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Enhanced Biodegradation of 2,4,6-Trinitrotoluene (TNT) with Various Supplemental Energy Sources

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Abstract The biodegradation of 2,4,6-trinitrotoluene (TNT) was performed on a laboratory scale using *P. putida* originally isolated from explosive-contaminated soil. One hundred mg/l of TNT was completely degraded within 20 h under optimum conditions. Various supplemental energy sources (carbon sources, nitrogen sources, and surfactant) were tested, with the main objective of identifying an inexpensive source and enhancing the degradation rate for large-scale biodegradation. Based on the degradation rate, molasses was selected as a possible supplemental carbon source, along with NH_4Cl and Tween 80 as a nitrogen source and surfactant, respectively. The degradation rate increased about 3.3 fold when supplemental energy sources were added and the degradation rate constant increased from 0.068 h^{-1} to 0.224 h^{-1}. These results appear to be promising in application of the process to TNT-contaminated soil applications.

Key words: Biodegradation, energetic materials, TNT, *Pseudomonas putida*

One of the major problems facing the industrialized world today is the contamination of soil, groundwater, and air with hazardous and toxic chemicals. Agricultural and industrial waste also introduce a wide variety of xenobiotic aromatic compounds into the biosphere [3]. In particular, 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are organic energetic compounds classified by the U.S. EPA as priority pollutants: TNT is a known mutagen and can cause pancytopenia as a result of bone marrow failure [1]. Oral LD_{50}s in rats are near 1 g/kg/day, and fatal cases of toxic jaundice and aplastic anemia were recorded among munitions workers during WWs I and II [17]. Mutatox and green alga bioassays have also confirmed TNT to be the most toxic of nitroaromatic explosives [7].

The number of contaminated sites where such compounds are found is increasing due to demilitarization activities and the disposal of obsolete munitions. TNT is the major explosive used by the military because of its low melting point, chemical stability, and low sensitivity to impact, friction, and high temperature. It is estimated that TNT alone is produced in amounts close to 2 million pounds a year and is now threatening human life through the food chain [19].

Several technologies have been evaluated for the treatment of TNT laden soils and sediments. Some have been demonstrated in the field, while others are still being tested on a bench and laboratory scale. Such technologies include incineration, composting, chemical oxidation, adsorption, and bioremediation. Incineration is the most effective and widely used method as a remediation alternative, but this method is expensive due to the costs involved in soil excavation, transport, and energy incineration [14]. The limitation related to composting is the maintenance of optimum environmental conditions for biological activity. In addition, large amounts of compost materials are mixed with only about 10% waste. The chemical oxidation of explosive waste is often difficult because of the need to contain any unreacted materials. Plus, problems with adsorption include the retention of untreated nitrocompounds on the granular activated carbon, incomplete degradation of TNT, and the requirement for added nutrients. Therefore, recent researchers have focused on less expensive alternative technologies, such as bioremediation. The treatability of a toxic compound by biological means is dependent on the compound being biodegradable. Although TNT is persistent in the environment, it is still susceptible to microbial attack. The biological removal of TNT and other explosives has

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already been proven to be feasible [5, 10]. Traditionally, many researchers on the bioremediation of TNT have involved anaerobic bacteria and fungi [8, 9], whereas studies exploring the capabilities of aerobic bacteria have begun only recently to appear in literature [5, 12].

Accordingly, the purpose of the current research was to develop a practical way to use *Pseudomonas putida* to bioremediate TNT-contaminated soil and identify an inexpensive supplemented energy source for the industrial scale treatment of explosive-contaminated soil. An attempt was also made to enhance the biodegradation process and reduce the biodegradation time by the addition of a surfactant.

An enrichment culture was developed from the explosives-contaminated soil using Stanier's Medium [2, 16]. Succinate (0.5%) and TNT were then added to the medium. To confirm the degradation of TNT, the bacteria were cultured in a modified heterotrophic medium (MHM) [5] at pH 7.0 containing 100 mg/l of TNT and a supplemental energy source. The following supplemental carbon sources were used (0.5%): acetate, asparate, citrate, glucose, glycerol, malic acid, molasses, pyruvate, and succinate. The supplemental nitrogen sources were KNO_3 and NH_4Cl (0.25 g/l). The culture flasks were incubated on a shaking incubator (150 rpm) at 30°C under aerobic conditions (Certomat BS-1, B. Braun Biotech International, Melsungen, Germany).

The culture turbidity was measured by the absorbance at 600 nm using a Uvikon XS spectrophotometer (Bio-Tek Instruments, Milano, Italy). The residual TNT was analyzed using high performance liquid chromatography (HPLC). The samples were prepared by mixing 0.7 ml sample with 0.7 ml acetonitrile. The mixture was then vortexed and centrifuged at 3,000 rpm for 5 min and the supernatant filtered through a 0.45 μm PTFE syringe (Gelman, MI, U.S.A.). The filtrate was used for the HPLC analysis of the TNT by a Varian liquid chromatograph equipped with a model 230 ternary gradient pump, model 410 autosampler, data module, and model 310 UV-VIS detector set at 254 nm. The mobile phase was methanol:water (50:50, v/v) and 10 μl samples were injected onto a Restek C-18 column at 30°C. The flow rate of the solvent was 1.0 ml/min. The concentration of TNT was measured using standard TNT.

