Biotechnol. Bioprocess Eng. **2002**, 7: 105-111

Biological Removal of Explosive 2,4,6-Trinitrotoluene by *Stenotrophomonas* sp. OK-5 in Bench-scale Bioreactors

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Abstract The biological removal of 2,4,6-trinitrotoluene (TNT) was studied in a bench-scale bioreactor using a bacterial culture of strain OK-5 originally isolated from soil samples contaminated with TNT. The TNT was completely removed within 4 days of incubation in a 2.5 L bench-scale bioreactor containing a newly developed medium. The TNT was catabolized in the presence of different supplemented carbons. Only minimal growth was observed in the killed controls and cultures that only received TNT during the incubation period. This catabolism was affected by the concentration ratio of the substrate to the biomass. The addition of various nitrogen sources produced a delayed effect for the TNT degradation. Tween 80 enhanced the degradation of TNT under these conditions. Two metabolic intermediates were detected and identified as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene based on HPLC and GC-MS analyses, respectively. Strain OK-5 was characterized using the BIOLOG system and fatty acid profile produced by a microbial identification system equipped with a Hewlett Packard HP 5890 II gas chromatograph. As such, the bacterium was identified as a *Stenotrophomonas* species and designated as *Stenotrophomonas* sp. OK-5.

Keywords: 2,4,6-trinitrotoluene; explosive; biodegradation; *Stenotrophomonas* sp.

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is a nitroaromatic explosive that is released into soil and water ecosystems mainly due to military activities. As a result, many sites used for TNT production have become seriously contaminated with the explosive. TNT has also been reported to be toxic to microorganisms [17], green algae [25], fish and animals [27]. As such, the fate of TNT residues in environmental ecosystems has become a serious concern to environmental scientists due to the fear that TNT and its metabolites can be introduced into the food chain [6, 13].

The microbial degradation of TNT has already been extensively studied in the white-rot fungus, *Phanerochaete chrysosporium* [5,10,26]. Several other bacteria, such as *Serratia marcescens* [1], *Pseudomonas fluorescens* [2], *Desulfovibrio* [21], *Enterobacter cloacae* [11], and the fungus *Phanerocheate chrysosporium* [20] have also been found to be capable of utilizing TNT as a sole source of nitrogen.

TNT degradation in soils has been demonstrated in previous studies [6,18]. Composting was very recently attempted for the bioremediation of TNT-contaminated soils in a reactor system [3]. Biochemical and genetic knowledge on the microbial degradation of TNT has been well documented [4,8]. However, the utility of such knowledge has not been evaluated for possible treatment processes of industrial waste streams where TNT may represent a serious disposal problem.

The current authors previously evaluated the degradation of TNT in flask-level experiments using a *s*-triazine-degrading bacterium isolated from contaminated soil [22]. Yet the current report demonstrates that *Stenotrophomonas* sp. OK-5 is capable of growth with TNT, plus a medium developed for rapid TNT degradation was evaluated in a bench-scale bioreactor before its application to effluent treatment.

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MATERIALS AND METHODS

Bacterial Isolation and Maintenance

Soil samples collected from a TNT-contaminated site were incubated with shaking for an enrichment culture under aerobic conditions. From the enriched bacterial mixture, a pure strain, designated OK-5, was isolated based on its excellent ability to degrade TNT as a sole source of carbon and nitrogen. Strain OK-5 was routinely maintained on a TNA medium [23] containing 50 mg of TNT owing to its tendency to lose its TNT-degrading ability during cultivation in complex media. For TNT degradation, the cells were grown in a liquid basal salts medium composed of 100 mg TNT, 1.74 g

K_2HPO_4, 0.6 g NaH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.001 g $CaCl_2$, 0.001 g $FeCl_3$, 0.0001 g $MnCl_2$, 0.0001 g $ZnSO_4$, 1 mL Tween 80, several trace metals, and 0.36 g supplemented carbon source, per liter. Either glucose, fructose, molasses, or citrate (each final concentration, 2.0 mM) was added as the supplemented carbon source. The medium was adjusted to pH 7.2 before autoclaving. The subcultures were maintained with 10% inocula. The bacterium was incubated aerobically with shaking at 30°C.

