Journal of Energetic Materials
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713770432

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To cite this Article Pal, Nirupam and Christodoulatos, Christos(1995) 'Fungal degradation of 2,4-Dinitrotoluene and nitroglycerin in batch and fixed-film bioreactors', Journal of Energetic Materials, 13: 3, 259 — 282
To link to this Article: DOI: 10.1080/07370659508019388
URL: http://dx.doi.org/10.1080/07370659508019388

FUNGAL DEGRADATION OF 2,4-DINITROTOLUENE AND NITROGLYCERIN IN BATCH AND FIXED-FILM BIOREACTORS

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ABSTRACT

Energetic materials, which are the main components of propellants and other explosive mixtures, are characterized as hazardous and must be disposed in an environmentally sound way. Microbially mediated destruction of energetic chemicals shows great promise for converting these substances to innocuous products. A number of fungal species namely *Phanerochaete chrysosporium*, *Penicillium corylophilum*, *Aspergelus fumigatus*, and *Geotricum candidum* have the ability to attack energetic materials and completely mineralize them into carbon dioxide and water. This paper presents results on the biodegradation of 2,4-Dinitrotoluene and nitroglycerin by *P. chrysosporium* in completely mixed batch reactors and in continuous flow immobilized bed systems. The fungus was able to degrade both compounds and the observed intermediates in the presence of a co-substrate.

Journal of Energetic Materials Vol. 13, 259-282 (1995)
Published in 1995 by Dowden, Brodman & Devine, Inc.

INTRODUCTION

Due to recent demilitarization activities in the United States, Eastern Europe, and the former Soviet Union, a large quantity of propellants, explosives, and pyrotechnics (PEP) are to be disposed in an environmentally safe manner.[3] The major constituents of PEP are nitroaromatics and nitroaliphatics such as nitrocellulose, nitroglycerin (NG), trinitrotoluene (TNT), 2,4-Dinitrotoluene (2,4-DNT), 1,3,5-triazo-135-trinitrocyclohexane (RDX), 1,3,5,7-tetraazo-1357-tetranitrocyclohexane (HMX) etc. The manufacturing, cleaning and repackaging of explosives requires large quantities of water which become contaminated during processing. For years, explosive-contaminated wastewater (pink water) was discharged on the ground or in lakes resulting in a widespread soil and groundwater contamination at ordinance manufacturing and storage facilities. The current method of disposal of mutition wastes such as rocket and gun propellants, is Open Burning/Open Detonation (OB/OD).[7] Due to the problems associated with production of NO_x, alternative chemical and biological treatment technologies to OB/OD are currently under investigation.[3,6] Microbially mediated decomposition of these compounds has produced encouraging results at the bench-scale and pilot-scale levels and may become the technology of choice for the treatment of certain types of energetic.

Experiments conducted in our laboratory using *P. chrysosporium* in batch and continuous flow reactors showed that the fungi cannot utilize 2,4-DNT or NG as

sole carbon sources and a co-substrate is required to induce production of the enzymes responsible for the decomposition of the energetic compounds. The produced intermediates of both 2,4-DNT and NG are also biodegradable and the compounds are completely mineralized.

DEGRADATION OF 2,4-DNT

The nitroaromatic compound 2,4-DNT, a priority pollutant, is a precursor for the manufacturing of 2,4,6-trinitrotoluene and polyurethane foam, and extensively used in manufacturing of propellants and explosives.[4,5] Wastes from these manufacturing processes contain 2,4-DNT which is found to be toxic to various forms of life including mammals.[5] Approximately 9% of the organic content of pink water comes from 2,4-DNT.[4]

The potential of *P. chrysosporium* to decontaminate explosives-contaminated soil has been documented in the literature.[6] Valli *et al.*[11] reported a mechanism for complete mineralization of 2,4-DNT by *P. chrysosporium.* After 24 days of incubation, 34% of the substrate was mineralized, as recorded by radiolabeled $^{14}CO_2$. Three intermediates namely 2-amino 4-nitrotoluene, 4-amino 2-nitrotoluene, and 2,4-diaminotoluene were identified, with 2-amino 4-nitrotoluene showing the highest yield. Furthermore, 1,2-dimethoxy-4-nitrobenzene and 2,4-diaminotoluene were identified as the metabolites for 2-amino-4-nitrotoluene. Subsequently, these intermediates are methylated to methoxy compounds and to

trihydroxy benzene before getting degraded via the ketoadipic pathway. Two enzymes namely lignin peroxidase (LiP) and manganese peroxidase (MnP) are involved in the initiation of the reaction. The intermediates are further degraded by the mycellium or by mycellium bound enzyme(s).

