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TNT and RDX Degradation by Cell-Free Extracts of *Clostridium acetobutylicum*

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The U.S. Army generates large volumes of explosives-contaminated wastewater during munitions production and demilitarization operations. Contaminants such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are environmentally hazardous and must be removed from the wastewater.

This research examined the transformation of TNT and RDX in cell extracts of *Clostridium acetobutylicum*. *C. acetobutylicum* was grown in thioglycollate medium at 37 °C. Cells were harvested by centrifugation and frozen in 1-gram packets at -80 °C for later use. Cells (100 mg) were placed in 30-ml serum bottles and incubated anoxically under an N_2 headspace in phosphate buffer containing lysozyme. Following lysis, TNT was added to the lysed cells. Periodically the contents of the serum bottle were analyzed for TNT and the formation of TNT degradation intermediates. TNT was completely degraded in 60 minutes without observing any TNT reduction products in the cell extract supernatant. The addition of pyruvate to the serum bottles increased the TNT degradation rate 10-fold. RDX was completely degraded in 1-g cell extracts after 60 minutes, but not in 100-mg cell extracts. No TNT degradation and <25 percent RDX degradation occurred in cell extracts incubated aerobically. These studies indicate that cell extracts of *C. acetobutylicum* are capable of degrading TNT and RDX.

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Foreword

This study was conducted for the Construction Engineering Research Laboratory (CERL) by The Nitrate Elimination Company, Inc., under 611102BT25 "Environmental Research COE," work unit J09, "Bacterial Enzymes Involved in the Biodegradation of Explosive and Nitroaromatic Compounds." The technical reviewer was Stephen J. Maloney, Environmental Processes Branch (CN-E).

The work was performed by The Nitrate Elimination Company, Inc., for CN-E, which is part of the Installations Division (CN) at CERL. The CERL Principal Investigator for this research was Neal Adrian. Ellen Campbell is Vice President and Chief Operating Officer for the Nitrate Elimination Company, Inc. (Lake Linden, MI). Dr. Ilker R. Adiguzel is Chief, CN-E, and Dr. John Bandy is Chief, CN. The technical editor was Linda L. Wheatley, Information Technology Laboratory.

The Director of CERL is Dr. Michael J. O'Connor.

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1 Introduction

Background

The U.S. Army generates large volumes of wastewater contaminated with explosives through munitions production operations (load, assemble, and pack) and demilitarization or washout operations. This wastewater is commonly referred to as pinkwater. Pinkwater typically contains 2,4,6-trinitrotoluene (TNT), which is photochemically active, resulting in the color giving pinkwater its name (Concurrent Technologies 1995). Pinkwater is the highest volume hazardous waste generated by the U.S. Army Industrial Operations Command, which produced 77 million pounds of pinkwater in 1998.

Hazardous contaminants such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are also found in pinkwater; however, this research focused on TNT. TNT and its metabolites represent an environmental hazard because they exhibit considerable toxicity toward humans, fish, algae, and microorganisms (Rieger and Knackmuss 1995). Because of this toxicity, the explosive compounds and related nitroaromatic compounds must be removed from pinkwater. The standard technology used by Army ammunition plants for treating pinkwater is carbon adsorption (Concurrent Technologies 1995). Unfortunately, this generates a secondary hazardous waste, carbon loaded with adsorbed explosives, which must be either regenerated for reuse or disposed of by incineration.

The Army is investigating alternative technologies for treating pinkwater, including biological treatment (Concurrent Technologies 1995). Recent studies with a fluidized bed granular activated carbon bioreactor for treating TNT were promising (VanderLoop et al. 1998). The success of this study prompted a 1-year demonstration of a pilot-scale fluidized bed reactor for treating pinkwater at the McAlester Army Ammunition Plant in McAlester, OK. The evaluation demonstrated the cost effectiveness of using a biological system rather than carbon adsorption. Furthermore, the reactor system for treating pinkwater was reliable and consistently obtained removal efficiencies >99 percent for TNT.

Biochemistry of Degradation of Nitroaromatic Compounds

The transformation and degradation of TNT by microorganisms has been well characterized (Myers and Townsend 1997; Lewis et al. 1996; Won et al. 1974). In aerobes or facultative anaerobes, the partial reduction of TNT is catalyzed by nitro reductase, which appears to be flavoprotein (Bryant and DeLuca 1991).