Morphological, Physiological, Biochemical Tests, and Identification of TNT-degrading Bacterium

Early log-phase cells from cultures grown at 30°C were used for the morphological, physiological, and biochemical tests. The morphological characteristics were tested by a Gram stain [12]. The cell motility was checked on motility media [9], by observing the number and morphology of the flagella using Mayfield and Inniss' method [19]. Other physiological and biochemical characteristics were tested according to protocols found in Methods for General and Molecular Bacteriology [12]. A GN2 MicroPlate™ (BIOLOG, Hayward, CA, USA) was used to characterize strain OK-5 based on substrate utilization profiling. The identification was performed using a Microbial Identification System equipped with a Hewlett Packard HP 5890 II gas chromatograph.

Operations of Bioreactor

Subsequent experiments on TNT utilization were carried out using a 2.5 L bottom driving-type bench-scale bioreactor (Model KF-2.5, KoBioTech Co., Incheon, Korea), which was fitted with a water condenser (5°C) and operated at 30°C with a stirring rate of 150 rpm and airflow rate of 1.3 L/min. A 10% (v/v) inoculum of the test culture initially grown in shaking flasks was used. Technical-grade TNT was used as the initial substrate in the bench-scale bioreactor experiments. The cell turbidity was read at 660 nm in a Jasco spectrophotometer (Model V-550, Japan)

Analytical Methodology

A standard stock solution of TNT for the reverse-phase HPLC was prepared by adding 10 mg of analytical-grade TNT to a 100 mL volumetric flask. When completely dissolved, the volume was made up with HPLC-grade water. The HPLC system consisted of a pump (Shimadzu LC-10A, Japan), injector fitted with a 20 μL loop, UV detector (254 nm), and integrator. A commercial Zorbax ODS reverse column (250 mm × 4.6 mm, particle size 5 μm) was eluted with a mobile phase, which contained 20% (v/v) isopropanol and 80% water. The flow rate of the mobile phase was 1.0 mL/min. The samples collected from the bench-scale bioreactor were centrifuged at 3,500 × *g* for 10 min to sediment the bacterial cells. Aliquots (1 mL) of the supernatants were diluted with HPLC grade water before filtration through a 0.45 μm Gelman Arco LC25 disposable syringe filter. For peak identification, the retention times of the unknown peaks were compared with those of authentic reference compounds. Standard curves were constructed by plotting the peak areas versus known amounts of the authentic standards.

For the GC-MS analyses, 50 mL of a centrifuged culture sample (8,000 × *g*, 20 min) was acidified to pH 3 with 6 N HCl, followed by extraction with an equal volume of ethyl acetate twice. The solvent was removed under a vacuum and the residue redissolved in dichloromethane. The MS data was obtained using a Hewlett-Packard 5970 mass selective detector equipped with a Hewlett-Packard gas chromatograph. A HP 5-MS capillary column (25 m by 0.2 mm) was used and programmed from 80°C to 300°C at 10°C /min. The injector temperature was 250°C. The carrier gas was helium at 0.8 mL/min and the injection volumes were 2 μL.

Chemicals

The technical- and analytical-grade TNT were obtained from an explosives manufacturing company. The analytical-grade 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) were purchased from Absolute Standards Chemical Co. (Hamden, CT, USA), and the HPLC-grade isopropanol and water from Fisher Scientific Co. (Fair Lawn, NJ, USA).

RESULTS AND DISCUSSION

Physiological and Biochemical Characterization of Strain OK-5

Strain OK-5 was selected from among several TNT-degrading bacteria for its ability to rapidly degrade TNT under aerobic conditions. Strain OK-5 was found to be Gram-negative, coccobacillus-shaped, catalase-positive, oxidase-negative, motile, phenylalanine deaminase-negative, tryptophanase-negative, urease-negative, and negative for starch utilization, indole formation, and H_2S formation. The physiological and biochemical characteristics of strain OK-5 obtained from the BIOLOG system are listed in Table 1. An analysis of the carbohydrate utilization profiles based on the GN2 MicroPlate™ and an analysis of the fatty acid composition by GC-MS also placed OK-5 as a *Stenotrophomonas* species with confidence over 96%.

