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Pacific Northwest National Laboratory
Operated by Battelle for the U.S. Department of Energy

Bio-Treatment of Energetic Materials Using White-Rot Fungus

M. M. Shah

November 1998

Prepared for the U.S. Department of Energy
under Contract DE-AC06-76RLO 1830

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Summary

The nitramine explosive, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), is used by militaries around the world in high yield munitions and often in combination with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Improper handling and disposal of manufacturing wastewater may lead to environmental contamination. In the past wastewater was collected in disposal lagoons where it evaporated, and deposited large amounts of explosives on the lagoon floor. Although lagoon disposal is no longer practiced, thousands of acres have been already contaminated. RDX and, to a lesser extent, HMX have leached through the soil subsurface and contaminated groundwater (1,2). Likewise, burning of substandard material or demilitarization of out-of-date munitions has also led to environmental contamination.

The current stockpile of energetic materials at DOE sites requires resource recovery or disposition (RRD). A related challenge exists in the clean-up of the DOE sites where soil and ground water are contaminated with explosives. Current technologies such as incineration, molten salt process, supercritical water oxidation are expensive and have technical hurdles. Open burning and open detonation(OB/OD) is not encouraged by regulatory agencies for disposal of explosives. Hence, there is need for a safe technology to degrade these contaminants. The fungal process does not employ open burning or open detonation to destroy energetic materials. The fungal process can be used by itself, or it can augment or support other technologies for the treatment of energetic materials. The proposed enzyme technology will not release any air pollutants and will meet the regulations of Clean Air Act amendments, the Resource Conservation and Recovery Act, and the Federal Facilities Compliance Act.

The goal for this project was to test the ability of white-rot fungus to degrade HMX. In our study, we investigated the biodegradation of HMX using white-rot fungus in liquid and solid cultures. The degradation of HMX was studied at 1, 10, 100 and 1000 ppm levels. In all cases, HMX was degraded. In general, the rate of degradation of HMX increased with increase in HMX concentration. Because of encouraging findings, further optimization of this method and eventual field testing of this technology is recommended. This research was performed in collaboration with Utah State University.

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Introduction

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The current stockpile of energetic materials at DOE sites requires resource recovery or disposition (RRD). A related challenge exists in the clean-up of the DOE sites where soil and ground water are contaminated with explosives. Current technologies such as incineration, molten salt process, supercritical water oxidation are expensive and have technical hurdles. Open burning and open detonation(OB/OD) is not encouraged by regulatory agencies for disposal of explosives. Hence, there is need for a safe technology to degrade these contaminants. The fungal process does not employ open burning or open detonation to destroy energetic materials. The fungal process can be used by itself, or it can augment or support other technologies for the treatment of energetic materials. The proposed enzyme technology will not release any air pollutants and will meet the regulations of Clean Air Act amendments, the Resource Conservation and Recovery Act, and the Federal Facilities Compliance Act.

Concerns regarding the environmental fate of nitramine compounds, such as HMX and RDX, are now arising because of their relative recalcitrance. Both chemicals have been classified as "toxic to aquatic organisms" (3). The U.S. Environmental Protection Agency (EPA) has a recommended lifetime exposure of 0.1 mg/l (4). The EPA has also recommended long-term health advisories for HMX exposure for a 10-kg child and a 70-kg adult at 5 mg/l and 20 mg/l, respectively (5). The Office of the Surgeon General has recommended a 24-hr average maximum allowable concentration of 0.3 mg/l for RDX (4).

Bioremediation has been proposed as a safe, cost-effective method of cleaning explosive-contaminated sites (6–8). Many methods for remediation of 2,4,6-trinitrotoluene (TNT) have been developed. These include soil slurry bioreactors, a two stage anaerobic/aerobic submerged soil bioreactor, and bacterial or fungal composting (9–18). The two stage anaerobic/aerobic bioreactor has been shown to degrade RDX, but only composting has been successfully demonstrated to degrade HMX (13–15).