The major drawbacks for the utilization of this fungal culture for commercial applications are (i) its slower degradation rate compared to bacterial processes; (ii) the high dependability of the degradation rate on nutrient conditions, the optimum nutrient conditions are not known; (iii) its sensitivity to shear stress; and (iv) incomplete information on the mechanism of degradation. Recently, Pal *et al.*[7] have shown that in case of trichlorophenols, the degradation rate can be substantially increased using a continuous reactor system where the shear stress effects can be eliminated and a continues supply of energy can be maintained. These researchers have shown that the optimal carbon to nitrogen ratio (C:N) varies between 60 to 80, and the volumetric flux is around 1.0 mL/(cm^2xmin). Recent studies showed that the first step in the mineralization process is initiated by extracellular enzymes or free radicals forming a complex.[1] The complex is subsequently mineralized by biomass to innocuous products.[1]

Although a number of studies have been performed on the biodegradation of 2,4-DNT using both bacterial and fungal cultures, the objectives of these studies were focused on the microbial or biochemical aspects of the degradation process rather than engineering design and implementation of the technology. A series of

studies were performed by the authors with the objective to maximize the degradation rate of 2,4-DNT by manipulating the process parameters and by optimizing the reactor configuration. The studies performed in this work are discussed below.

P. chrysosporium (ATCC 24725) was obtained from the American Type Culture Collection (ATCC) and maintained on yeast malt agar. The fungus was grown in growth medium (Table I), in Erlenmeyer flasks on a gyratory shaker at 30°C.

TABLE I
Composition of Growth Medium and Induction Medium

Components	Growth Medium	Induction Medium
Glucose	6.0 g	0.5-0.9 g
KH_2PO_4	2.0 g	2.0 g
$NaNO_3$	0.2 g	15-40 mg
$MgSO_4$	0.5 g	0.5 g
$CaSO_4$	0.1 g	0.1 g
Mineral Salt Solution*	5 mL	5 mL
Thiamin Hydrochloride	5 mg	5 mg
Deionized Water	1.0 L	1.0 L

*The composition of mineral salt solution is given elsewhere.[1]

After growing the microorganisms for five days, the glucose and nitrogen concentration dropped to about 30 mg/L and less than 1 mg/L respectively. The

pH dropped to about 3.0. This fungal culture was used as inoculum in all experiments.

Proposed Reaction Scheme and Mathematical Model

Armenante *et al.*[1] have recently shown that the degradation of 2,4,6-TCP by *P. chrysosporium* is a process that requires the simultaneous presence of both the mycelium and the extracellular enzyme system released by the mycelium to initiate degradation. These investigators have also shown that the rate limiting step in the process is the concentration of extracellular protein, and that the concentration of mycelial biomass does not affect the rate of degradation, although the presence of some biomass is necessary for degradation to occur. Accordingly, the following reaction scheme is proposed for the biodegradation of 2,4-DNT:

$$C + E_{ex} \Leftrightarrow C - E_{Ex} \text{ (Complex)} \quad (1)$$

$$C + E_{ex} \Rightarrow E_{Ex} + \text{Intermediate (s)} \quad (2)$$

$$\text{Intermediate} + E_{\text{Cell bound}} \Leftrightarrow \text{Intermediate} + E_{\text{Cell bound}} \text{ (Complex)} \quad (3)$$

$$\text{Intermediate} + E_{\text{Cell bound}} \Leftrightarrow \text{Products} + E_{\text{Cell Bound}} \quad (4)$$

A justification for the proposed scheme is as follows. It has been reported by many investigators that the fungus is able to break down large molecules, such as lignin, having molecular weights in excess of 600 kilodaltons.[1] Such large

molecules are unlikely to be transported across the cell membrane. This leads to the conclusion that the first step in the degradation process is typically an extracellular enzymatic or free radical reaction. Since 2,4-DNT cannot support fungal growth as a sole carbon source (it can support its nitrogen requirement), one can assume that the second step in the reaction scheme occurs on surface bound enzymes or by the biomass itself. However, the mechanism of nitrogen transfer is not yet elucidated, which may involve intracellular transfer of the intermediate(s). A schematic of the proposed mechanism is given in Figure 1.

A Michaelis-Menten model can be used here to describe the rate of degradation of the target compound according to the above reaction scheme.

$$r_c = \frac{V_p P C}{K_m + C} \tag{5}$$

Where r_C is the degradation rate, C is the concentration of the target compound, P is the concentration of extracellular protein(s) (proportional to the extracellular enzyme concentration, E_{EX}), and V_P and K_m are the Michaelis-Menten parameters.

