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Degradation of Explosive 2,4,6-Trinitrotoluene by *s*-Triazine Degrading Bacterium Isolated from Contaminated Soil

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2,4,6-Trinitrotolene (TNT) is a military explosive and a predominant contaminant of soil and ground water at many sites of TNT production and processing in many countries. TNT is recalcitrant and has been shown to be toxic to aquatic and terrestrial organisms in the environment (Won et al. 1976). TNT can be degraded, mineralized, or conjugated into more complex products by a variety of microorganisms (Lewis et al. 1997). The pathway of TNT degradation has been elucidated in several bacteria including *Serratia marcescens* (Mantpas et al. 1997). The most extensive degradation of TNT was exhibited by white-rot fungus, *Phanerochaete chrysosporium* (Fernando et al. 1990; Michels and Gottschalk, 1994; Stahl and Aust, 1995). TNT degradation in soils has been performed in most previous studies (Comfort et al. 1995; Knackmuss, 1996; Shelley et al. 1996; Hawari et al. 1997). Although information exits on the biochemistry and genetics of TNT degradation by microorganisms, the utility of this information has not been evaluated for possible treatment processes of industrial waste streams where TNT may represent a serious disposal problem.

In the present work, we explored the feasibility of using a bacterium for TNT degradation, with the ultimate aim of application for the industrial treatment. The bacterium was to determine the ability to degrade TNT under various experimental conditions. The formation of 2-ADNT (2-amino-4,6-dinitrotoluene) and 4-ADNT (4-amino-2,6-dinitrotoluene) as major intermediates of TNT degradation and their confirmation by HPLC and GC-MS are reported.

MATERIALS AND METHODS

The bacterium, designated as M91-3, originally isolated from an agricultural soil which had a previous history of annual atrazine application, was used in this work (Radosevich et al. 1995). The bacterium was maintained in liquid basal salts medium composed of (per liter) TNT, 100 mg; glucose, 0.35 g, K_2HPO_4, 1.75 g, NaH_2HPO_4, 0.6 g, $MgSO_4$, 0.25 g, and trace metals. The medium was adjusted to pH 7.2 before autoclaving. Subcultures were maintained with 10% inocula. The bacterium was incubated aerobically with shaking at 30°C for 28 days. Growth was monitored by following optical density at 660 nm and by microscopic examination.

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TNT was analyzed by HPLC (Shimadzu LC-10A, Japan) and GC-MS. For HPLC analyses, a Zorbax ODS reverse column (250 mm x 4.6 mm, particle size 5 µm) was eluted with a mobile phase which contained 20% (vol/vol) isopropanol and 80% water. The flow rate of the mobile phase was 1.0 ml/min. For GC-MS analyses, a centrifuged culture sample (8,000 x *g,* 20 min) was acidified to pH 3 with 6 M HCl, followed by extraction twice with equal volume of ethyl acetate. The solvent was removed under vacuum and the residue was redissolved in dichloromethane. MS data were obtained with 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.

Technical– and analytical-grade TNT were obtained from an explosive manufacturing Co. (S. Korea). Analytical-grade 2-amino-4,6-dinitrotoluene (2–ADNT) and 4–amino–2,6-dinitrotoluene (4-ADNT) were purchased from Absolute Standards (Hamden, CT, USA), and HPLC-grade isopropanol and water from Fisher Scinetific Co. (Fair Lawn, New Jersey, USA).

RESULTS AND DISCUSSION

The degradation of TNT was studied with the soil bacterium (designated as M91-3) which was initially enriched with atrazine, *s*-triazine herbicide. The isolate was obtained on plates containing solid media and TNT. Microscopic examination of the TNT-grown isolates revealed that all were Gram-negative and rod-shaped cells. Fatty acid analyses of isolate grown on Trypticase Soy Agar plates were performed to the MIS (Microbial Identification System), and the isolate indicated that the bacterium could be assigned to *Stenotrophomonas maltophilia.* In initial experiments, the degradation of TNT in basal salts media under aerobic conditions by *s*-triazine degrading bacterium, M91-3 was performed. The bacterium was able to degrade TNT as well as atrazine and simazine.