Bacterial Growth and TNT Degradation in Bench-scale Bioreactor

Strain OK-5 was initially tested for the degradation of TNT under aerobic conditions in 250 mL Erlenmeyer flasks. OK-5 was able to grow with up to 100 mg of TNT per liter, which was the highest concentration tested. Ten percent inocula from a fully-grown bacterial culture, which had a cell density of approximately 7.5 × 10^7 cells per mL, were added to the bench-scale bioreactor for TNT degradation. Changes in the optical density

Table 1. Physiological and biological characterization of the isolate using the BIOLOG analysis system

Physiological & biochemical tests		Physiological & biochemical tests	
Water	-	*p*-hydroxyphenylacetic acid	–
α-cyclodextrin	–	Itaconic acid	–
Dextrin	+	α-ketobutyric acid	+
Glycogen	–	α-ketoglutaric acid	+
Tween 40	+	α-ketovaleric acid	–
Tween 80	+	D,L-lactic acid	+
N-acetyl-D-galactosamine	+	Malonic acid	+
N-acetyl-D-glucosamine	+	Propionic acid	+
Adonitol	–	Quinic acid	–
L-arabinose	–	D-saccharic acid	–
D-arabitol	–	Sebacic acid	–
Cellobiose	+	Succinic acid	+
i-erythritol	–	Bromo succinic acid	+
D-fructose	–	Succinamic acid	–
L-fucose	–	Glucuronamide	–
D-galactose	–	Alaninamide	+
Gentiobiose	+	D-alanine	+
α-D-glucose	+	L-alanine	+
m-Inositol	–	L-alanylglycine	+
α-D-lactose	–	L-asparagine	+
Lactulose	–	L-aspartic acid	–
Maltose	+	L-glutamic acid	–
D-mannitol	–	Glycyl-L-aspartic acid	+
D-mannose	+	Glycyl-L-glutamic acid	+
D-melibiose	–	L-histidine	–
β-methyl D-glucoside	–	Hydroxy-L-proline	–
Psicose	–	L-leucine	+
D-raffinose	–	L-omithine	–
L-Rhamnose	–	L-phenylalanine	–
D-Sorbitol	–	L-proline	+
Sucrose	–	L-pyroglutamic acid	–
D-trehalose	+	D-serine	–
Turanose	–	L-serine	+
Xylitol	–	L-threonine	+
Methylpyruvate	+	D,L-camitine	–
Mono-methylsuccinate	+	γ-aminobutyric acid	–
Acetic acid	+	Urocanic acid	–
Cis-aconitic acid	–	Inosine	–
Citric acid	+	Uridine	+
Formic acid	–	Thymidine	–
D-galactonic acid lactone	–	Phenylethylamine	–
D-galacturonic acid	–	Putrescine	–
D-gluconic acid	–	2-aminoethanol	–
D-glucosaminic acid	–	2,3-buthanediol	–
D-glucuronic acid	–	Glycerol	–
α-hydroxybutyric acid	+	D,L-α-glycerolphosphate	–
β-hydroxybutyric acid	–	Glucose-1-phosphate	–
γ-hydroxybutyric acid	–	Glucose-6-phosphate	–