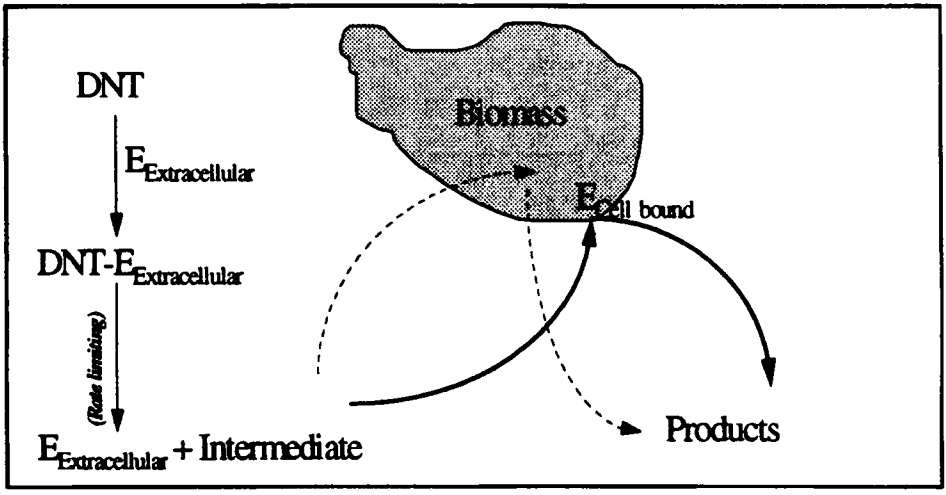


FIGURE 1
Degradation mechanism of 2,4-DNT by *P.chrysosporium*

Therefore, for a well mixed-reactor, the steady-state mass balance yields:

$$\frac{P\tau}{C_0 - C} = \frac{K_m}{V_P C} + \frac{1}{V_P} \qquad (6)$$

The above equation is valid when the enzyme deactivation is neglected or the retention time (τ) is much less than the life time of the enzyme.[1,7] Equation (6) can be used to estimate the Michaelis-Menten parameters, V_p and K_m, from experimental data.

Batch Experiments in Shaker Flasks

Batch experiments were conducted in shaker flasks. A stock solution of 200± 10 mg/L 2,4-DNT (98% purity, Sigma Chemical Co.) was prepared. Aliquots of the stock solution were added to the shaker flasks containing growth medium as required. Control flasks containing growth medium, inoculum, and acid/base for pH adjustments, were autoclaved and supplemented with 2,4-DNT. All flasks (including controls) were incubated at 30°C on a gyratory shaker rotating at 150 rpm. Samples of different volumes (2 or 5 mL) were taken from the shaker flasks to measure 2,4-DNT, nitrogen, and glucose concentrations.

The results of a typical shaker flask study (conducted in triplicate) are depicted in Figure 2. The results show that 2,4-DNT can be degraded by *P. chrysosporium* in the presence of glucose however, the fungi cannot utilize 2,4-DNT as the sole carbon source. During the batch study, a number of intermediates were observed and two of these intermediates were identified as 4-methyl 3-nitro aniline and 2-amino 4-nitrotoluene. Similar intermediates were reported by Valli *et al.*[11]

The results of the batch studies showed that the rate of fungal degradation of DNT is significantly lower compared to the degradation rate by bacterial species observed by other researchers.[2] Lower rate of degradation has attributed to depletion of energy source and accumulation of inhibitors.[1,2]

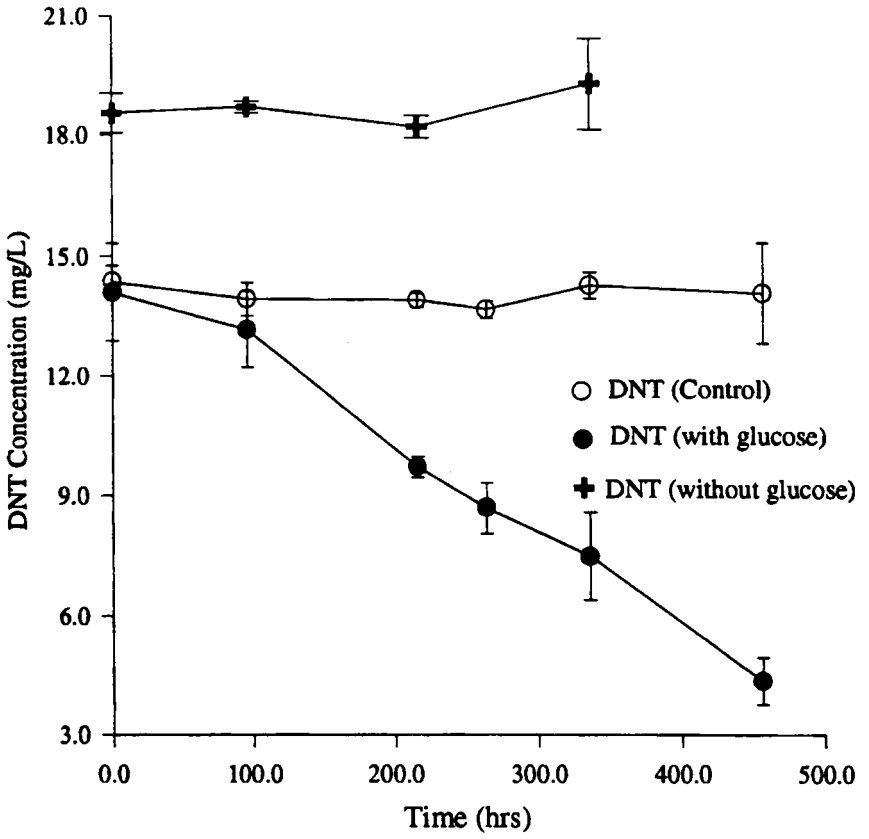


FIGURE 2
Concentration profile of 2,4-DNT in batch reactor.