Change in turbidity associated with the biodegradation of TNT (100 mg/liter) is shown in Figure 1 for the culture M91-3. Complete depletion of TNT was achieved in this experiment within 28 days. 2-Amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) were detected as the major intermediates in the growth medium (Figure 18). Their concentrations were transient, reaching levels as high as 5.6 mg of 2-ADNT and 13.6 mg of 4-ADNT per liter.

To evaluate the effect of different TNT concentrations, the M91-3 culture was cultivated in the basal salts media with 25, 50, and 100 mg/liter of TNT. Complete degradation of TNT was proceeded, but TNT degradation was retarded with higher TNT concentrations in the culture (Figure 2). These results indicate that TNT is the substrate for the growth of microorganisms, but increasing TNT concentrations give the inhibitory effect because of its toxicity.

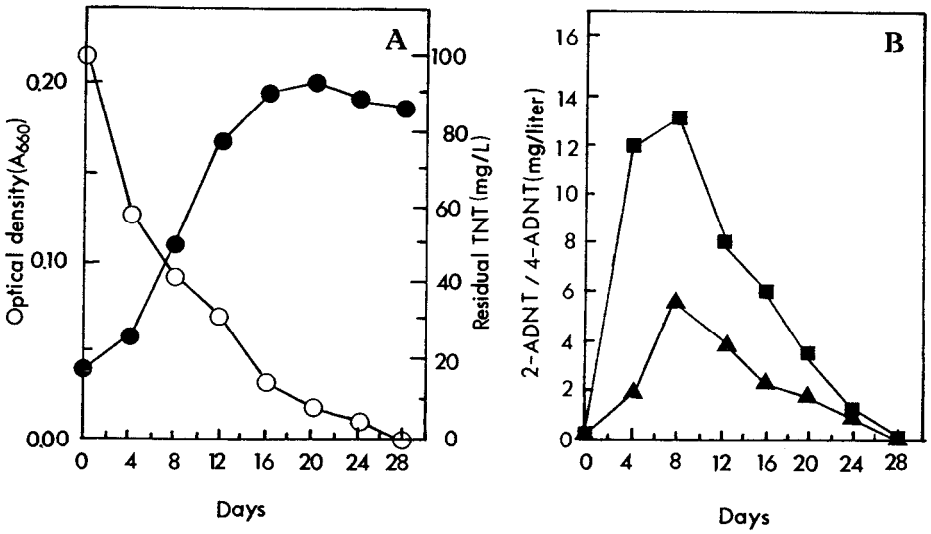

Figure 1. Growth of test culture, measured as cell density (○), associated with degradation of 100 mg/liter of TNT (●) (A), and the parallel formation of 2-ADNT (▲) and 4-ADNT (■) (B).