In anaerobes, a more complete degradation of TNT can be achieved (Preuss and Rieger 1995). The degradation of TNT to products such as triaminotoluene (TAT) has been accomplished with a crude cell extract of *C. acetobutylicum* using pyruvate as a metabolite to drive the reduction process (Hughes, Rudolph, and Bennett 1997). This process is considered a reductive degradation of TNT since it was carried out under anaerobic conditions and no oxygen is available to drive an oxidative process. It has been suggested that this reduction may involve the enzyme hydrogenase and the electron carrier ferredoxin or flavodoxin (Preuss and Rieger 1995). However, the process catalyzed by hydrogenase does not lead to complete TNT destruction and another enzyme is proposed to participate. In strict anaerobes such as *Clostridia*, which completely reduce TNT to TAT, there does not appear to be a specific enzyme for carrying out this series of reactions. The steps involved in the conversion of diaminonitrotoluene (DANT) to TAT, processes that do not occur in facultative anaerobes, are the formation of a hydroxylamine group followed by its reduction to the amino group to yield TAT. The conversion of DANT to diaminohydroxylaminotoluene (DAHAT) intermediate is catalyzed by the "ferredoxin-forming enzymes" such as hydrogenase (driven by hydrogen gas) and CO-dehydrogenase (driven by carbon monoxide). Since the DANT conversion to DAHAT can be catalyzed by ferredoxin alone after chemical reduction, the ferredoxin-forming enzymes do not appear to be specific catalysts for the nitro group reduction. It is possible that this chemical reaction involves a nitro radical group and rearrangements of the partially reduced intermediates. However, it should be noted that the rate of the reaction is 10 times greater in the presence of the ferredoxin-forming enzymes.

The final step, in which DAHAT is reduced to TAT, is not well understood. No enzyme has been isolated for catalyzing this final step. However, since whole cells of *Clostridia* catalyze the conversion of DANT to TAT, there is likely to be an enzyme responsible for catalyzing this last step in the reduction process. The reductant used will most likely be an iron-sulfur protein such as ferredoxin, but this has not yet been demonstrated. It appears that more research is needed to clarify how this last step is carried on in the anaerobic bacteria. See Spain (1995) for a recent exposition on the TNT reduction pathway.

Objectives

The objective of the current work was to begin characterization of the degradation of TNT and RDX catalyzed by enzymes from *Clostridium spp.* The overall goal is to develop an enzymatic reactor that will degrade TNT, RDX, and HMX and that is more cost effective than carbon adsorption. The advantages of an enzyme-based reactor as opposed to a reactor using live cells are that the process can be more stringently controlled, the addition of a carbon cosubstrate is not required, and the reactor effluent would contain a lower biological oxygen demand because no biomass is produced during the operation of the reactor. Enzymatic degradation of TNT and other nitroaromatic explosives has the potential to be the method of choice for treating groundwater and wastewater contaminated with explosives. The first step toward such an enzyme-based reactor for treating explosives contaminated wastewater (pinkwater) is described in this report.

Approach

A literature review was conducted of laboratory studies investigating TNT degradation. The review indicated that *Clostridia spp.* are particularly adept at degrading explosives, including TNT, and were good candidates for further study of the enzymes degrading explosives.

Clostridium acetobutylicum was obtained from a commercial culture collection. Cells were grown, harvested, and frozen at -80 °C until used. Cell extracts were obtained by lysing cells anoxically under an N_2 headspace in 30-ml serum bottles. RDX or TNT was added to the cell extract. The cell extract supernatant was assayed for RDX, TNT, and TNT-reduction products by high pressure liquid chromatography (HPLC). The results were analyzed and conclusions drawn based on the results of this stage of work.

Mode of Technology Transfer

Findings from this research will be incorporated into subsequent studies and into ongoing Exploratory Development (6.2) work in treatment of munitions wastewater.

2 Materials and Methods

Chemicals

TNT and RDX were obtained from Chem Service, Inc., West Chester, PA. An analytical standard (Standard A) containing TNT and related nitroaromatic compounds was obtained from PolyScience, Niles, IL. The mixture contained 1,3,5-trinitrotoluene, 1,3-dinitrobenzene, nitrobenzene, 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene, and 2- , 3-, and 4-nitrotoluene. All compounds were at a concentration of approximately 2 mg/ml. A second mixture (Standard B from Accu-Standard, New Haven, CT) contained 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, tetryl, RDX, and HMX. All compounds in the Standard B mixture were at a concentration of approximately 1 mg/ml. All other chemicals used were reagent grade or better, and purchased through Research Organics, Cleveland, OH, or Fisher Chemical, Pittsburgh, PA. Biochemicals were purchased from Sigma Chemical Company. Cells were grown in Difco thioglycolate media, purchased from Fisher Chemical.

Source and Growth of Cultures Used for Enzymatic Degradation Studies

Three species of *Clostridium* — *C. acetobutylicum*, *C. histolyticum*, and *C. rubrum* — were obtained from Presque Isle Cultures, Presque Isle, PA. Growth curve information was obtained under microaerobic growth conditions. *C. acetobutylicum* was chosen for experiments because of its ease of growth and literature citations describing its TNT-degrading properties (Lewis et al. 1996; Hughes, Rudolph, and Bennett 1997).