P. putida was isolated from soil contaminated with explosives. The bacterium has been found to grow well in enriched media, such as a tryptic soy broth. However, since enriched media are too expensive for industrial operations, an alternative cost-effective medium is required. Various supplemental carbon and nitrogen sources were examined as possible energy sources, and the growth with various energy sources was monitored by measuring the absorbance at 600 nm. Thus, *P. putida* was cultured in a MHM with various supplemental carbon sources and TNT of 100 mg/l. Growth of microorganisms was observed with various supplemental carbon sources, yet no growth was observed in the killed control. Figure 1 shows the degradation of TNT with the various supplemental carbon sources after 30 h. Normally, the aromatic ring is not cleaved and degradation to CO_2 does not occur. However, the current study showed that *P. putida* isolated from explosive-contaminated soil caused an extensive transformation of TNT in a reasonably short period of time. All the supplemental carbon sources, except for asparate, biodegraded over 90% of the TNT within 30 h. In particular, molasses was found to be a good supplemental carbon source, and it is one of the cheapest sources of carbon. The composition of molasses is very complex (containing 30% glucose and

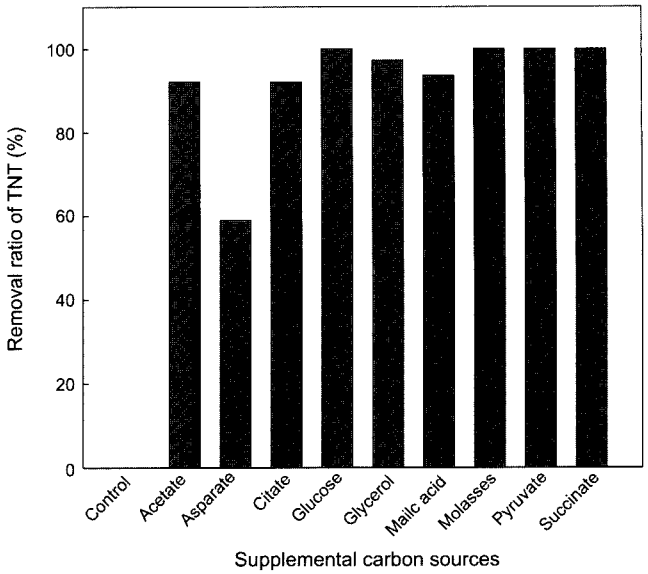

Fig. 1. Performance of bacterial cultures with different supplemental carbon sources.

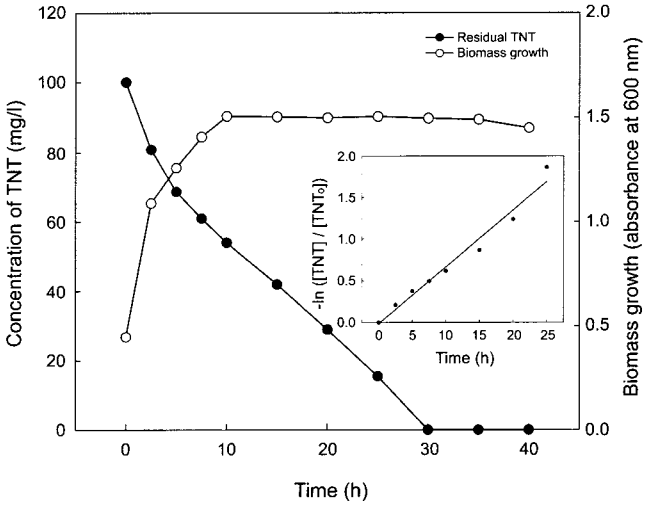

Fig. 2. Degradation of TNT and growth of *P. putida* with molasses.

43% sucrose in the current study, etc.), making it conducive to the growth of many types of bacteria.

Figure 2 shows the biodegradation of TNT and growth of microorganisms when molasses was added. In this case, the TNT was completely degraded within 30 h. This biodegradation of TNT followed first-order kinetics, therefore, the reaction rate constant was calculated from the equation (1). Considering the first-order reaction relationship, the reaction rate constant, $k_{obs,\,TNT}$, was then calculated from the slope value of the plot t vs $-\ln(TNT/TNT_0)$.

$$-\ln\frac{[TNT]}{[TNT]_0}=k_{obs,\,TNT}\cdot t \quad (1)$$

where $[TNT]_0$, [TNT], t, and $k_{obs,\,TNT}$ represent the initial concentration of TNT, concentration of TNT at time t, reaction time, and observed first-order reaction rate constant for the biodegradation of TNT, respectively.

