anaerobic bacteria. Fig. 5 shows variation of the number of anaerobic bacteria. The number of anaerobic bacteria in the initial sample was on the order of 10^5 CFU (Colony Forming Unit)/mL of soil slurry. Maximum growth was observed at about the order of 10^9 CFU/mL at 30 days, and then remained constant for the experimental duration. In case of co-substrate control, total anaerobic bacterial number was less than ten-fold compared with that of co-substrate addition. A similar trend was observed by Fuller and Manning [23], who reported that the CFU in the molasses-fed slurry phase bioreactor increased rapidly by three orders of magnitude compared with the experiment without molasses. Robertson and Jjemba [24] evaluated the bioavailability of TNT by a bacterial consortium. The microbial consortium was isolated from a TNT-contaminated sediment and was acclimated to grow on TNT by maintaining it on an inorganic salt solution containing 200 mg TNT per liter. They reported that the consortium was identified to consist of *Acinetobacter* sp., *Enterobacter* sp., and *Pseudomonas* sp. Approximately 35% of TNT was mineralized over 20 weeks in instances where the consortium was present.

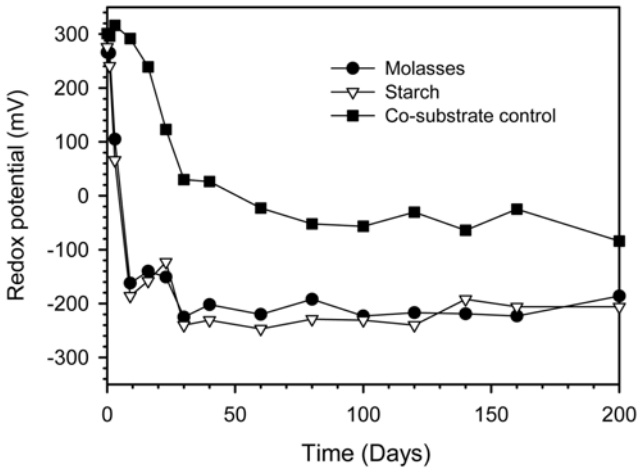

Fig. 4. Variation of redox potential with co-substrate addition.

Table 2. Experimental conditions

Experiment	Condition
Molasses	Molasses 0.3% (w/v)
Starch	Starch 0.3% (w/v)
Abiotic control	Molasses 0.3% (w/v) and 2,000 mg $HgCl_2$/kg sample
Co-substrate control	No co-substrate addition

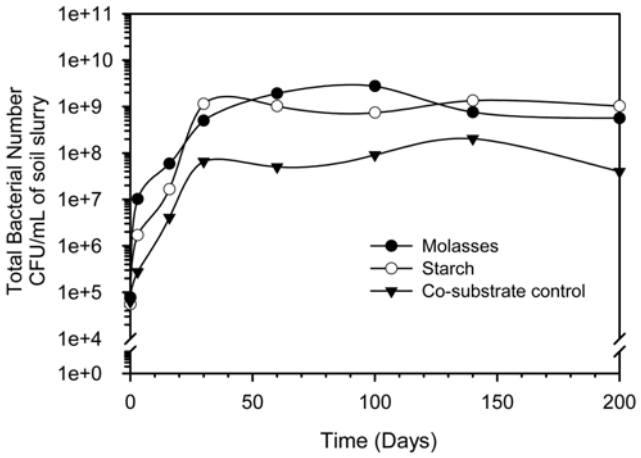

Fig. 5. Variation of the number of anaerobic bacteria in soil slurry enumerated by the anaerobic roll tube method.

2. Kinetics

Degradation kinetic parameters based on the first order kinetic models are presented in Table 3. The degradation of TNT was described with high correlation coefficient for the first order model. In case of molasses addition, overall first order degradation rate was 0.0161/day (half-life=43.1 day) which indicated the most active degradation of TNT. As described in Fig. 2, the TNT was little changed in the early stage of operation until 60 days in case of co-substrate addition, but thereafter more rapidly decreased. Therefore, kinetic model parameter of overall reaction was divided into two parts based on 60 days and presented in Fig. 6 and Table 3. The first order degradation rates (k_1=0.0056 for molasses, k_1=0.0033/day for starch) before 60 days were slower than those thereafter (k_2=0.0237 for molasses, k_2=0.0136/day for starch). Anaerobic slurry phase bioreactors have been operated without acclimation of soil microorganisms from the start. Lower degradation rate in early stage might be