Uniform batches of *C. acetobutylicum* cells for enzymatic studies were grown microaerobically at 37 °C in closed, 1-l media bottles, in standard thioglycolate medium (yield 36 g cells/l culture). Cells were harvested in air (via centrifugation) in late log phase, frozen at -80 °C in 1-g packets, and stored for later use.

Analytical Methods

HPLC analysis of TNT and its metabolites was performed based on a method developed by Waters Corporation, Milford, MA (Winslow, Weichert, and Baker 1991; Bouvier, Steriti, and Oehrle 1994). This method was chosen for its simplicity, low toxicity of mobile phase, and ease of sample preparation. HPLC assays were performed using a Waters 600 pump, Rheodyne injector, and Waters 410 fixed wavelength detector operated at 254 nm. Researchers used a Waters Nova-Pak C8 column, 3.9 by 150 mm, with guard column. The mobile phase was water/isopropanol 82:18, at a flow rate of 1.0 ml/min. A 20-µl injection loop was used throughout. Early experiments were quantified by measuring peak height (Kipp and Zohnen strip chart recorder) — a Waters Data Module Integrator 730 was used for later experiments. Separation and quantitation for both Standards A and B mixtures are quite good. The limit of detection in this system was 125 ng TNT per ml (approximately 0.5 µM), and 250 ng/ml (approx. 1.0 µM) for RDX. Sensitivity for metabolites varies within this range.

Standards of the DANT isomers were not obtained for this project. However, the analytical method used is similar to other published methods for nitroaromatics shown to be effective for these compounds (e.g., Walsh and Jenkins 1990), and it is possible that these metabolites can be detected with this method. When a similar reverse phase column and buffer system is used, with the exception that acetonitrile is the organic component of the mobile phase, the diamino compounds (which are less polar and more hydrophilic) elute before the TNT and other metabolite peaks, near the void volume. No such peaks were observed in the chromatograms described here.

Sample preparation was performed using Waters Porapak RDX Sep-Pak cartridges. These cartridges enable simple, reproducible extraction and concentration of nitroaromatic and nitramine explosives and many of their degradation products. Use of these cartridges made sample preparation for HPLC analysis easy and reproducible. In addition, since the total reaction mixture is processed, samples were uniform, reducing sampling errors. Cartridges were used as directed by company product literature. Briefly, each cartridge (one per sample or standard) is fitted with a disposable 10 ml "slip-tip" syringe and washed with 9 ml of HPLC-grade methanol followed by 9 ml of deionized water (d-H_2O). Next, the sample (centrifuged to remove cell debris) or standard was made to a total volume of 10 ml (using a buffer consisting of 150 mM potassium dihydrogen phosphate (KH_2PO_4)/0.3 mM ethylenediamine tetra-acetic acid (EDTA), pH 7.5). It was applied to the cartridge via syringe, followed by a wash step of 9-ml d-H_2O. Finally, the nitroaromatics are eluted from the cartridge with 9 ml of HPLC-grade methanol. The sample is ready for direct analysis by HPLC. Once

the samples are in the methanol stage, they can be stored for later analysis — repeat assays showed no change of metabolite concentration over 2 wk. Samples were analyzed on the same day as the experiment in some cases and, after it was determined that sample stability was adequate, 1 to 5 days after the experiment.

Centrifuged cell extracts processed through these cartridges can be analyzed by HPLC without any further preparation. Because the purpose of this project was to study enzymatic degradation of TNT with the goal of eliminating TNT and its toxic metabolites, no further efforts were made to identify compounds other than by comparison of retention time of peaks in purchased Standards A and B. No DANT isomer standards were tested in this system, so it cannot be stated with certainty that they were not produced.

Preparation of Enzyme Abstract From *C. acetobutylicum* for Degrading TNT

The specified amount of thawed cells (10, 100, or 1000 mg) is placed into a 30-ml serum bottle on ice. To these cells is added 1.0 ml of ice-cold buffer (150 mM potassium phosphate buffer/0.3 mM EDTA, pH 7.5) containing 0.6 mg of lysozyme. Bottles are kept on ice to prevent lysis from occurring until anaerobic conditions are obtained. Anaerobic conditions were created in each reaction vessel using a refrigerated trap and vacuum pump with digital vacuum gauge for evacuation, followed by purging with standard purity N_2 gas (99.998 percent nitrogen; oxygen in the range of 10 ppm), repeated three times and left under a nitrogen atmosphere. Gas and vacuum are introduced by a needle piercing the sidewall of the stopper, which in turn is attached to tubing with a "Y" fitting. One end of the "Y" goes to the N_2 tank and the other to the vacuum source. A vacuum of 75 to 150 millitorr is reached for each bottle, followed by 6 to 8 seconds of purging with the nitrogen.