at 660 nm associated with cell growth and the degradation of TNT in the bench-scale bioreactor are shown in Fig. 1. One hundred milligram of TNT per liter was completely degraded within 4 days of incubation. The HPLC and GC-MS analyses revealed that 2-ADNT (2-amino-4,6-dinitrotoluene) and 4-ADNT (4-amino-2,6-dinitrotoluene) were the major intermediates formed following TNT degradation by strain OK-5. However, since their concentrations were miniscule and transient, the TNT degradation and parallel formation of 2-ADNT/4-ADNT were not plotted. Several researchers have already reported that two major metabolites, 2-ADNT and 4-ADNT, were detected during TNT degradation by certain bacteria, such as *Serratia*, *Pseudomonas*, and *Enterobacter* spp. [1,5,21]. However, few reports would seem to exist on TNT degradation by *Stenotrophomonas* species. *Stenotrophomonas* species have been intensively studied in the medical field because they are often found in patients suffering from diseases, such as leukemia and AIDS. Furthermore, *Stenotrophomonas* species have also been discovered in soil seriously contaminated with aromatic compounds necessary for the synthesis of herbicides, pesticides, plastics, explosives, etc for industrial, agricultural, or military purposes. Accordingly, *Stenotrophomonas* is becoming the main species of interest for many environmental microbiologists.

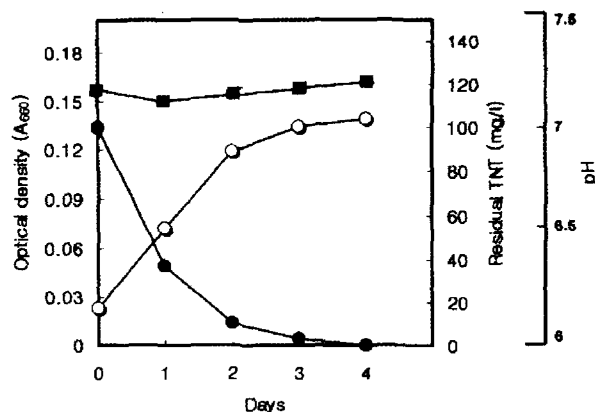

Fig. 1. Cell culture of strain OK-5 tested for its ability to degrade TNT as sole source of carbon and nitrogen. The cells were grown on a minimal medium containing 100 mg TNT, then the cell density was measured at 660 nm (○). The rate of TNT degradation (●) and change of pH (■) over the incubation time course of this experiment were determined in bench-scale bioreactors.

Effect of Supplemented Carbon and Nitrogen Sources on TNT Degradation

TNT degradation by strain OK-5 was studied in the presence of different supplemented carbon sources (Fig. 2). The supplemented carbon was found to stimulate the bacterial growth and TNT degradation in the current study. Strain OK-5 grew faster in the presence of fructose or molasses than in the presence of glucose, or citrate. Similarly, the degradation rate of TNT in the presence of molasses or fructose was 4-9 times higher than that in the presence of the other carbon sources tested. The molasses-fed culture took 4 days to degrade 100 mg/L of TNT and the fructose-fed culture degraded approx