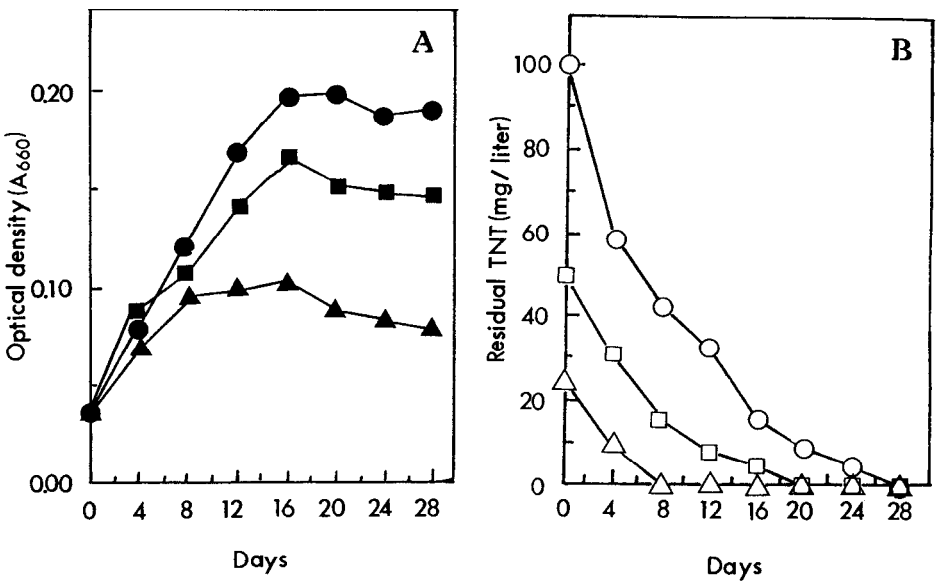

Figure 2. Time course of (A) growth and (B) degradation of TNT by test culture. The media contained 25 (▲, △), 50 (■, □), 100 (●, ○) mg TNT/liter.

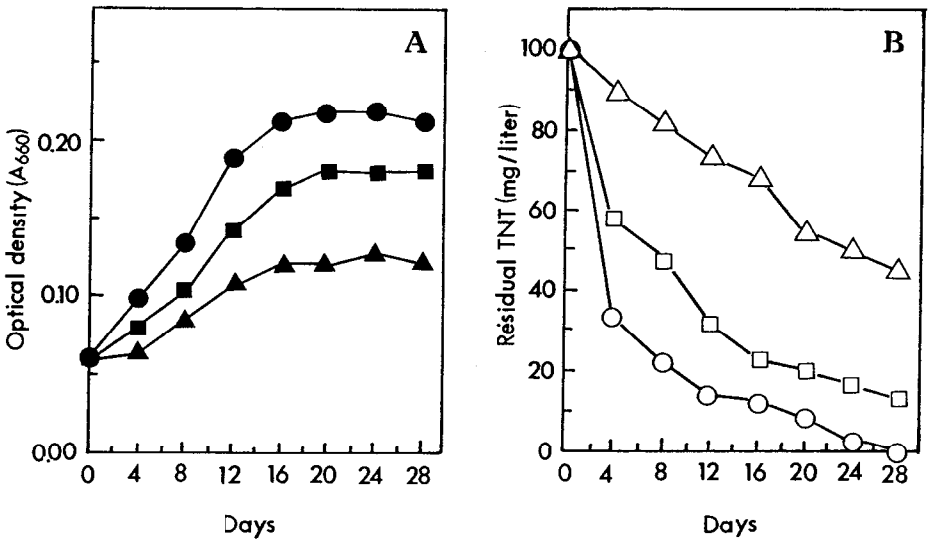

Figure 3. Time course of (A) growth and (B) degradation of TNT by test culture. The media were adjusted at pH 6.2 (▲, △), 7.2 (●, ○), or 8.2 (■, □) respectively.

The effect of pH on the degradation of TNT by M91-3 culture is shown in Figure 3. The optimal pHs for the degradation of TNT seem to be 7.2 or above pH. No residual TNT was detected at pH 7.2 and about 90% of TNT was utilized at pH 8.2 after 28 days of incubation. However only 50% of TNT degradation was observed at acidic condition (pH 6.2). These results suggest that microorganisms enriched from soil were adapted to the pH because the initial pH of soil collected from environment was around 7.2.

Figure 4 shows the effect of additives on the degradation of TNT by M91-3 culture. Addition of glucose stimulated the degradation of TNT and showed the complete TNT degradation within 28 days of incubation (Figure 4A). However no TNT degradation was observed without glucose. It suggests that glucose acts as a cometabolite for the degradation of TNT by M91-3. Effective degradation of TNT was monitored in the addition of fructose as a supplemental carbon source. Complete degradation of TNT with fructose was achieved within 16 days of incubation (Figure 4B). Tween 80 on the degradation of TNT is shown in Figure 4C. Complete depletion of TNT has achieved in this experiment within 8 days of incubation. This suggests that Tween 80 is a biosurfactant used to promote the release of enzymes from the microorganisms and the dispersion of TNT in the aqueous solution.