Once all the bottles have been treated, they are placed on a orbital shaker (ambient temperature, ~ 25 °C) and incubated for 15 min, with gentle agitation, to lyse cells. Lysozyme concentration required for complete cell lysis was determined by measuring protein release (Bio-Rad assay) until endpoint. The aerobic control bottle is treated exactly as the samples, except that it is treated with lysozyme in the presence of air (i.e., it is not put through the evacuation and gassing procedure). Following the 15-min lysis period, TNT or RDX solution is added as described in the next section.

Procedure for Enzymatic Degradation of TNT

Following the 15-min cell lysis incubation described above, 1.8 ml of 25 µM (5.65 mg/L) TNT is added — final TNT concentration in the vessels is 15 µM (3.39 mg/L). For "assisted" reactions, 0.2 ml of 15 mM pyruvate (final concentration of 1.0 mM) is also added. Additions are made from "anaerobic" stocks prepared by an evacuation and nitrogen gassing procedure similar to that used for sample bottle preparation, using gastight syringe transfers to maintain the nitrogen atmosphere in the reaction bottle. Bottles are mixed to homogeneity, then incubated at room temperature (~25 °C) on a rotation platform at approximately 40 rpm for specific lengths of time (ca. 10 or 15 min). Two controls are included in every experiment. The first is a zero time point, in which air is permitted to enter the reaction bottle immediately after addition of the TNT (or RDX) solution. Second is an aerobic control, in which cells are lysed in the presence of air, and incubated with the same RDX, TNT, or TNT/pyruvate ("assisted") solution as the experimental samples, while the anaerobic experimental samples are processed. In all cases the aerobic control is permitted to react for as long as the longest time point in any particular experimental condition. This step is critical to proving that an anaerobic enzymatic degradation process is the reason that the nitroaromatic compounds are disappearing from the reaction mix. Reactions are terminated by opening the stoppers, allowing oxygen into the bottle. Total reaction volume is 3 ml.

At the end of the reaction period, each serum bottle is opened and the reaction supernatant is recovered from cell debris by centrifugation. Supernatant (approximately 3 ml volume) is brought up to 10 ml with d-H_2O and applied to an RDX Sep-Pak cartridge. The cartridge is washed with 9 ml of d-H_2O, then eluted with 9 ml of HPLC-grade methanol. A 20-µl sample of this eluate is analyzed by HPLC.

3 Results and Discussion

Optimization of Cell Growth

The standard growth conditions and protocol for the *C. acetobutylicum* cells used in all experiments were optimized during the first 3 months of the project. The protocol adopted was:

- Day 1 – a screw cap tube containing 9.0 ml of thioglycolate media is inoculated with 1 ml of stock culture, which is stored at 4 °C.
- Day 2 – 1.0 ml of the first tube is used to inoculate a second 10-ml culture tube.
- Day 3, after 24 h – the contents of this culture tube are used to inoculate 100 ml of the same media in a 250-ml screw cap bottle.
- Day 4 – contents of the Day 3 stage are used to inoculate a 1-l bottle of media. This step is timed so that cell harvest can occur 12 h after inoculation.

Average yield from this protocol is 28 to 35 g of cells (wet cell weight). These cells are recovered by centrifugation and washed twice in a phosphate buffer before being frozen at -80 °C in 1-g packets in Saran Wrap for later use.

Development of Experimental Protocols

Use of the Waters RDX Sep-Paks enabled direct HPLC analysis of the lysed-cell reaction mixtures. This saved time and also avoided potential loss of target compounds during the extensive sample processing required by alternative methods for recovery of analytes from complex matrices, such as lysed cell extracts. Validation of the Sep-Pak technique used for sample preparation and HPLC analysis of TNT and metabolites in cell extract was done by spiking studies. Lysed cell extract was spiked with TNT at a concentration of 1 mM, processed through a Sep-Pak cartridge, and analyzed by HPLC. This procedure was repeated for both Standards A and B. Recovery was reproducible at ~65 percent. Recovery of compounds is 90 percent from stock solutions of standards and 65 percent from lysed-cell extracts as determined by spiking cell extracts with known amounts of standard TNT/metabolite solutions (see Figure 1).