The first step in this degradation process is initiated by extracellular enzyme(s) released by the fungus. The generation of these enzymes is an energy consuming process, hence in a substrate depleted system the fungal culture cannot secrete any enzymes.[1,2,7] However, when glucose is added to a substrate depleted system, degradation is re-established.[7] Therefore, for unhindered degradation of 2,4-DNT

a continuous supply of energy is essential. On the other hand, when substrates are in abundance, the fungi shift its metabolic activity from the secondary phase to growth phase and no lignolytic enzymes are produced.[1] These two opposing mechanisms must be optimized to attain continued high degradation rates which cannot be achieved in conventional batch reactors. In addition, conventional stirred tank reactors are unsuitable since they exert significant shear stress on this filamentous microorganism.[7]

Experiments in Continuous Packed-Bed Reactors

A schematic of the set up used in the continuous flow experiments is shown in Figure 3 (length 45.7 cm and internal diameter 10 cm). The reactor was equipped with a feed pump, an air sparger at the bottom, and an external recirculation loop (including a circulation pump). Clear polyethylene terephthalate (PET) flakes (irregular in shape and size, cross-sectional area = 2-15 mm^2; thickness ≅ 0.5 mm) were obtained from the Polymer Recycling Plant, Rutgers University, New Brunswick, NJ.

After cleaning and autoclaving, the flakes were used as random packing material (void volume: 50-60%). The temperature in the reactor was maintained at 30°C by circulating water from a water bath through the reactor jacket. The reactor was continuously fed from a feed tank at flow rates between 0.5 and 3.0 mL/min.

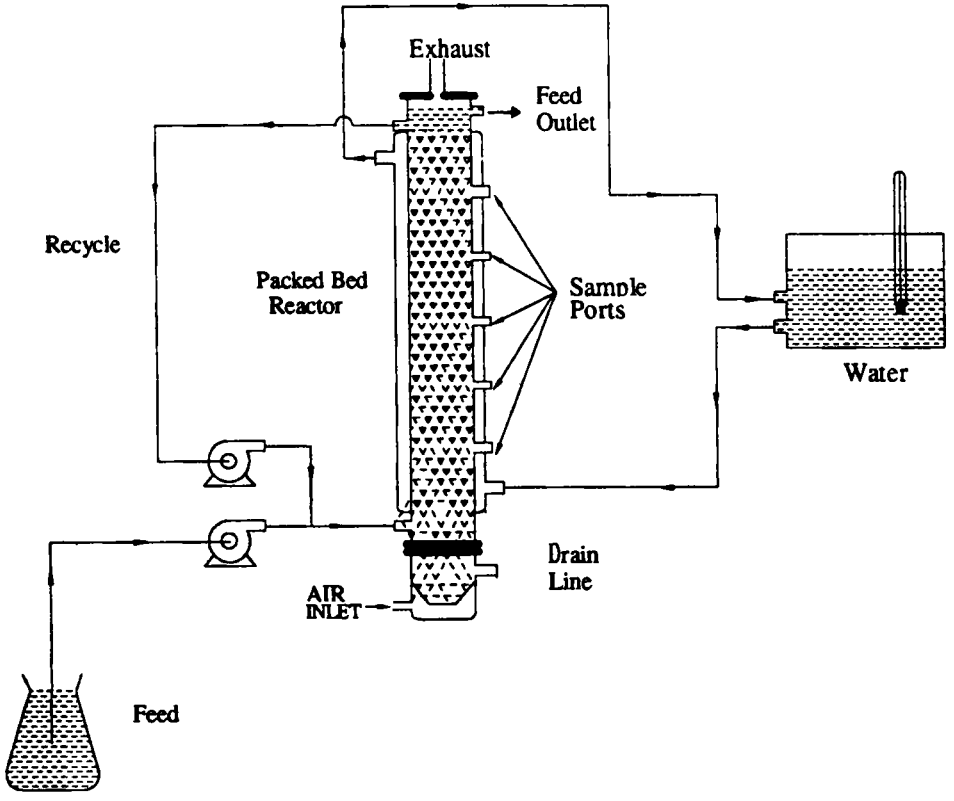


FIGURE 3
Schematics of the fixed-film bioreactor.