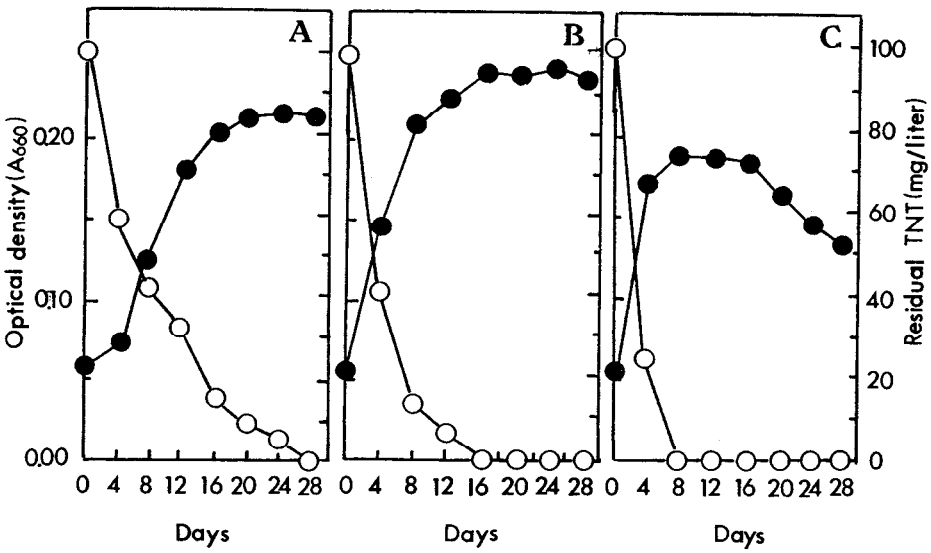

Figure 4. Time course of growth (●) and degradation (○) of TNT by test culture in media which contained glucose (A), fructose (B), and Tween 80 (C) as the additive.

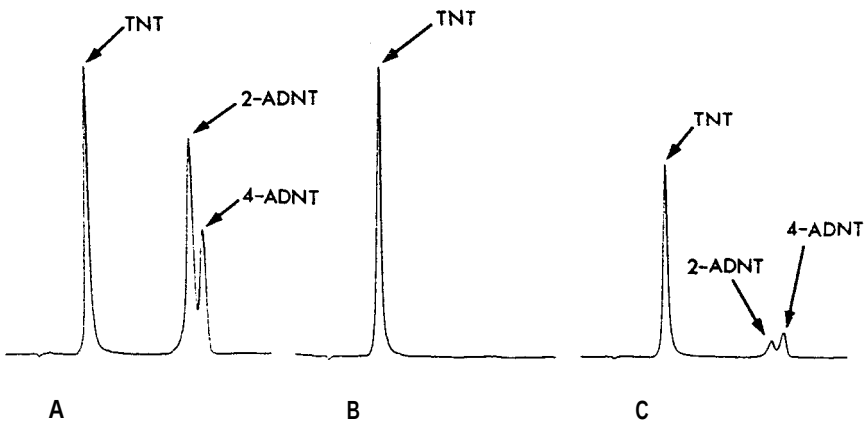

Figure 5. HPLC chromatograms of authentic standards of TNT, 2-ADNT and 4-ADNT (A), a supernatant of the test culture initially (B) and after 8 days of incubation (C). The retention times were 20.25 min for TNT, 33.97 min for 2-ADNT and 35.61 min for 4-ADNT.

The detection of the parent compound and its intermediates was based on HPLC methodology. The chromatograms shown in Figure 5 demonstrate that TNT and metabolic intermediates 2-ADNT and 4-ADNT can be successfully resolved under these analytical conditions. An authentic standard mixture had retention times (*Rt*) of 20.25 min for TNT, 33.97 min for 2-ADNT and 35.61 min for 4-ADNT in the HPLC chromatogram (Figure 5A). The three peaks obtained from the samples of culture supernatants were in complete agreement with those of authentic standards.