The first step of biological degradation of nitramines appears to involve the reduction of the nitro groups to form nitroso compounds (19,20). These compounds are stable under anoxic conditions and can be further reduced to unstable hydroxyl amino compounds that rapidly undergo hydrolytic ring cleavage reactions (21). These intermediates can also react with organic matter and be bound to soil humics (5). Under aerobic conditions, the nitroso compounds are quickly reoxidized. However, aerobic degradation of HMX has been reported (14,15).

White-rot fungi have been shown to degrade many environmentally persistent, insoluble compounds, including lignin, DDT, polychlorinated biphenyls, and polynuclear aromatic hydrocarbons (22,23). Also, they have been shown to degrade a number of explosives including trinitrotoluene (TNT) (17,23–25) and RDX (23,25). Relatively high concentrations of TNT have been degraded by developing a bioreactor whose system is relatively insensitive to TNT toxicity (17). White-rot fungi have also been shown to have the ability to degrade mixtures of chemicals (22,23). Extensive degradation of pollutants appears to require enzymes that are produced during secondary metabolism. Primary among these are reductases, oxidases, and peroxidases. Cellobiose dehydrogenase (CDH) is a principal extracellular reductase which is produced by many of these fungi, and its role in biodegradation is currently being investigated. This enzyme has been shown to catalyze the formation of oxygen radicals, including the very reactive hydroxyl radical (26). The extracellular oxidases produce H_2O_2 which is the substrate for the peroxidases. The peroxidases oxidize a number of compounds to form free radicals which have long been implicated in the biodegradation of pollutants.

This study investigated HMX degradation by *Phanerochaea chrysosporium* under growth conditions that produced CDH and fungal peroxidases in liquid and solid state conditions. It has been reported that white-rot fungi have a unique enzyme system, and they are capable of degrading wide variety of recalcitrant chemicals, including lignin, DDT, PCBs and explosives.

Fungal technology has three special features:

- It can be applied to degrade a mixture of structurally diverse explosives.
- It is a safe and simple process to operate as process can be performed at room temperature and atmospheric pressure using water as solvent.
- It requires minimal capital cost.

Material and Methods

Chemicals. HMX (greater than 99% pure as judged by high-pressure liquid chromatography [HPLC]) was kindly provided by Thiokol Corporation (Brigham City, UT). Other chemicals were of analytical grade and were obtained from Sigma Chemical Corporation (St. Louis, MO).

Analytical. Quantitation of HMX concentration was performed with a Beckman System Gold HPLC system consisting of pumps, a 96-vial autosampler, and a UV-wavelength detector. Integrations were performed using Beckman System Gold software. Samples (5-35 µl) were separated with a 5 µm LC-CN reverse-phase column (length, 25 cm; diameter, 4.6 mm; Supelco Corporation, Bellefonte, PA). The mobile phase consisting of methanol:water (70:30 vol/vol) was delivered at a flow rate of 1.0 ml/min. HMX elution was monitored at 230 nm.

Organism. Phanerochaete chrysosporium (ATCC 24725) was obtained from the American Type Culture Collection. The fungus was maintained on 2 percent malt agar slants at room temperature.

HMX Extraction. Liquid cultures were extracted at room temperature with ethyl acetate to collect HMX for HPLC analysis. Ethyl acetate (10 ml) was added to liquid cultures which were then vigorously shaken for 30 seconds. The ethyl acetate and water phases were allowed to separate in a separatory funnel and the organic phase removed and the process repeated two times. Extraction efficiency was estimated to be 99.9 percent. Sawdust microcosms were extracted at 70°C with ethyl acetate to collect HMX for HPLC analysis. Ethyl acetate (~35 ml) was added to sawdust microcosms that were then incubated in sealed bottles at 70°C for an hour. The ethyl acetate was decanted from the bottles and the process repeated two times. Extraction efficiency was estimated to be 99.8 percent.