The external recirculation rate was between 6 to 20 mL/min, and was always much larger than the feed rate to ensure that the liquid content of the reactor was well mixed. To confirm this, a separate study was conducted in which a step change in the concentration of a tracer in the feed stream was imposed while the tracer concentrations at three sampling ports along the reactor were measured.

The flow regime was found to approximate that of a continuously stirred tank reactor (CSTR).

To immobilize the fungi on the packed-bed, a 5-day old fungal culture broth from shaker flasks was transferred to the reactor with the simultaneous addition of PET flakes. The fungus was allowed to grow for an additional period (2 to 10 days depending on the experiment) while continuously feeding growth medium and maintaining external recirculation. Then, the feed was switched from growth medium to induction medium. These media differed only in their glucose and $NaNO_3$ compositions (Table I). The induction medium was supposed to stimulate the secretion of extracellular enzymes by limiting the availability of nutrients to the fungus[1,7]. The induction medium fed to the reactor was supplemented with DNT. Samples from different ports along the length of the reactor were analyzed for DNT, nitrogen, glucose, pH, nitrate, and total protein concentrations. The attainment of steady state condition was confirmed when no significant change in outlet concentrations was observed throughout a period of at least 3 to 4 days.

Typical results for a packed-bed reactor is shown in Figure 4. At steady state 96.5% of the 2,4-DNT initially fed to the reactor was mineralized in 19.1 hours. The biomass loss from the bed was negligible (0.36 mg/h to 0.78 mg/h). Small concentrations of glucose (10-60 mg/L) and nitrogen (0.2-1 mg/L) were detected in the outlet. Rarely, we were able to detect the presence of any intermediates during chromatographic analysis.

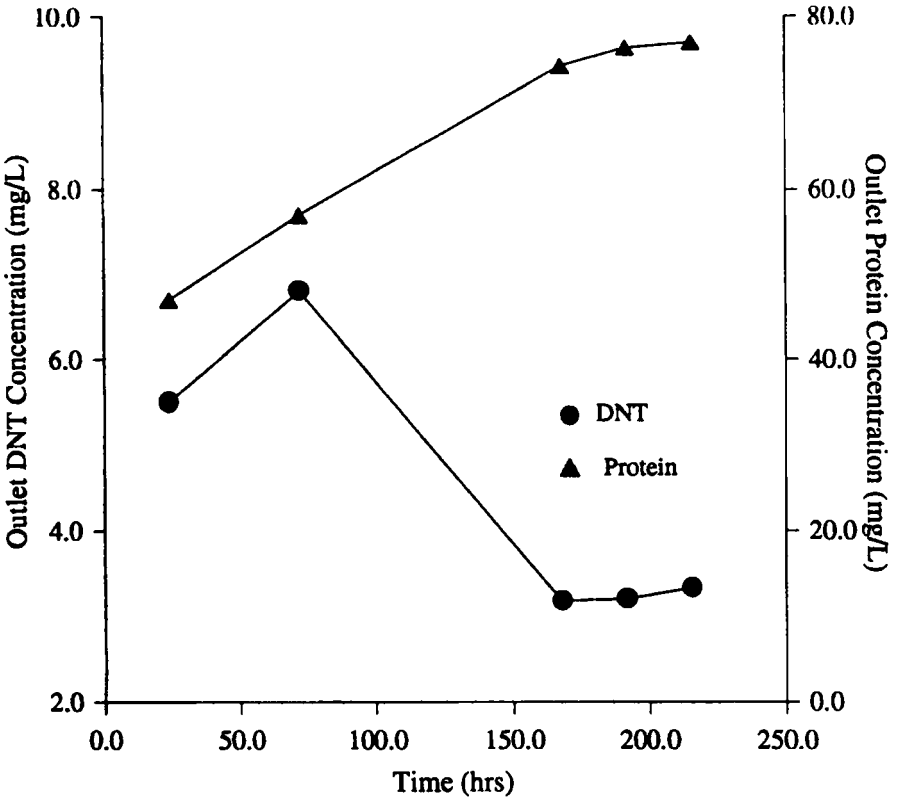


FIGURE 4

Development of steady state outlet concentrations in continuous packed-bed reactor (Inlet DNT concentration = 106 ± 0.9 mg/L, glucose = 600 mg/L, nitrogen 3.0 mg/L, retention time 19.1 hours)