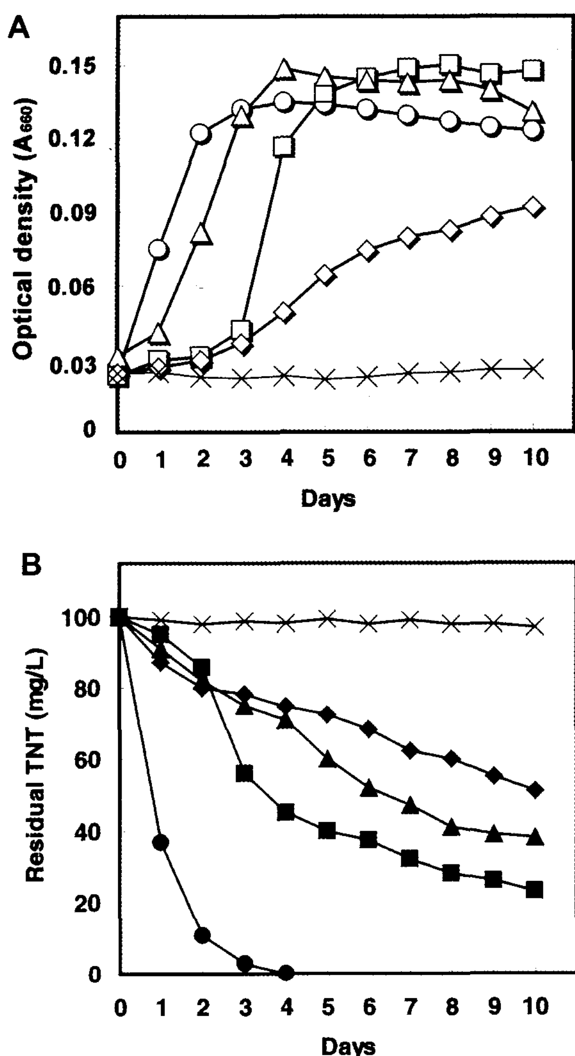

Fig. 2. Cell culture of strain OK-5 tested for growth (A) and TNT degradation (B) in presence of molasses (○,●), glucose (△,▲), fructose (□,■), or citrate (◇,◆) as supplementary carbon source in bench-scale bioreactors. No supplemented carbon was added to the medium (x,x).

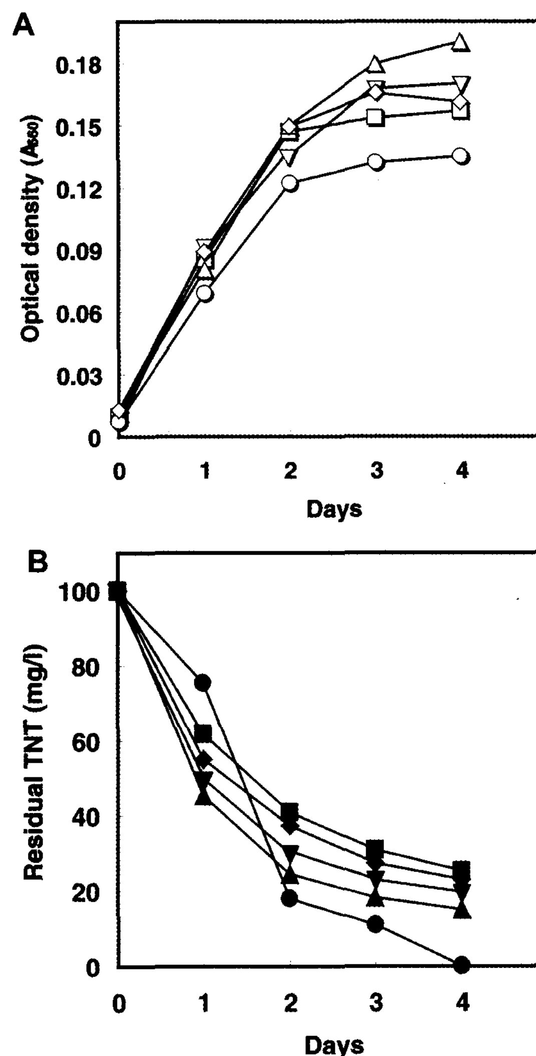

Fig. 3. Cell culture of strain OK-5 tested for growth (A) and TNT degradation (B) in presence of 2 mM $(NH_4)_2SO_4$ (◇,◆), NH_4Cl (□,■), KNO_3, or urea (△,▲) as supplementary nitrogen source in bench-scale bioreactors. No supplemented nitrogen was added to the medium (○,●).