Culture Conditions. P. chrysosporium was grown in liquid medium composed of 1.2 mM ammonium tartrate, trace metals, thiamine, (1 mg/l), 50 mM sodium tartrate buffer (pH 4.5) and either 1 percent glucose or cellulose (21). The cellulose medium also contained 0.1 percent glucose. The culture media were sterilized by filtration through a cellulose acetate membrane filter (pore size 0.22 um). The cellulose and culture bottles were sterilized by autoclaving (121°C, 21 psig) for 20 minutes. Samples of the culture medium (9 ml) were dispensed into 250 ml Wheaton bottles with Teflon-lined caps. Cultures were inoculated with a spore suspension of *P. chrysosporium* (1 ml, 0.5 absorbance at 650 nm) and grown at 37°C. The cultures were grown under ambient atmosphere for 3 days. Thereafter at 3-day intervals, the headspace was exchanged with air.

For sawdust microcosms, 100 g of Betachip sawdust (Northeastern Products Corp., Warrensburg, NY) was mixed with 148.5 ml of water, autoclaved (121°C, 21 psi) for one hour and inoculated with a spore suspension of *P. chrysosporium*. The cultures were incubated in quart-sized jars at 37°C for 2 weeks. Prior to the addition of HMX slurry, 14.1 g (wet weight) of inoculated sawdust was aseptically transferred into 250 ml Wheaton bottles. HMX slurries that contained 0.1 mg/ml, 1.0 mg/ml, and 10 mg/ml HMX were mixed with the inoculated sawdust to final (analyzed) concentrations of 13 mg/kg, 42 mg/kg, 160 mg/kg, 450 mg/kg, 910 mg/kg, and 1500 mg/kg f sawdust. The microcosms were incubated at 37°C, and the headspace of each microcosm was exchanged with air every 3 days.

For soil microcosms, Betachip sawdust was prepared as described above. Garden soil from Utah State University's greenhouses was dried, mixed and sieved through a 2.0 mm screen. This soil (10.0 g) was added to 250 ml Wheaton bottles. HMX slurry (1 mg/ml) was added to the soil and mixed. Inoculated sawdust (4.4 g wet weight) was aseptically transferred into the 250 ml Wheaton bottles. The soil was mixed with the sawdust, sterile research grade 2 water (4.0 ml) was added and the microcosms mixed for a final time. Microcosms were incubated at 37°C and the headspace of each microcosm exchanged with oxygen every 3 days.

Results

HMX biodegradation in nitrogen-deficient liquid-medium cultures of *P. chrysosporium*, conditions that result in the production of peroxidases, was investigated. HMX was added as a slurry in water to an initial culture concentration of 0.8 mg/l, 79 mg/l, and 780 mg/l HMX, as determined by HPLC, and designated as sets A, B, and C, respectively. The lowest HMX concentration (0.8 mg/l) in this experiment was chosen for study because HMX would be completely soluble at this concentration while only a very small fraction of the total HMX would be soluble in the cultures containing 79 mg/l and 780 mg/l HMX. HMX degradation reached 66 ± 13%, 35 ± 13%, and 25 ± 3% with degradation rates of 0.024 ± 0.005 ppm/day, 1.3 ± 0.5 ppm/day, and 8.8 ± 1.2 ppm/day within 22 days for sets A, B, and C, respectively (Table 1). The maximum rate of degradation was 8.8 ± 1.2 ppm/day in set C. Degradation rates in this and all subsequent experiments were calculated as the amount of degradation that occurred from the beginning of the experiment not the amount that occurred between samplings. The greatest amount of degradation occurred within the first six days in sets A and B but occurred since day 9 in set C (Figure 1). This apparent lag in degradation at higher concentrations was previously observed during degradation of the structural homolog RDX. The quantitation limit for the HPLC assay was approximately 0.35 mg/l, so the amount of HMX remaining in set A is a conservative approximation. The exact amount of HMX present was somewhat lower than these values. The detection limit for HMX in the HPLC assay was between 0.1 and 0.05 mg/l.