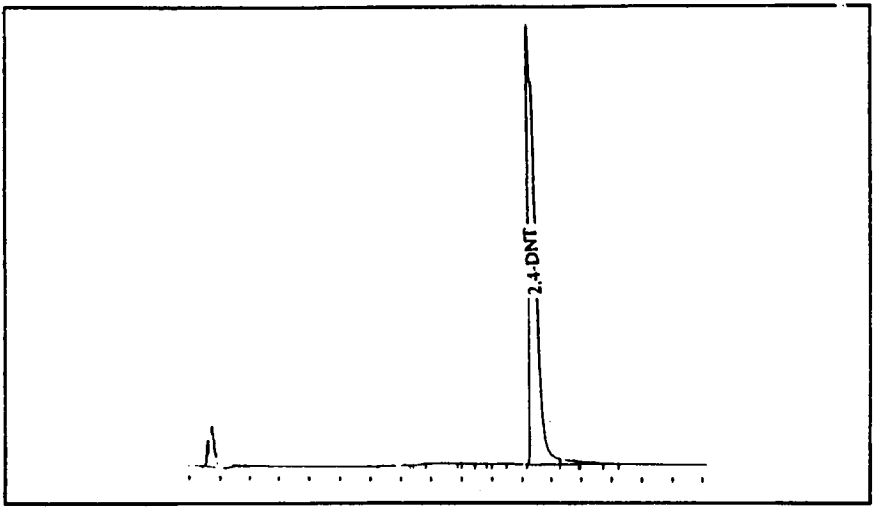
Because the system was not buffered, the pH of the feed and the outlet differed significantly (4.6 and 5.1 respectively).

A comparison of this results with that of batch study results shows that the specific degradation rates were very different in the two systems examined here

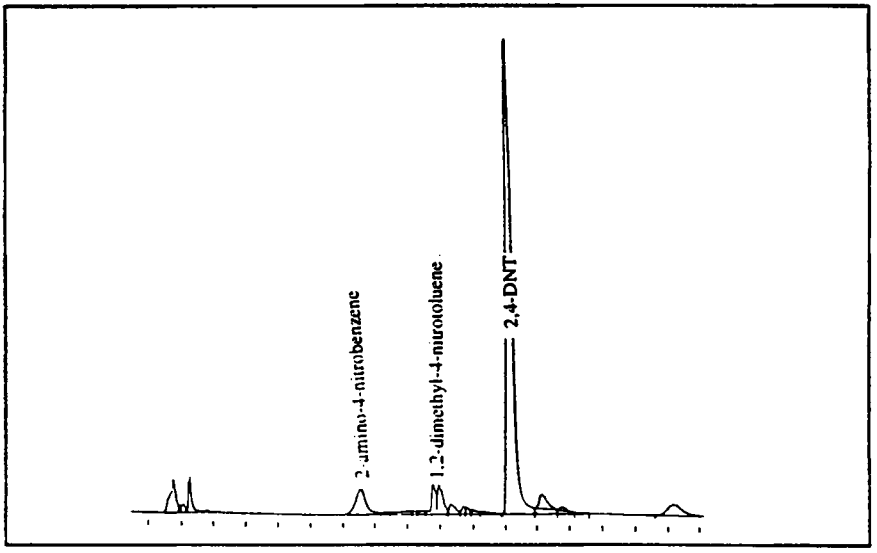
(0.0208 mg 2,4-DNT/(L × h) in shake flasks vs. 5.34 mg 2,4-DNT/(L × h) in the packed-bed reactor). This increase in degradation rate can be attributed to the preference of the fungus to be attached to a solid support,[7] and the constant availability of low levels of carbon and nitrogen in the packed-bed reactor (which likely enable the fungus to synthesize fresh extracellular enzyme to initiate the attack on the target molecule).

In addition, any effects related to shear-stress was eliminated in this reactor configuration. Therefore one can observe two orders of magnitude increase in degradation rate in packed-bed reactor compared to suspended growth system in shaker flask. The degradation rate in packed-bed bioreactor is a strong function of residence time, pH and nutrient conditions.[7]

Further studies have shown that improper parameters not only reduce the degradation rate but a large number of undegraded intermediates appear in the outlet stream during chromatographic analysis. At lower glucose concentration, a significant amount of metabolized products are detected (Figure 5) where as at higher glucose feed concentration, complete mineralization of DNT is achieved. However the concentration of glucose inside the reactor determines the outlet concentration of the intermediates which can be manipulated by changing the residence time using much lower levels of glucose in the feed stream.



Inlet glucose= 6.0 gm/L



Inlet glucose= 0.6 gm/L

FIGURE 5
Chromatograms showing effect of inlet glucose concentration on degradation of byproducts as identified in the outlet liquids

Modeling Studies

Experiments were conducted in a packed-bed reactor at a fixed pH of 5.6. Figure 6 shows the data obtained at a pH of 5.6. Each data point in this figure represents a steady-state condition.