76% of TNT at the same incubation time point, whereas the others required a longer time to degrade the same concentration of TNT. No growth was observed in the uninoculated controls and in the cultures where TNT was the sole source of carbon and nitrogen during the incubation period, even after 30 days of incubation. In these experiments, molasses had the greatest effect on the rate of TNT degradation by strain OK-5. Molasses, a byproduct of sugar production, is one of the cheapest sources of carbons. In addition to its large content of sugar, molasses also contains some unknown nitrogenous substances, vitamins, and trace elements [7]. Therefore, molasses could be effectively used in large-scale applications for the treatment of TNT-containing wastewater. Fig. 3 shows the effect of the supplemented nitrogen on the degradation of TNT by strain OK-5. The addition of various nitrogen sources, such as $(NH_4)_2SO_4$, NH_4Cl, KNO_3, or urea resulted in a slow rate of TNT degradation over the incubation period. Few studies have been published on the effect of supplemented nitrogen on the bacterial degradation of TNT. According to the previous report on the microbiological degradation of *s*-triazines, which is structurally related to TNT, the molar ratio of carbon-nitrogen (C-N ratio) was considered as one of the important factors for *s*-triazines degradation [14]. Hogrefe *et al.* [14] also reported that when the C-N ratio was 2, only minimal cell growth was observed with a slight degradation of *s*-triazines. However, when the C-N ratio was increased to 12, this enhanced the degradation of *s*-triazines. The degradation rate of *s*-triazines was not altered by additional carbon sources. Accordingly, these results indicate that the C-N ratio should be

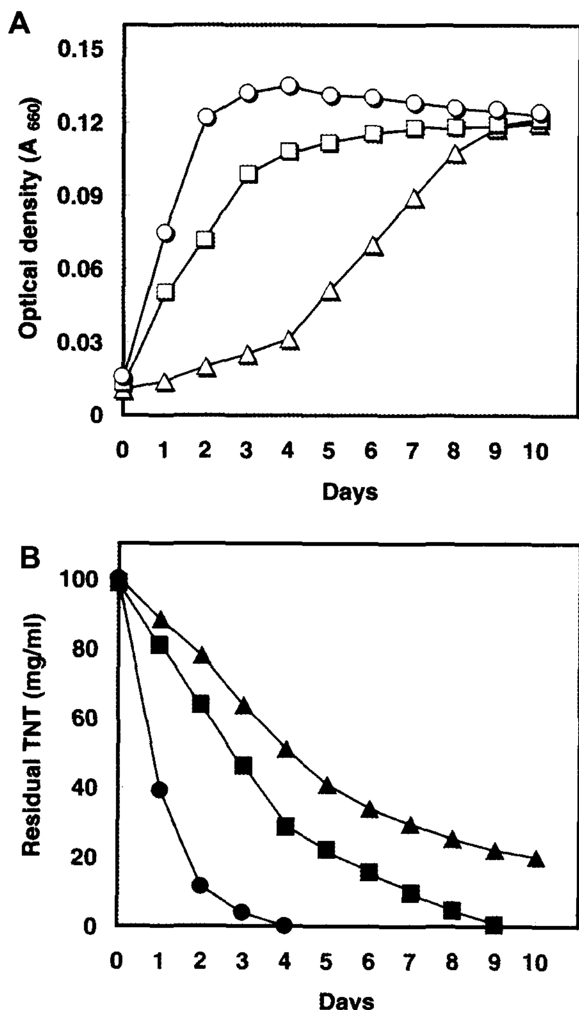

Fig. 4. Cell culture of strain OK-5 tested for growth (A) and TNT degradation (B) in absence of Tween-80 (△,▲), or presence of 0.5 mL Tween-80 (□,■) and 1 mL Tween-80 (O,●) in bench-scale bioreactors.

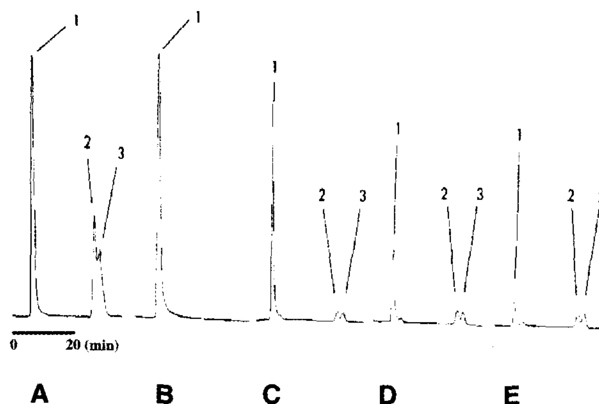

Fig. 5. HPLC chromatograms of standard mixture of TNT (peak 1), 2-ADNT (peak 2), and 4-ADNT (peak 3) (A). The bacterial cultures were centrifuged at the incubation timepoint of 0 days (B), 1 day (C), 2 days (D), and 3 days (E), respectively.

