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Disappearance of 2,4-Dinitrotoluene and 2-Amino,4,6-Dinitrotoluene by *Phanerochaete chrysosporium* Under Non-Ligninolytic Conditions

M. M. Jackson,[1] L.-H. Hou,[1] H. N. Banerjee,[1] R. Sridhar,[2] S. K. Dutta[1]

[1] Department of Biology and Cancer Center, Howard University, 415 College Street, N.W., Washington, DC 20059, USA
[2] Department of Radiotherapy and Cancer Center, Howard University, Washington, DC 20060, USA

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Nitroaromatic compounds such as 2,4,6-trinitrotoluene (2,4,6-TNT) and 2,4-dinitrotoluene (2,4-DNT) are extensively associated with the manufacture of explosives and are found in dyes which are considered hazardous environmental pollutants. These nitroaromatic compounds are most often found at military sites. 2-Amino,4,6dinitrotoluene (2-Am,4,6-DNT) is the most common biotransformation product of TNT, and is induced by numerous soil microbes. A significant proportion of these contaminants are found in soil and groundwater due to continual leaching. This is of great concern since nitroaromatic compounds have been found to be mutagenic in bacterial and mammalian cells (Banerjee and Dutta, 1997; Rieble et al. 1994) and found to be carcinogenic in animal studies (Haetter 1985; Rickert et al. 1983). Studies have shown that *Phanerochaete chrysosporium* has the ability to degrade nitroaromatic compounds such as 2,4,6-TNT (Bumpus and Brock 1988; Fernando et al. 1990, Stahl and Aust 1993) and 2,4-DNT (Valli et al. 1992). The use of this fungus for developing bioremediation strategies has recently been reviewed by Payne (1998) and Kalafut et al. (1998). This fungus degrades various environmental pollutants such as polychlorinated biphenyls (PCB), anthracene, triphenylmethane dyes, pentachlorophenol (PCP), and benzo[a]pyrene (Bumpus and Brock 1988; Cripps et al. 1990; Hutterman et al. 1989; Mileski et al. 1988; Toze 1996). In nature, *P.chrysosporium* thrives on woody substrates and effectively degrades lignin. The lignin degrading system involves a peroxidase system composed of lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as an H_2O_2,-degrading peroxidase (Gold et al. 1989; Kirk and Farrell 1987). Recent studies have shown that the decomposition of various pollutants such as 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), phenanthrene, 2,4,5-trichloro-phenoxyacetic acid, BTEX (benzene, toluene, ethyl benzene, and xylenes) and 2,4,6-TNT (Kohler et al. 1988; Spiker et al. 1992; Sutherland et al. 1991; Yadav and Reddy 1992; Dutta et al. 1998) by this fungus does not involve extracellular peroxidase, under non-ligninolytic conditions. Information on TNT disappearance cannot be extrapolated indiscriminately to 2,4-DNT or 2-Am-4,6-DNT metabolism, It could be due to unique enzymes involved in disappearance of TNT. This study was conducted to examine whether the disappearance of 2,4-DNT and 2-Am-4,6-DNT occurred upon incubation with *P. chrysosporium* cultures grown under non-ligninolytic conditions as well as under ligninolytic conditions.

MATERIALS AND METHODS

P. chrysosporium BKM-F1767 was obtained from C.A. Reddy at Michigan State University, East Laming, MI. 2,4-DNT and 2-Am-4,6-DNT were purchased from

Correspondence to: S. K. Dutta, Department of Biology, Howard University, 415 College Street, N.W., Washington, DC 20059, USA

Aldrich Chemical, St. Louis MO. The stock solutions were prepared in methanol (10 mg/ml). The purity of the chemicals was determined by HPLC and was found to be greater than 98% in all cases as was specified by the suppliers. An aqueous suspension of *P. chrysosporium* conidia was prepared from three week old malt extract agar slants. Suspensions of conidia were inoculated in low-nitrogen (LN) (Dass and Reddy 1990) and malt extract (ME) medium (Difco). Adequate controls were run in experiments using heat killed fungus in order to demonstrate that the biotransformation of the two nitroaromatic substrates involves metabolism by live fungus. The stability of the aromatic compounds in the culture medium devoid of the fungus was also studied as further controls. 100 ppm of 2,4-DNT and 10 ppm of 2-Am-DNT were added to two-day old cultures grown in ME medium or six-day old cultures in LN medium. Samples were taken out each day for eight days after addition of these chemicals. Samples were centrifuged to pellet the mycelia and supernatants collected for analysis.