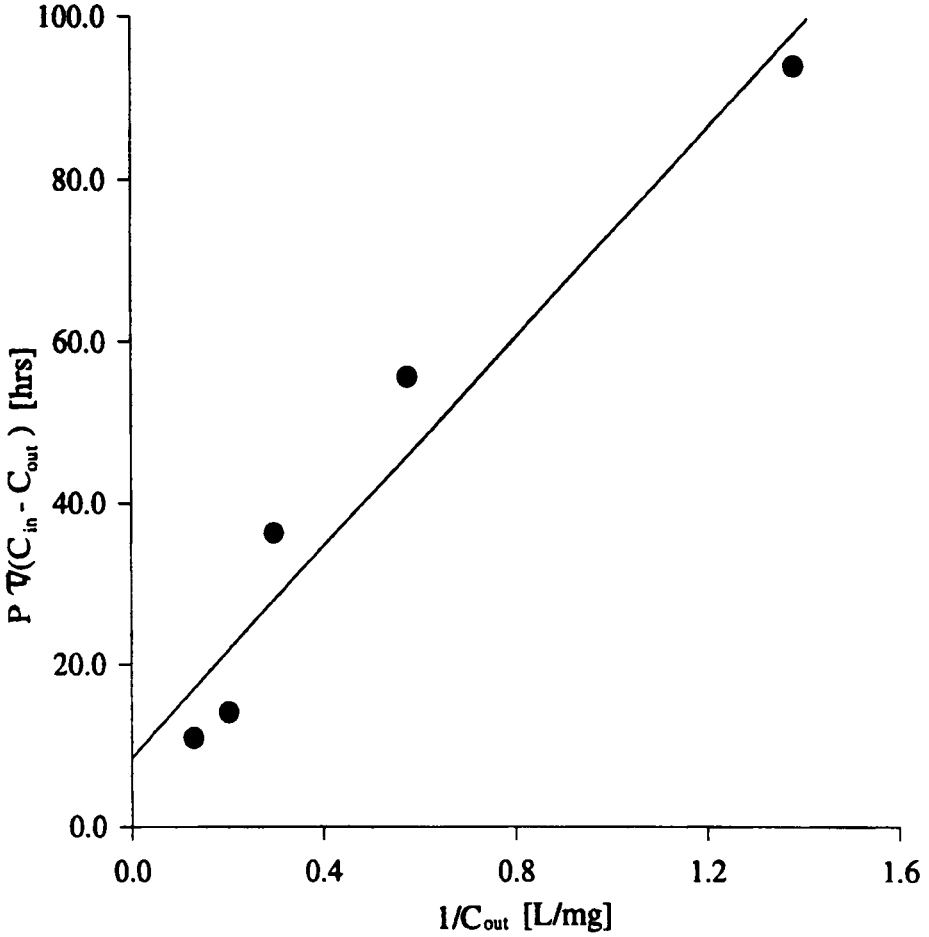


FIGURE 6
Estimation of model parameters using Equation 6.

Linear regression was used to determine the Michaelis-Menten parameters V_P and K_m in equation 6. The values of the Michaelis-Menten parameters were calculated from the slope and intercepts of such plots. The correlation coefficient for the line was 0.93. Figure 6 shows that the model adequately describes the experimental data. Since variables such as shear stress, nutrient concentration, and pH can affect the value of these parameters only selected data sets were used in the regressions. Specifically, the data regressed came from experiments in which shear stress effects were negligible (i.e., the volumetric flux was below 1.0 $mL/(cm^2 \times min)$), the glucose concentration was less than 50 mg/L, the nitrogen concentration was low enough to prevent inhibitory effects (less than 2 mg/L), and the inlet C:N ratio was between 60 and 80. As for pH, different data sets were considered in which the pH was kept constant throughout the experiment.

Table II

V_{max} and K_m Values for DNT at pH 4.6 and 5.6

pH	V_{max} (1/hr)	K_m (mg/l)
4.6	0.042	7.67
5.6	0.117	7.58

The resultant values of V_{max} and K_m for pHs of 4.6 and 5.6 are given in Table II. The results show that V_P is a strong function of pH however K_m remains

unchanged for all practical purposes. These results indicate that probably a single enzyme is involved in the degradation process.[10]

DEGRADATION OF NG

Literature reveals that nitroglycerin (glycerol trinitrate) is an inhibitory non-growth substrate.[8] Servant *et al.* has studied the biodegradation of NG by *Phanerochaete chrysosporium*.[9,10] In this study occurrence of a number of enzymes were observed with subsequent disappearance of NG. The only hydrolytic process appeared to be a glutathione S-transferase-soluble system producing nitrate and isomeric glycerol dinitrate from NG. In subsequent steps, NG-reductase reacts with dinitrates, the dinitrates are subsequently transformed to glycerol mononitrate. No further transformation has been shown. In another study, production of NO from nitrate has also been documented.[9] The authors are conducting experiments in order to determine the rates and extent of NG degradation by fungal cultures.

Experiments in Batch Systems

Experiment were performed using *P. chrysosporium* in fungal media with nitroglycerin as the sole carbon source and in the presence of 0.1% glucose. In this experiment three flasks were prepared, two were inoculated with fungi and the

third served as the control. The concentration profile is given in Figure 7. Significant degradation was observed within 10 days in both flasks.