considered as a key factor in TNT degradation. However, a previous report by the current authors on aniline utilization by *Burkholderia* sp. HY1 showed that the rate of aniline degradation decreased as the amount of glucose increased [15]. As with the TNT used in the current study, aniline can also be used as both a carbon and nitrogen source by several microorganisms. The difference in the effect of secondary carbon sources on the rates of aniline- or TNT-degradation would seem to suggest that the TNT metabolism in strain OK-5 may be regulated by a different mechanism from that found in the aniline-degrading bacterium, strain HY1.

Effect of Tween 80

The effect of Tween 80 on the degradation of TNT was determined. The TNT was completely depleted after 4 days of incubation in the presence of Tween 80. However, without Tween 80, the TNT was only partially biodegraded (~85%) during up to 10 days (Fig. 4). Sabatini *et al.* [24] reported that bioavailability enhancement of apolar pollutants by added surfactants including Tween 80 was found to greatly improve the rate of biodegradation. As such, Tween 80 was used as a surfactant to promote the release of enzymes from the microorganisms and aid in the dispersion of TNT in the aqueous solution. Since many enzymes are located in the cell membrane, the identification of agents, such as Tween 80, that can increase the cell permeability efficiently while not impairing the enzyme system is of great importance [16]. Although it was not within the scope of the present work to determine whether or not the enzymes involved in the TNT degradation were located in the cell membrane, the current results did indicate that the addition of Tween 80 stimulated the TNT degradation in the media.

Detection of Intermediates by HPLC

The intermediates formed by the TNT degradation were detected based on authentic standard peaks resolved by HPLC. The chromatograms shown in Fig. 5 demonstrate that the initial substrate, TNT, and the expected intermediates, 2-ADNT and 4-ADNT, could be successfully resolved under these analytical conditions. The peaks obtained in the culture samples were in complete agreement with those of authentic standards. Plots of the concentration vs. the peak area for each of the three compounds displayed linearity within 0.5-100 mg per liter range. Accordingly, these results confirm that *Stenotrophomonas* sp. OK-5 was able to degrade TNT as a sole source of carbon and nitrogen.

Verification of Intermediates Using GC-MS

The MS data is shown in Figs. 5 and 6 for a culture sam-

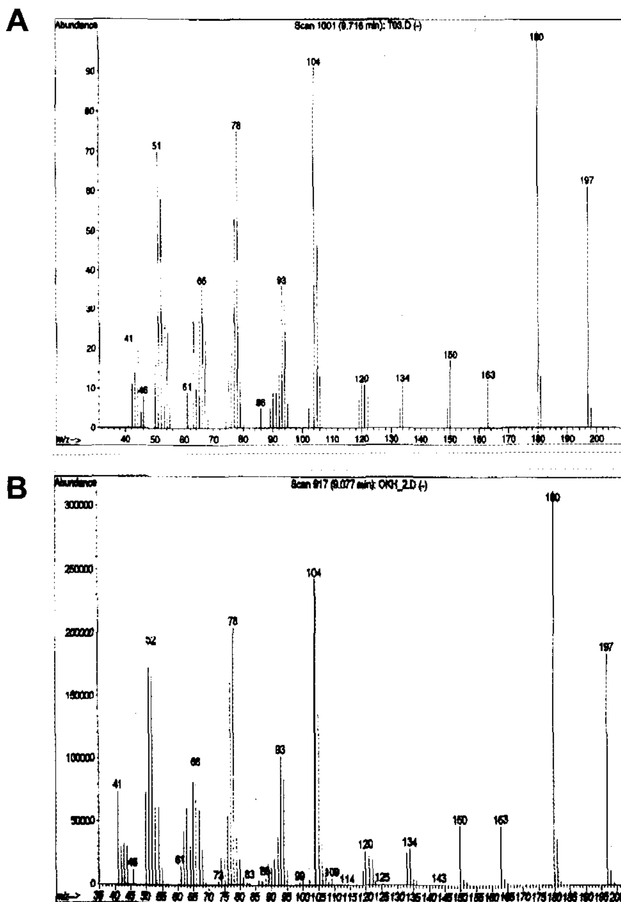

Fig. 6. MS analysis of authentic 4-ADNT (A) and bacterial culture sample (B). The fragmentation pattern (Rt = 9.916 min) and molecular ions (m/z = 197) obtained by the TNT degradation were consistent with those of authentic 4-ADNT.