Table 1. HMX Degradation in Nitrogen-Deficient Glucose Cultures of *P. Chrysosporium*

Set	HMX (mg/l)	Degradation (percent)	Rate (ppm/day)
A	0.8	66 ± 13	0.024 ± 0.005
B	79	35 ± 13	1.3 ± 0.5
C	780	25 ± 3	8.8 ± 1.2

Very good fungal growth was observed in nitrogen deficient cultures. A mycelial mat was completely formed within three days, and the onset of sporalation began within 6 days. Lignin peroxidase activity was first observed on day 4 (~30 U/l), peaked on day 6 (150 U/l), and was still present on day 20 (~20 U/l). Manganese-dependent peroxidase activity (~100 U/l) was first detected on day 3 and mirrored the lignin peroxidase activity in pattern.

HMX biodegradation in nitrogen deficient cultures grown on cellulose as the primary carbon source was similar to that in cultures which contained glucose as the principle carbon source. Cultures which initially contained 11 mg/l, 94 mg/l, and 990 mg/l HMX were designated as sets A, B, and C, respectively. HMX degradation reached 25 ± 37%, 10 ± 5%, and 25 ± 6% with degradation rates of 0.13 ± 0.09 ppm/day, 0.4 ± 0.1 ppm/day, and 11.5 ± 0.4 ppm/day within 22 days for sets A, B, and C, respectively (Table 2). Maximum rate of degradation was 16 ± 2 ppm/day in set C. Degradation principally began after a lag period of 7–10 days and correlated with fungal growth (Figure 2). Fungal

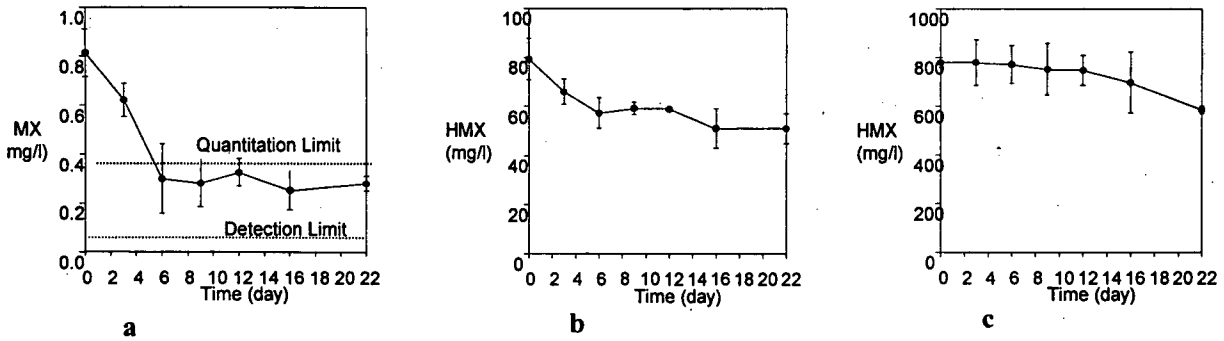

Figure 1. HMX Biodegradation in Nitrogen Deficient Cultures of *P. chrysosporium*. Cultures (10.0 ml) were prepared and maintained as described in Methods. HMX slurry (1.0 ml) was added to final concentrations of 0.8, 79, and 780 mg/l for graphs a, b, and c, respectively. Periodically, microcosms were sacrificed, extracted with ethyl acetate and analyzed for HMX by HPLC as described in Methods. Data represents the average and standard deviation of the quadruplicate microcosms.