The biodegradation efficiencies were measured based on changes in the TNT concentration. In this case, the biodegradation rate of TNT was found to follow the first-order relationship and the reaction rate constant was 0.068 h^{-1} (r^2=0.97).

Figure 3 shows the effect of the supplemental nitrogen sources on the biodegradation of TNT by *P. putida*. The addition of KNO_3 and NH_4Cl led to an increase in the degradation rate and reaction rate constants to 0.073 h^{-1} (r^2=0.97) and 0.091 h^{-1} (r^2=0.97), respectively. The removal rate of TNT with NH_4Cl as supplemental nitrogen source was about 1.3 times faster than that without it. Therefore, this result seems to suggest that the C-N ratio should be considered as an important factor in the biodegradation of TNT.

Figure 4 shows the growth of *P. putida*, degradation of TNT, and reaction rate constant, when a surfactant (Tween

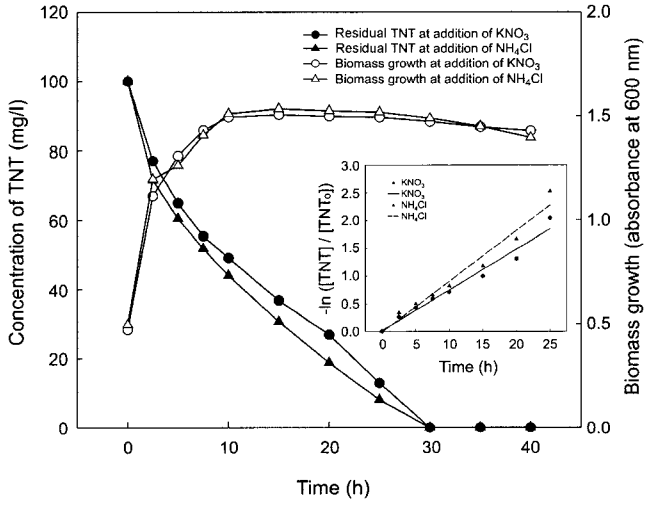

Fig. 3. Degradation of TNT and growth of *P. putida* with nitrogen sources.

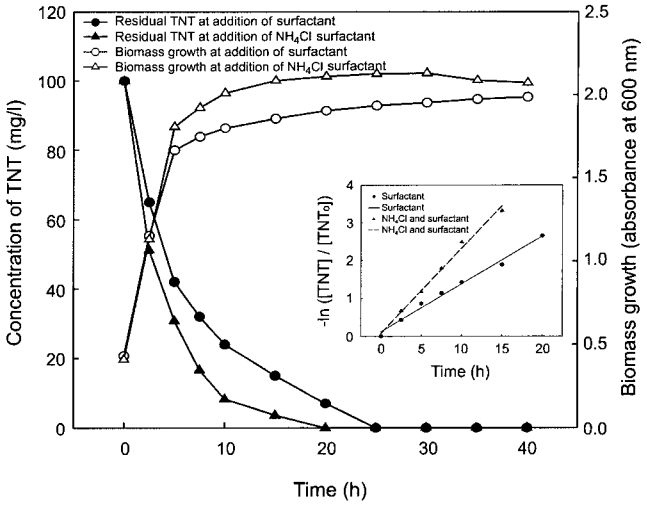

Fig. 4. Degradation of TNT and growth of *P. putida* with surfactant.

80) was included with and without NH_4Cl. The TNT removal rate was the fastest with both NH_4Cl and the surfactant, and the TNT was completely degraded within 20 h. Boopathy and Manning [4] previously reported that the bioavailability enhancement of pollutants by the addition of Tween 80 greatly improved the rate of biodegradation. Other studies have also shown that biological systems dosed with a surfactant exhibit enhanced rates of contaminant degradation [6, 18].

In the current study, the reaction rate constant was 0.224 h^{-1} (r^2=0.99) under optimum conditions. Because of NH_4Cl and a surfactant, the degradation rate constant was about 3.3 times higher than that in Fig. 2. Therefore, these results indicate that a surfactant can be used to promote the release of enzymes from the microorganisms and aid in the dispersion of TNT in the reactor.

In addition, surfactants contain a hydrophobic group that distorts the structure of water, thereby increasing the free energy in the system. Due to this increase in free energy, surfactants concentrate at the surface or interface and orient their hydrophobic group to reduce the free energy in the system. As a result, the surfactant reduces the interfacial free energy, thereby reducing the resistance to mass transfer. Furthermore, the surfactant can reduce the free energy by orienting the hydrophobic groups internally to form micelles, into which the contaminant is partitioned. This partitioning can increase the contaminant concentration in the aqueous phase above its solubility limit. Consequently, there are two primary mechanisms whereby surfactants can reduce the free energy of a system, i.e. adsorption at the interface and micelle formation [11, 13, 15].

Accordingly, the current study identified optimum conditions for the biodegradation of TNT in a laboratory scale test by using the molasses and the surfactant. These

results appear to be promising in the application of the process to explosive contaminated soil and ground water.

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