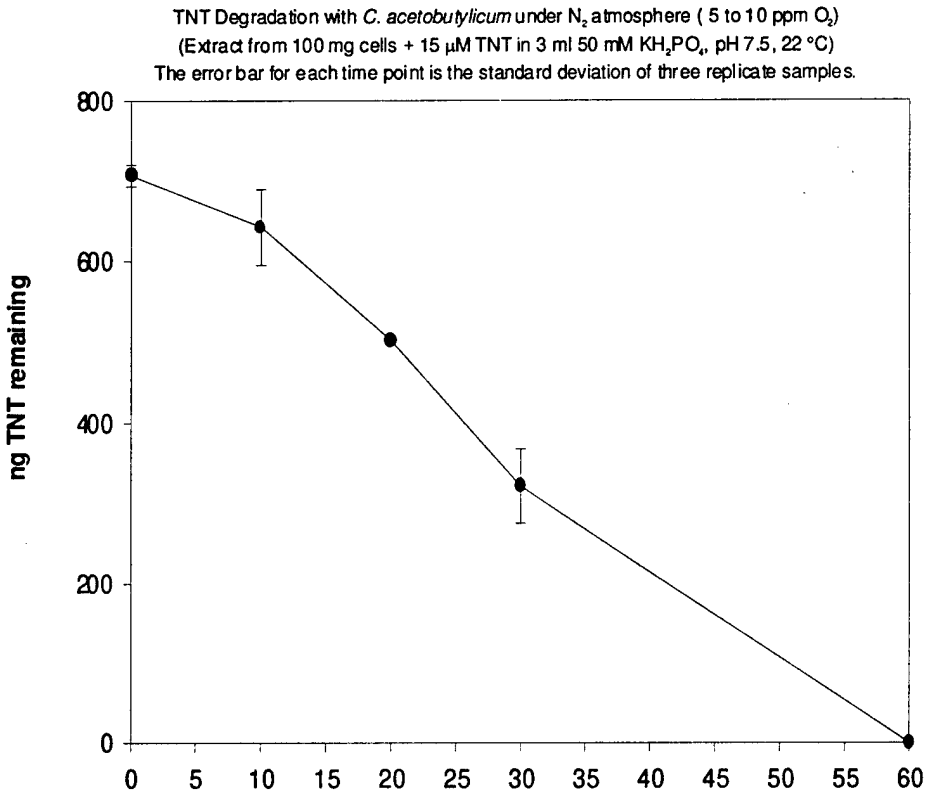

Figure 1. TNT degradation in serum bottles by cell extracts of *C. acetobutylicum* incubated under anaerobic conditions.

The next target in development of the standard experimental protocol was to demonstrate that an anaerobic environment could be maintained in the reaction vessels (the 30-ml serum bottles) for the entire course of the experiment. This was done using 1 mM of methyl viologen (MV) as an indicator. MV was added to the stoppered bottles, which were evacuated and purged (three times) with N_2. Using a gastight syringe, 50 µl of a 200-mM solution of the reducing agent sodium dithionite (also known as sodium hydrosulfite) was added. MV shows as a deep blue color in an anaerobic environment; the color disappears in the presence of oxygen. The reaction protocol developed for this study is capable of maintaining an oxygen-free environment in the serum bottles for at least 12 h.

In the early experiments, an attempt was made to maximize initial TNT concentration in each reaction bottle in order to enhance concentration and detection of any metabolites or degradation products. A stock solution of TNT at ~1 mM was used in these experiments. One gram of cells was used for the experiments with the high TNT concentration. The control bottles contained all reagents, but were not evacuated and gassed with N_2; these are called aerobic controls in the protocols.

Next, to enhance the degradation of TNT, a series of experiments were done with added MV and dithionite (sodium hydrosulfite) to add reducing capacity to the mixture. Table 1 shows degradation of TNT over time, with and without added dithionite reductant.

Table 1. Recovery of TNT in cell extracts of *C. acetobutylicum*.

Reaction Contents	Buffer (1.25 ml)	1 mM* TNT (1.75 ml)	1 g lysed cell extract	200 mM dithionite (200 µl)	1 mM methyl viologen	%TNT recovered
Bottle 1	X	X				100
Bottle 2	X	X		X		0
Bottle 3	X	X	X			19
Bottle 4	X	X	X	X		0
Bottle 5	X	X	X	X	X	0

Note: Cell extracts were placed in 30-ml serum bottles, brought to a total volume of 3.0 ml, and incubated under anoxic condition containing a nitrogen atmosphere.

* 1 mM = 226 mg/L

Table 1 shows nearly 100 percent recovery of TNT from Bottle #1. No TNT or breakdown products were seen in Bottle #2. Bottle #3 showed that TNT degradation was occurring, with only 19 percent recovery of the initial TNT added. Again no peaks were seen for the contents of Bottles #4 and #5, which demonstrated total disappearance of TNT and all detectable nitroaromatic metabolites in less than 1 min (the time it took to stop the reaction); no detectable compounds were present in the anaerobic zero time point. Unfortunately, TNT appears to be abiotically degraded by dithionite, an observation previously reported with other reducing agents (Budavari 1996). This is clearly not a desirable mechanism for destruction of TNT on any large scale, so experimentation with formulations containing dithionite was terminated. However, this experiment clearly demonstrated that the lysed cells are able to degrade TNT in an anaerobic environment based on Bottle #3, which had no dithionite. It should be noted that 1 mM TNT, the concentration used in the above experiments, is greater than the solubility of TNT in water, so that recovery of nondegraded TNT was not completely reproducible in experiments using this TNT concentration.