Table 2. HMX Degradation in Nitrogen-Deficient Cellulose Cultures of *P. Chrysosporium*

Set	HMX (mg/l)	Degradation (percent)	Rate (ppm/day)
A	11	25 ± 3	0.13 ± 0.09
B	94	10 ± 5	0.4 ± 0.1
C	990	25 ± 6	11.5 ± 0.4

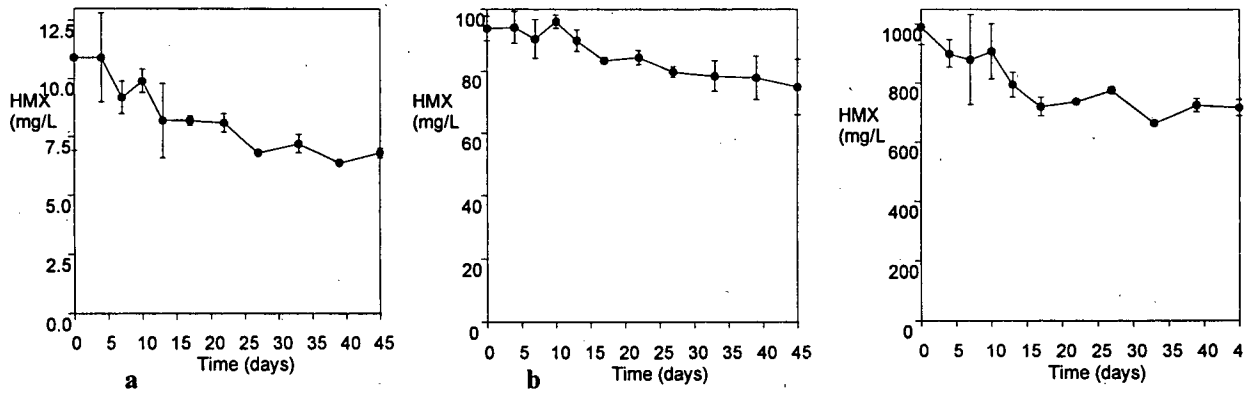

Figure 2. HMX Biodegradation in Nitrogen Deficient cellulose Cultures of *P. chrysosporium.* Cultures (10.0 ml) were prepared. HMX slurries (1.0 ml) were added to initial concentrations of 11, 94, and 990 mg/l for graphs a, b, and c, respectively. Data represent the average and standard deviation of the quadruplicate microcosms.

growth in these cultures was quite slow. The fungus colonized cellulose on the bottom of the culture bottle during the first week, then began to form a mycelial mat on the liquid surface during the second week. A weakly formed mat covered only ~70 percent of the surface of the cultures by the end of the third week. Additional cellulose (2 percent) was added to cultures on day 28 in an attempt to stimulate growth and biodegradation. Additional growth and degradation was minimal.

HMX biodegradation in sawdust inoculated with *P. chrysosporium* was similar to that in liquid. Cultures which initially contained 1 3 mg/kg, 42 mg/kg, 160 mg/kg, 450 mg/kg, 910 mg/kg, and 1490 mg/kg HMX were designated as sets A through F, respectively. HMX degradation reached 60 ± 4%, 28 ± 17%, 46 ± 6%, 20 ± 5%, 10 ± 3%, and 16 ± 9% with degradation rates of 0.19 ± 0.01 ppm/day, 0.3 ± 0.2 ppm/day, 1.8 ± 0.2 ppm/day, 2.2 ± 17 ppm/day, 2.2 ± 0.7 ppm/day, and 6 ± 3 ppm/day within 40 days in sets A through F, respectively (Table 3). The maximum rate of degradation was 13 ± 2 ppm/day in set E (data not shown). Between days 20 and 40, HMX degradation increased from 42 ± 4 to 60 ± 4%, 24 ± 17 to 28 ± 17%, 38 ± 7 to 46 ± 6%, 14 ± 5 to 20 ± 5%, and 8 ± 9 to 16 ± 9% in microcosms that initially contained 13, 42, 160, 450, and 1500 mg/kg. Degradation decreased from 20 ± 2 to 10 ± 3% in the microcosm which initially contained 910 mg/kg. Degradation is reported from parallel microcosms since whole microcosms were sacrificed for HMX quantification. Variations in growth between microcosms in part account for the differences in degradation detected. Also note that HMX was visible on the bottom of the culture bottles in sets D, E and F. There was insufficient sawdust to completely adsorb the HMX slurry which may adversely affect the rate of degradation in these sets since some of the HMX may not be in contact with the fungus. HMX biodegradation in sawdust did not have an observable lag phase (Figure 3).