Table 3. Degradation kinetics based on the first order kinetic model

Experiment	k_1^* (1/day)	r_1^*	k_2^{**} (1/day)	r_2^{**}	k^{***} (1/day)	r^{***}	Half-life (day)
Molasses	0.0056	0.90	0.0237	0.99	0.0161	0.96	43.1
Starch	0.0033	0.83	0.0136	0.99	0.0106	0.98	65.4
Abiotic control	-	-	-	-	0.0005	0.78	1,389.3
Co-substrate control	-	-	-	-	0.0025	0.94	227.3

* k_1 and r_1 : First order kinetic constant and correlation coefficient from 0 day to 60 days.
** k_2 and r_2 : First order kinetic constant and correlation coefficient from 60 days to 200 days.
*** k and r : Overall first order kinetic constant and correlation coefficient from 0 day to 200 days.

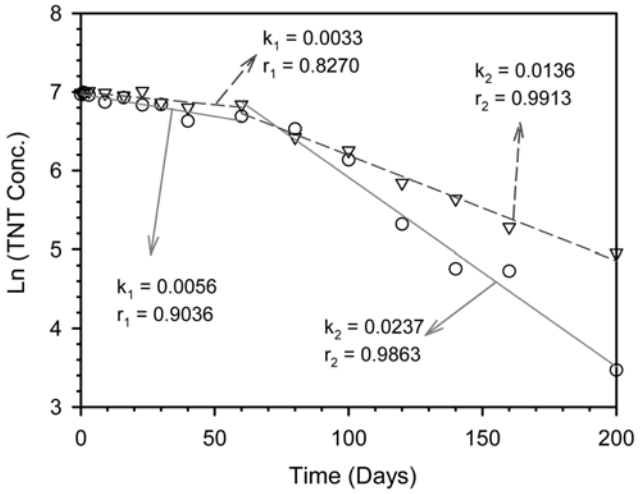

Fig. 6. Kinetics based on the first order kinetic model with co-substrate addition (Circle and solid dash mean molasses addition experiment and reversed triangle and short dash mean starch addition experiment).

caused by the adaptation of native soil microorganisms.

Initial lower degradation rate in this research was very similar to the result of Boopathy [25]. His study was conducted to evaluate the possible biological treatment of HMX (High Melting eXplosive: Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine) -contaminated soil by using native soil bacteria present at the contaminated site in a slurry phase bioreactor. The HMX-contaminated soil needed at least three weeks acclimation period for soil bacteria to metabolize HMX. Boopathy [26] also has been reporting that native soil bacteria could remove TNT by a cometabolic process. Various co-substrates have been used to find an inexpensive carbon source for large-scale bioremediation of TNT. Succinate, citrate, malic acid, acetate, glucose, sucrose, and molasses were used in his study as carbon sources for bacterial consortium transforming TNT.

3. Transformation Characteristics of TNT Metabolites

The predominant TNT metabolites observed in this research were 4-ADNT (4-Amino-2,6-Dinitrotoluene), 2-ADNT (2-Amino-4,6-Dinitrotoluene), and unknown metabolites. TNT is transformed to mono- and/or diaminonitrotoluenes [15]. Fig. 7 shows the variation of ADNT metabolites. The data was corrected by excluding abiotic transformation of ADNTs, which were confirmed from abiotic control experiment. Both abiotic and biotic transformations occurred in soils [27]. In case of co-substrate control, ADNTs were detected through the experimental period (Fig. 7(c)). However, the ADNTs