The next step was to refine the protocols so that no ambiguity remained regarding the issue of TNT disappearance. Solubility is approximately 0.01 percent (100 mg/l, or 0.44 mM) in water at 25 °C, but concentrations this high require boiling for total dissolution of the TNT, and even this concentration tends to precipitate over time. Since various temperatures were used during the procedure, including an incubation on ice, it became clear that it was critical to determine a TNT concentration that would stay in solution under all experimental conditions and over time. It was determined using the HPLC assay that 25 µM (5.65 mg/L)

TNT dissolves in water or buffer at room temperature with stirring, and stays in solution at 0 °C, is stable in solution for more than 7 days. This concentration is well within the detection limits of the method used for this study. Therefore, stock solutions of 25 µM (5.65 mg/L) TNT were prepared and used in all subsequent experiments. Volumes remained constant (1.8 ml of the TNT solution added to a total reaction volume of 3.0 ml), so that TNT concentration in the serum bottles for subsequent experiments was 15 µM (3.39 mg/L).

Table 2 synopsizes experiments done using these standard conditions. The first experiment showed complete degradation of TNT within seconds of the reaction initiation. The amount of cells used in the reaction was therefore reduced from 1 g to 100 mg. This resulted in complete disappearance of TNT in 60 min. Figure 1 shows the results of this experiment. Detection of degradation products is limited by the sensitivity of ultraviolet (UV) detection; however, only trace amounts of any compound can be seen at 0.005 absorbent units full scale (AUFS), and no traces elute at the retention time of any of the metabolites analyzed in the method used. The detection limit for TNT in the HPLC method used for this study is approximately 125 ng/ml (0.5 µM); limits for metabolites vary within the range of 0.5 to 10.0 µM. While neither standard mixture used contains either of the DANT compounds or TAT, Standard B did contain 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene — neither of these compounds accumulated in any of these experiments. In all experiments, TNT concentration in the aerobic controls remained unchanged, showing that anaerobic enzymatic reactions are the cause of TNT transformation.

Figure 2 shows the data in an alternate way, as micrograms (µg) of TNT remaining per gram of lysed (extracted) cells per unit time. Data are taken from a typical experiment (22 October 1998).

Table 2. TNT degradation by lysed *C. acetobutylicum* cells.

Experiment date 1998	TNT (µM)	Gram cells	Aerobic control %TNT left	0 min %TNT left	10 min %TNT left	15 min %TNT left	20 min %TNT left	30 min %TNT left	60 min %TNT left
10/19	6	1.00	0	ND				ND	
10/20	15	1.00	62	ND		ND			
10/22	15	0.10	95	96	81		67	39	0

Note: Cell extracts were placed in 30-ml serum bottles, brought to a total volume of 3.0 ml, and incubated under anoxic condition containing a nitrogen atmosphere.
ND = not determined

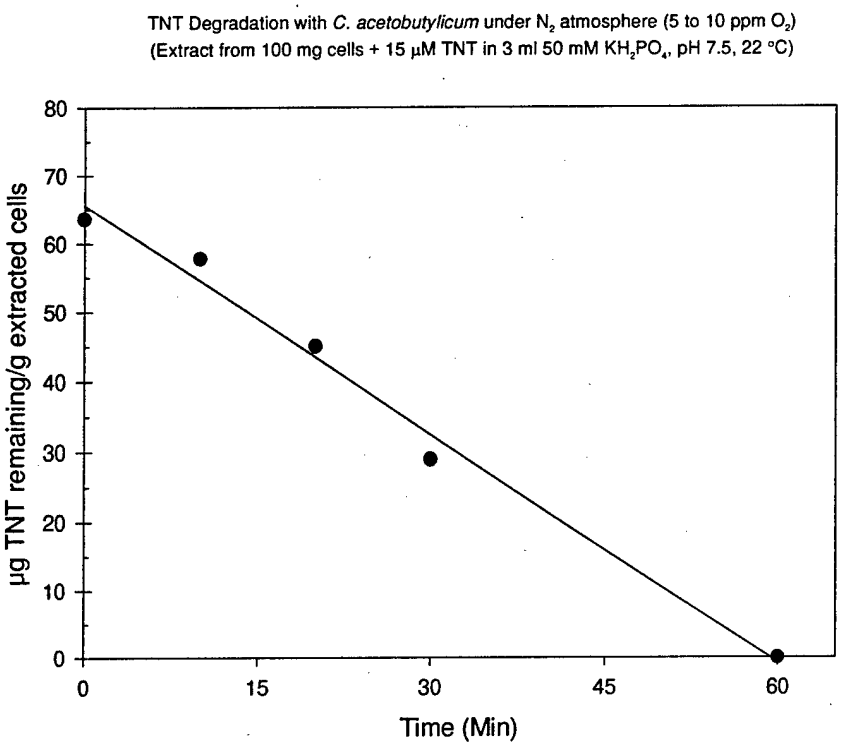

Figure 2. TNT degradation rate normalized to 1-g cell extracts of *C. acetobutylicum*, incubated in serum bottles under anaerobic conditions.