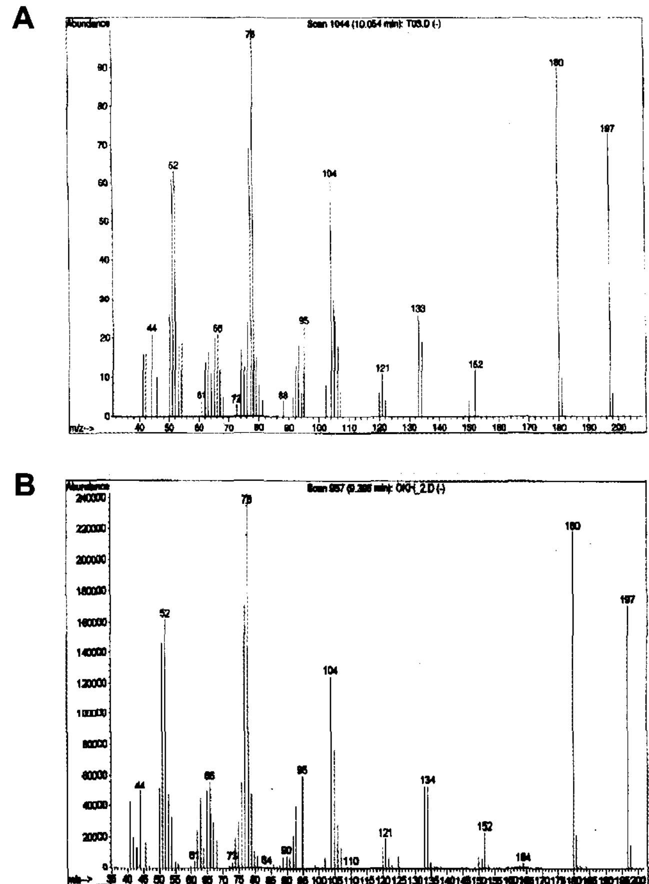

Fig. 7. MS analysis of authentic 2-ADNT (A) and bacterial culture sample (B). The fragmentation pattern (Rt = 10.054 min) and molecular ions (m/z = 197) obtained by the TNT degradation were consistent with those of authentic 2-ADNT.

ple analyzed after 2 days of incubation. The total ion chromatogram of this sample exhibited several major and minor peaks. Analyses of the major peaks obtained by MS identified 4-ADNT (9.916 min) and 2-ADNT (10.054 min) as the metabolites based on the mass/charge.

A small-scale system that completely degraded TNT in the newly developed media was also operated. This study showed that the soil bacterium *Stenotrophomonas* sp. OK-5 isolated from soil samples contaminated with TNT was able to rapidly degrade TNT as a sole source of carbon and nitrogen. The effective degradation of TNT was monitored to optimize the environmental factors, in particular, several supplemented carbon sources and Tween 80. Among the different supplemented carbon sources studies, molasses was found to produce the best result in terms of bacterial growth and TNT degradation, while Tween 80 had the greatest effect on the TNT degradation.

CONCLUSION

Stenotrophomonas sp. OK-5 was found to be capable of growth with TNT, and a special medium prepared for rapid TNT degradation was evaluated in a bench-scale bioreactor prior its application in effluent treatment. Further work will evaluate bioremediating TNT-contaminated soil using the same bacterium and investigate the treatment of wastewater containing TNT in different biosystems.

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[Received February 7, 2002; accepted April 16, 2002]