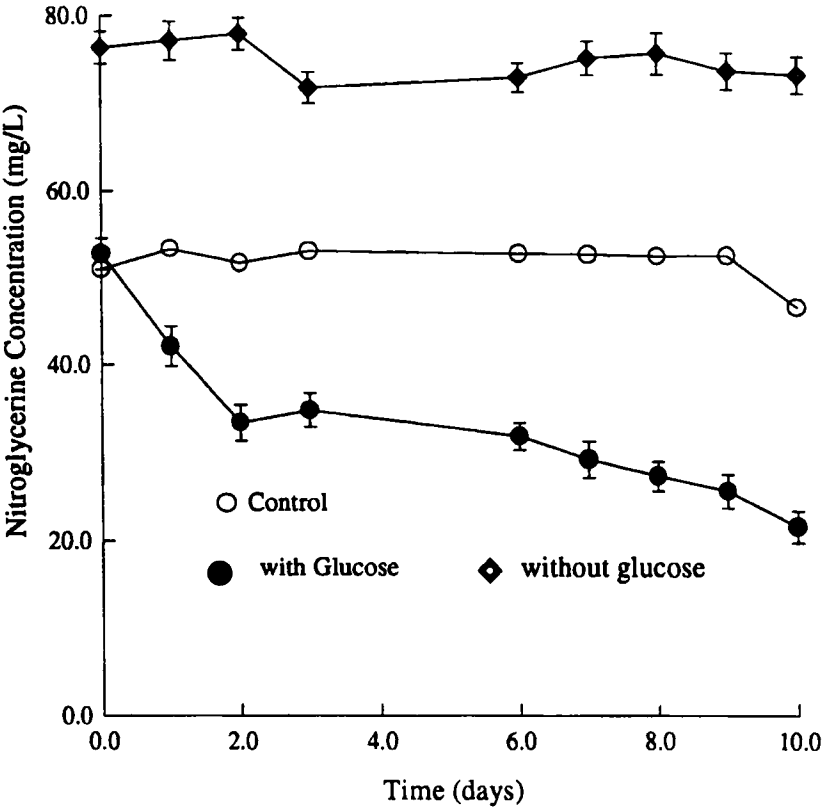


FIGURE 7
Biodegradation of NG by *Phanerochaete chrysosporium* in shaker flask.

Biodegradation of Nitroglycerin by *P. chrysosoporium* in Fixed-Film Reactor

As mentioned in case of 2,4-DNT, the rate of degradation can be significantly enhanced by optimizing the reactor configuration. In that case an immobilized-bed bioreactor was found to increase the degradation rate by two orders of magnitude. The same reactor configuration was also tested with NG using *P. chrysosoporium.*

Two reactors were used packed with polyethylene terephthalate (PET) and *P. chrysosporium* was immobilized over it. A stock solution of nitroglycerin along with fungal growth media and 1 gm/l glucose was fed continuously from the bottom of the reactor. The effluent was taken out from the top of the reactor. The inlet and outlet concentrations of both reactors were monitored daily. A retention times of 16 hours were maintained in both systems. The results of these two experiments are given in Figure 8.

The results show that nitroglycerin can be effectively degraded in a fixed film biological reactor immobilized with *P. chrysosporium.* One must note that similar to 2,4-DNT, the rate of degradation has been enhanced by approximately one order of magnitude (0.12 mg/L/hr in the batch reactor compared to 3.0 mg/L/hr in the packed-bed reactor). The degradation was confirmed by the formation of intermediates namely 1,3 dinitroglycerine, 1,2 dinitroglycerine, and 2-mononitroglycerine and confirmed by HPLC analysis matching the standard.

CONCLUSIONS

The fungus *P. chrysosporium* can effectively degrade 2,4-Dinitrotoluene and glycerol trinitrate and their corresponding intermediates but it requires a co-substrate as a primary carbon source.

The use of a continuous packed-bed reactor containing the immobilized fungus can enhance the degradation rate of 2,4-dinitrotoluene and nitroglycerine by orders of magnitude as compared to a batch reactor containing suspended biomass.

A Michaelis-Menten kinetic model for the degradation of 2,4-DNT, based on a reaction scheme involving a rate controlling step of formation of reaction intermediates, appears to satisfactorily describe the experimental data. The value of the Michaelis-Menten parameter V_P is strongly dependent on the system pH, however pH has no effect on K_m.

ACKNOWLEDGEMENTS

This work was performed at the James C. Nicoll Jr. Laboratory at Stevens Institute of Technology for Picatinny Arsenal, Dover, New Jersey, and it was sponsored by the U.S. Army AMCCOM under contract No. DAAA21-93-C-1018. The authors express their gratitude to Bruce Brodman from Picatinny Arsenal for his support and guidance throughout this project.

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