Metabolically Driven TNT Degradation by *Clostridium* Cell Extract

The TNT degradation by *C. acetobutylicum* cell extracts described in the last section was carried out with no added reductant. In the next set of experiments, pyruvate was tested as an additive to drive production of reductant using metabolic enzymes in the extract. These are termed "assisted assays." In the initial experiment of 26 October 1998, TNT was totally degraded within 10 min (see Table 3). No TNT remained after 10 min, and no metabolites were observed.

Table 3. Pyruvate-assisted TNT degradation results.

Experiment date 1998	Gram cells	Aerobic control %TNT left	0 min %TNT left	10 min %TNT left	20 min %TNT left	30 min %TNT left	45 min %TNT left	60 min %TNT left	Metabolites
10/26	0.100	85	27	0	ND	ND			No
10/27	0.010	100	92	16	70	69			Trace
11/04	0.010	100	97	71*	ND	61	30	ND	Trace

Note: Cell extracts were placed in 30-ml serum bottles, brought to a total volume of 3.0 ml, and incubated under anoxic condition containing a nitrogen atmosphere. Pyruvate at 1 mM added to accelerate reaction.

ND = not determined

*Sample taken at 15 min.

The amount of cells was further reduced to 10 mg per serum vial, and the experiment was repeated. This resulted in decrease of TNT over time, with possible metabolites detected at the mid-range time points. TNT and metabolites are entirely absent from the chromatogram in the 60-min sample. The experiment was repeated, and similar results were obtained. Table 3 summarizes these three experiments.

In Figure 2, degradation of TNT without added pyruvate is shown. Figure 3 compares the results of these conditions with the assisted assays in which 1 mM pyruvate was added. The rate of TNT degradation in Figure 3 is 1.1 µg TNT degraded/min/g of lysed *C. acetobutylicum* cells. The rate in the assisted experiment, with pyruvate, is 12.1 µg TNT degraded/min/g cell extract. Addition of pyruvate to the system therefore enhances the rate of degradation by a factor of about 10.

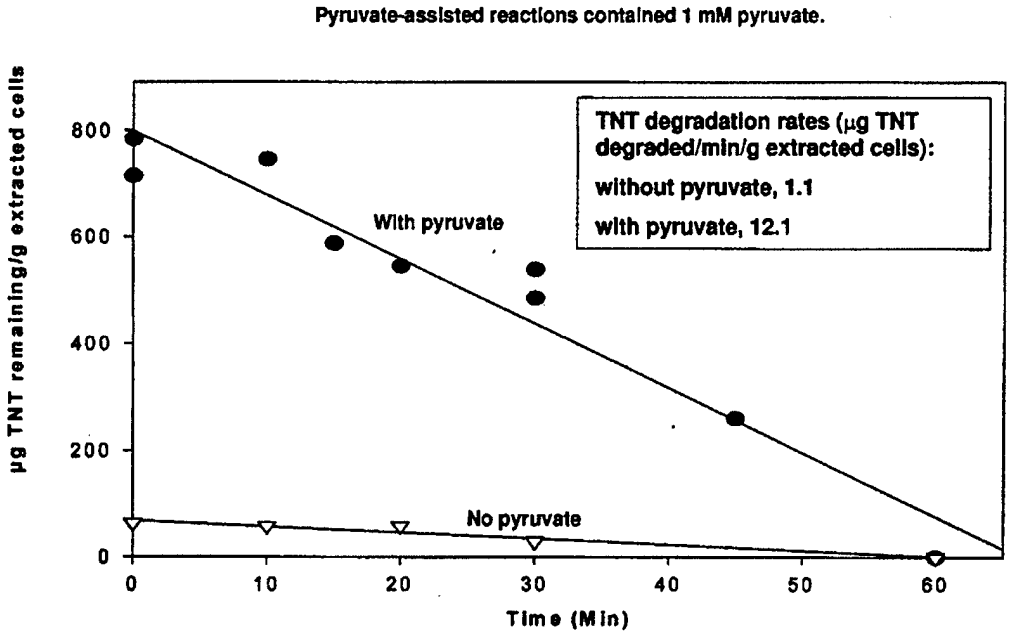

Figure 3. Pyruvate-assisted TNT degradation by *C. acetobutylicum*.