Fungal growth in sawdust was slightly slower than has been observed in previous experiments. Many of the characteristic indicators of a "good" biodegradation system were lacking or impaired. These include cohesiveness of the microcosm colonies, sporulation density, characteristic odors, volume reduction, and bio-bleaching of the wood. The microcosms rarely exhibited the odors which have been associated with good fungal biodegradation. Also, volume reduction was minimal, and only about 20 percent of the sawdust in the microcosms exhibited characteristic bio-bleaching.

Table 3. HMX Degradation in *P. chrysosporium* Inoculated Sawdust

Set	Initial HMX (mg/kg)	Degradation (%)		Rate (ppm/day)	
		20 days	40 days	20 days	40 days
A	13	42±4	60±4	0.27±0.02	0.19±0.01
B	42	24±17	28±17	0.5±0.4	0.3±.02
C	160	38±7	46±6	3.1±0.6	1.9±0.3
D	454	14±5	20±5	3.3±2.2	2.2±0.6
E	910	20±2	10±3	9.1±0.9	2.2±0.7
F	1500	8±9	16±9	6±7	6±3

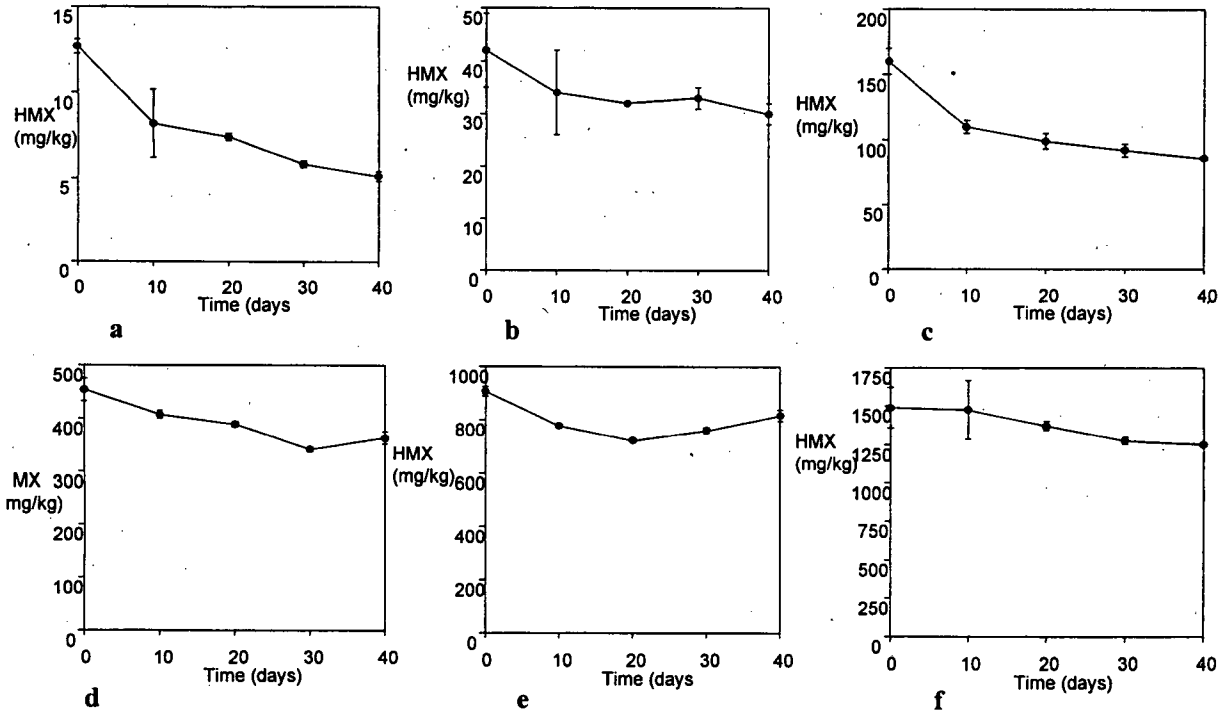

Figure 3. HMX Biodegradation in Sawdust by *P. chrysosporium*. Sawdust microcosms (6.0 g) were prepared and maintained as described in Methods. HMX slurry (1.0 ml) was added to final concentrations of 13 mg/kg, 42 mg/kg, 160 mg/kg, 910 mg/kg,, and 1500 mg/kg, for graphs a-f, respectively.

The average rate of HMX degradation in sawdust increased with increasing HMX concentration even though the fungal degradative system appeared to be suboptimal. The rate of degradation between day 0 and each time point was calculated and averaged (Figure 4). This was done to reduce the effects of inherent variations in growth and degradation found in parallel cultures grown on solid substrates. Variability in HMX degradation is noted from the standard deviation.