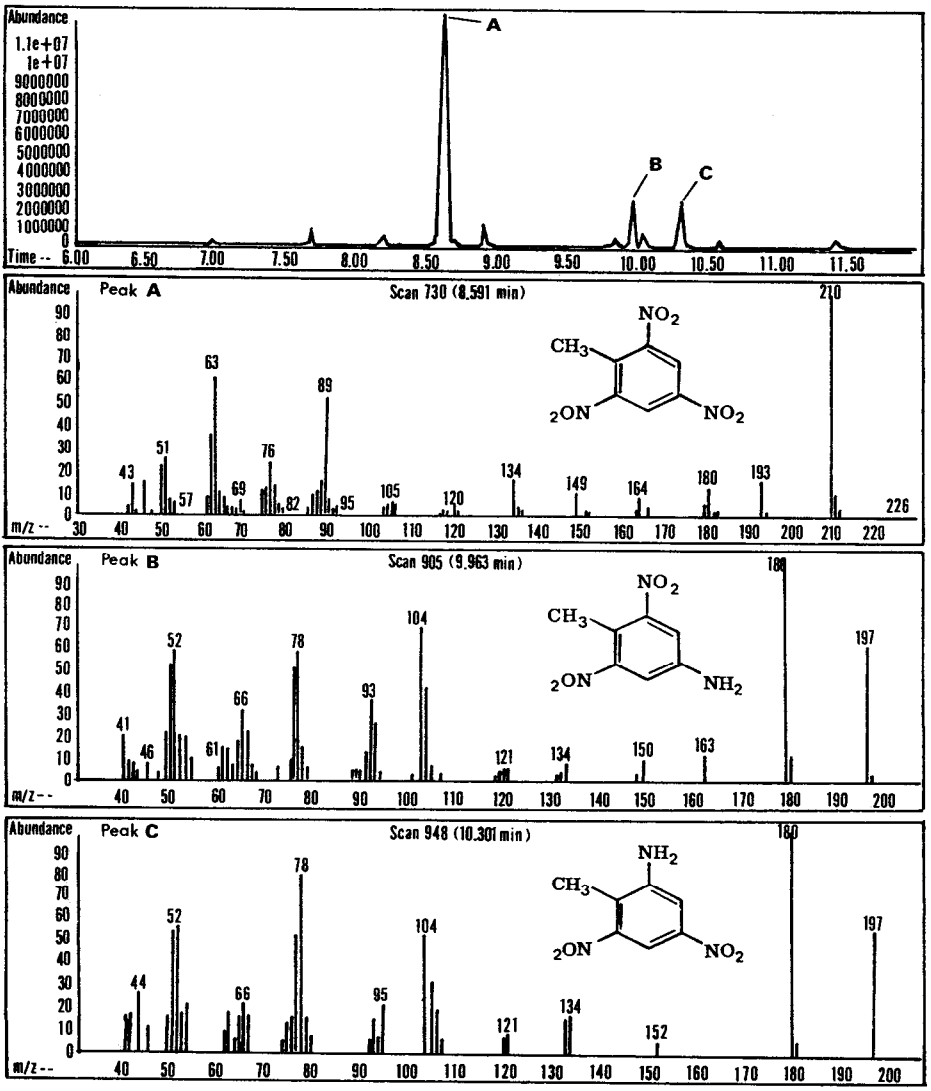

Figure 6. GC-MS data for a supernatant of test culture after 8 days of incubation. MS fragmentation patterns of the compounds representing the only three peaks (A, B and C) in the TIC are indicated and verified as TNT, 4-ADNT, and 2-ADNT in this work.

GC-MS data are shown in Figure 6 for a culture sample analyzed after 8 days of incubation. The total ion chromatogram (TIC) of this sample displayed four major and a few minor peaks. The major TIC peaks yielded positive identification based on mass/charge by MS for TNT (TIC peak A at 8.591 min), 4-ADNT (TIC peak B at 9.963 min), and 2-ADNT (TIC peak C at 10.301 min), These data are in keeping with the respective HPLC data shown in Figure 5C for this particular sample.

In conclusion, we established that the soil bacterium M91-3 is capable of aerobically degrading TNT. Effective degradation of TNT was achieved by the optimization of environmental factors - substrate concentration, pH, supplemental additives. Metabolic intermediates of TNT were detected and verified by HPLC and GC-MS. Further work will evaluate for bioremediating TNT-contaminated soil using the bacterium and investigate the wastewater treatment containing TNT in different biosystems.

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