RDX Degradation by *C. acetobutylicum* System

Finally, researchers wished to determine whether the same system might potentially be used for remediation of RDX. Table 4 summarizes these experiments. In the first experiment to determine whether RDX is reduced by the *C. acetobutylicum* cell extracts lysed-cell system, stringent conditions using 10 mg of cells but with pyruvate added did not show any degradation of RDX (1.5 µM [0.332 mg/L] in the reaction bottles) over 1 h. These experiments were repeated using 1

g of cells. Time permitted only one experimental run. However, as seen in Table 4, complete disappearance of RDX was attained within a 60-min reaction period. Detection limit for RDX in the HPLC assay was approximately 25 ng/ml (0.1 µM). These interesting results should be pursued in later experiments.

Table 4. Degradation of RDX by lysed *C. acetobutylicum* cells.

Experiment date 1998	Initial RDX Conc. (µM)	Gram cells	Aerobic control %TNT left	0 min %RDX left	10 min %RDX left	15 min %RDX left	20 min %RDX left	30 min %RDX left	60 min %RDX left
10/19	1.5	0.01	99	78	95	80	96	98	
10/20	1.5	1.00	78	61	ND	ND	ND	ND	0*

* Two samples.

ND = not determined; Conc. = Concentration

Note: Reaction is less efficient for RDX than for TNT. Pyruvate at 1 mM added to accelerate reaction. Cell extracts were placed in 30-ml serum bottles, brought to a total volume of 3.0 ml, and incubated under anoxic condition containing a nitrogen atmosphere.

4 Conclusions

This study showed that lysed cells of *C. acetobutylicum* can efficiently transform TNT. In the most striking results, 10 mg of lysed cells completely destroyed 45 nanomoles of TNT, with no detectable levels of metabolites, within 60 min. This is a much faster rate than has been reported in the literature or in CERL reports describing TNT biodegradation with intact microbial systems (Hwang, Chow, and Adrian 1998).

Degradation of RDX using the lysed cell system was also demonstrated. Future experiments might determine whether the system is also effective for HMX.

The long-term goal of this approach is an immobilized enzyme reactor system for removing TNT, RDX, HMX, and their harmful metabolites from water. The results obtained in this study are quite favorable to this goal. The straightforward sample preparation and HPLC analytical methods adopted for this study provided a dependable means for analysis of varying byproducts and metabolites of TNT degradation, RDX, and HMX, even from complex matrices such as lysed cell extracts. This method eliminated uncertainties in evaluation of results that can result from variance in extraction procedures. These methods also enabled experiments to be evaluated easily and in a timely manner.

Additional studies are needed to determine whether the DANT metabolites are created by the *C. acetobutylicum* lysed-cell system, but the preliminary results are favorable. Two samples were analyzed for TAT formation using an alternate HPLC method, and none was detected.

This study has shown that the rate of TNT degradation by extracts of *C. acetobutylicum* and supported by pyruvate is quite high. Since pyruvate is rather expensive and may be too labile (unstable) a biochemical for use on an industrial scale, it may be desirable to determine in a future study if hydrogen can be used as an effective replacement in bench and larger scale reactor systems.

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Abbreviations/Acronyms

AUFS	absorbent units full scale
CERL	Construction Engineering Research Laboratory
DAHAT	diaminohydroxylaminotoluene
DANT	diaminonitrotoluene
d-H_2O	deionized water
EDTA	ethylenediamine tetra-acetic acid
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high pressure liquid chromatography
KH_2PO_4	potassium dihydrogen phosphate
MV	methyl viologen
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
TAT	triaminotoluene
TNT	2,4,6-trinitrotoluene
UV	ultraviolet

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13. ABSTRACT *(Maximum 200 words)*

The U.S. Army generates large volumes of explosives-contaminated wastewater during munitions production and demilitarization operations. Contaminants such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are environmentally hazardous and must be removed from the wastewater.

This research examined the transformation of TNT and RDX in cell extracts of *Clostridium acetobutylicum*. *C. acetobutylicum* was grown in thioglycollate medium at 37 °C. Cells were harvested by centrifugation and frozen in 1-gram packets at -80 °C for later use. Cells (100 mg) were placed in 30-ml serum bottles and incubated anoxically under an N_2 headspace in phosphate buffer containing lysozyme. Following lysis, TNT was added to the lysed cells. Periodically the contents of the serum bottle were analyzed for TNT and the formation of TNT degradation intermediates. TNT was completely degraded in 60 minutes without observing any TNT reduction products in the cell extract supernatant. The addition of pyruvate to the serum bottles increased the TNT degradation rate 10-fold. RDX was completely degraded in 1-g cell extracts after 60 minutes, but not in 100-mg cell extracts. No TNT degradation and <25 percent RDX degradation occurred in cell extracts incubated aerobically. These studies indicate that cell extracts of *C. acetobutylicum* are capable of degrading TNT and RDX.

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