Approximately 80 percent of 45 mg/kg HMX in soil was degraded by the fungus within 45 days (Figure 5). The degradation occurred after a lag period of 20 days. This lag has been correlated to the time necessary for the fungus to colonize the soil microcosm and begin degrading sawdust present as the fungal nutrient source.

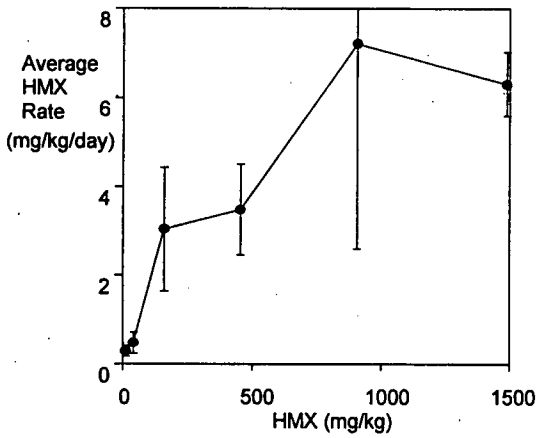

Figure 4. Effect of HMX Concentration on the Average Rate of its Degradation in Sawdust by *P. chrysosporium.* Data represent the average and standard deviation of rates of degradation between day 0 and each time point in Figures 12-18 versus the initial HMX concentration.

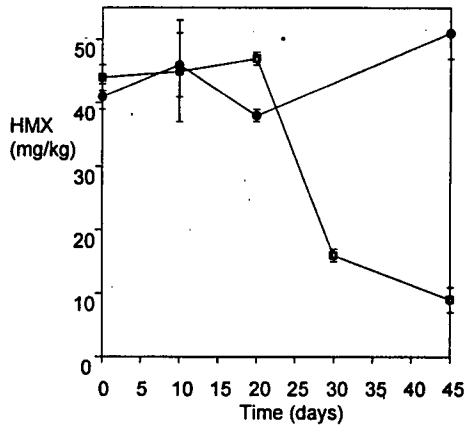

Figure 5. HMX Biodegradation in Soil by *P. chrysosporium*. HMX slurry (1.0 ml) was added to garden soil (10.0 g) to a concentration of 45 mg/kg which was then mixed with sterilized sawdust (4.0 g, circle) or sawdust (4.0 g, square) which had been inoculated 14 days prior with *P. chrysosporium* and water (4.0 ml). Microcosms were maintained at 37°C and head space gas exchanged with oxygen every 3 days. Microcosms were sacrificed, extracted with methanol and analyzed for HMX by HPLC as described in Methods. Data represents the average and standard deviation of the quadruplicate microcosms.