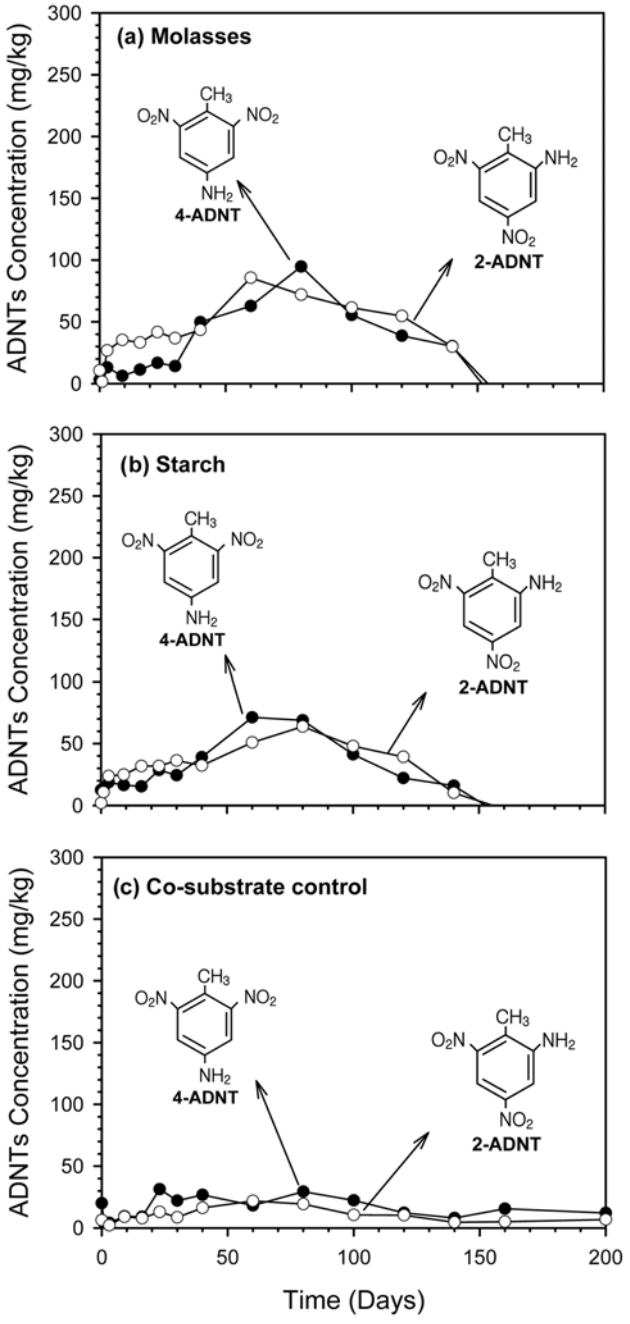

Fig. 7. Variation of ADNTs (aminodinitrotoluenes) concentration with co-substrate addition (Filled and open circles imply 4-ADNT (4-amino-2,6-dinitrotoluene) and 2-ADNT (2-amino-4,6-dinitrotoluene).

were transformed more slowly and detected in lower concentration than those of co-substrate addition, which showed an increase of ADNTs until 80 days and the disappearance of ADNTs at approximately 150 days. ADNTs generation for molasses addition was higher compared to starch addition. This means that more active transformation of TNT occurred in molasses addition. In case of co-substrate addition, 2-ADNT was observed higher than 4-ADNT until 40 days. Predominant amino isomer is 2-ADNT in anaerobic system [28].

CONCLUSIONS

In anaerobic slurry phase bioremediation, degradation efficiency of TNT in molasses and starch addition was approximately 97% and 87%, which is 50-60% higher than 38% without co-substrate addition. Molasses and starch addition enabled transforming TNT in contaminated soil into its metabolites. In the abiotic control with $HgCl_2$ of 2,000 mg/kg as biocide, there was little reduction in TNT, indicating that the degradation of TNT is a mainly biological process aided by soil microorganisms. The number of anaerobic bacteria in the initial sample was on the order of 10^5 CFU/mL of soil slurry. Maximum growth in co-substrate addition was observed at about the order of 10^9 CFU/mL at 30 days, and thereafter remained constant. Total anaerobic bacterial number in co-substrate control was less than ten-fold compared with that of co-substrate addition. In case of molasses addition, overall first order degradation rate was 0.0161/day, indicating the most active TNT degradation. The first order degradation rates (k_1=0.0056 for molasses, k_1=0.0033/day for starch) before 60 days were slower than those of thereafter (k_2= 0.0237 for molasses, k_2=0.0136/day for starch). Lower degradation rate in early stage might be due to adaptation of native soil microorganisms. ADNTs in molasses addition were generated more highly compared to starch addition, which means that more active TNT transformation occurred in molasses addition.

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