Discussion

Biodegradation by white-rot fungi seems to be a more practical approach to degrade HMX than traditional technologies. Wood rotting fungi evolved to degrade an insoluble substrate (wood). The fungi have been shown to degrade a number of insoluble pollutants, such as DDT, PAH, and synthetic polymers (22,27).

HMX degradation in two liquid media was studied. The only difference between the media was the source of nutrient carbon: glucose or cellulose. Nutrient-nitrogen deficient cultures that were grown on glucose produced a number of peroxidases that have been shown to be involved in the degradation of environmental pollutants (22). In the presence of cellulose, the cultures will produce cellulases and CDH in addition to the peroxidases. Cultures grow more slowly when cellulose is the primary carbon source. Typically, cultures grown on glucose become carbon limited around 18 days while cultures grown on cellulose become carbon limited around 30 days. Visual growth in the cellulose containing cultures slowed after 20 days. Additional cellulose that was added on day 28 had little effect on fungal growth and HMX degradation, which suggests that nutrient carbon was not the limiting factor. Other experiments in the laboratory on the degradation of DDT and PAH have suggested that cultures grown on cellulose have a higher nutrient nitrogen requirement. Degradation of DDT doubled in cultures grown on cellulose which initially contained 7.2 mM as opposed to 2.4 mM ammonium. Additions of 2.4–4.8 mM NH_4 have been shown to revive fungal growth and increase benzopyrene degradation rate by ~5–10 percent in other experiments.

Degradation of pollutants in liquid by white-rot fungi in general may be limited by their complex nutrient requirements. Complex nutrient sources, such as corn steep liquor or molasses, contain many easily oxidized chemicals that are substrates for the fungal peroxidases. While these are excellent nutrient sources for growth, they may completely inhibit pollutant degradation by the fungi. Use of a minimal nutrient medium, such as used in this study, will permit optimal fungal biodegradation but could be costly to set up and maintain.

Degradation of pollutants on wood sawdust may prove to be a cost-effective alternative for treating wastewaters. This is especially pertinent when the pollutant is essentially insoluble as is the case with HMX. Particulate HMX in wastewater may be physically adhered to the sawdust and degraded by white-rot fungi. The sawdust could be used as a filter prior to or after inoculation with the fungi. In the latter case, a trickle bed design bioreactor is proposed.

The ability of *P. chrysosporium* to degrade HMX in a sawdust matrix was evaluated to test the feasibility of this model. HMX was degraded in sawdust at least as well as in liquid. HMX degradation from ~10 ppm in sawdust and in liquid was 42 ± 4% and 25 ± 37% in 20 and 22 days, respectively. Degradation from ~950 ppm in sawdust and in liquid was 20 ± 2% and 25 ± 6% in 20 and 22 days, respectively. However, while liquid cultures require nutrient additions approximately every three weeks, sawdust microcosms do not. In general, HMX degradation in sawdust continued through the entire length of this study. Between 20 and 40 days, HMX degradation increased from 42 ± 4% to 60 ± 4%, 38 ± 7% to 46 ± 6%, 14 ± 5% to 20 ± 5%, and 8 ± 9% to 16 ± 9% in microcosms which initially contained 13 mg/kg, 160 mg/kg, 450 mg/kg, and 1500 mg/kg, respectively. Previous studies suggest that additional sawdust is necessary to continue degradation every 2–3 months, depending on fungal growth.

Conclusion

HMX biodegradation in soil by *P. chrysosporium* was also demonstrated. High concentration of HMX was also tolerated by fungus. We see a potential in developing this method to clean up HMX-containing water, soil and sediments. An ex-situ system will be required. Growth parameters such as fungal nutrients, metabolites, water, and oxygen all may play a role in the set up and maintenance of the degradation system. These relationships need to be defined further for HMX degradation. Optimizing these conditions will lead to improved degradation rates of HMX in bioreactors. Because of encouraging findings, further optimization of this method and eventual field testing of this technology is recommended.

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