Assays for total peroxidase activity were performed using 3,3',5,5'-tetramethylbenzidine (TMB, Microwell Substrate 1-Component, Kirkegard and Perry Laboratories, MD) as the substrate. Samples of extracellular fluid were concentrated 20-fold using a 30-kDa cutoff type YM-10 membrane concentrator (Amicon, Danvers, MA) in a centrifuge. The lower limit of this assay was 0.2 units/l. The upper limits were not observed in this study. The absorbance was measured at 620 nm after five minutes of 37°C incubation with 3,3'5,5'-tetramethylbenzidine in a volume of 500 ml.

Nitrite release assay was used to detect the release of nitrites in *P. chrysosporium* culture medium (LN and ME). Samples were assayed for the presence of nitrite by combining sulfanilamide 1% (w/v) in 1.5 N HCl and 0.02% N-(1-naphthyl)-ethylenediamide dihydrochloride mixed with one ml samples from the supernatant. Nitrite release was estimated by measuring the absorbance at 540 nm and converting into mM for uniform quantitative expression. Pure KNO_2 was used as a standard to make quantitative measurements of actual nitrite release in mM (Hewitt and Nicholas 1964). The lower limit of detection was 0.005 mM. We did not observe the upper limit of detection.

HPLC analyses were performed on a Hewlett-Packard series 1050 HPLC instrument equipped with a Supelco Supelocosil LC-18 (150 x 4.6 mm) column and an UV detector set at 254 nm. A mixture of methanol:water (46:54) at a flow rate of 1.0 ml/min was used for isocratic elution. In a typical experiment, cultures were extracted with methylene chloride and the organic layer filtered through anhydrous sodium sulfate to remove any residue of water. The methylene chloride extracts were evaporated free of solvent at room temperature using a rotary evaporator and the residue was dissolved in 1 ml of methanol. The samples were then filtered through a nylon membrane 0.45 mm syringe filter. Two microliters of this concentrated material were then used for HPLC analysis. Identity of each peak at specific retention time was confirmed by fortifying with the appropriate authentic nitroaromatic compound.

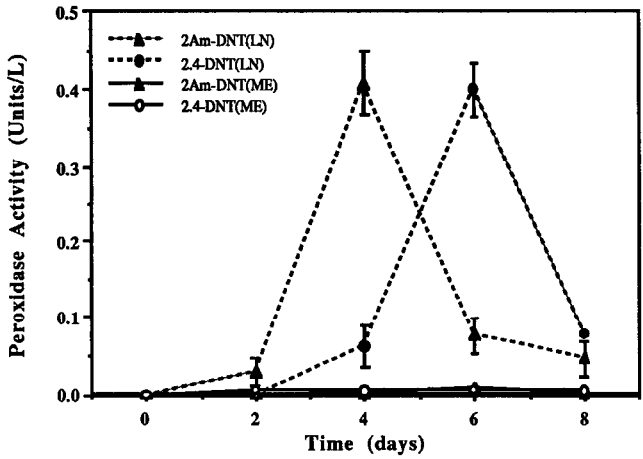

Figure 1. Peroxidase activity released into the culture medium during growth of *P. chrysosporium.* 2,4-DNT or 2-Am-4,6-DNT was added to the pregrown mycelia in LN medium and in ME medium. A small aliquot of the culture medium was removed for peroxidase assays daily for eight days after addition of chemicals. ---▲---, 2-Am-4,6-DNT in LN; ---●---, 2,4-DNT in LN; ----Δ---, 2-Am-4,6-DNT in ME; ---O ---, 2,4-DNT in ME.

RESULTS AND DISCUSSION

Peroxidase activity were conducted to confirm the presence or absence of the ligninolytic enzymes in culture medium. Figure 1 presents data as activity (units/ml) versus time in days. It graphically depicts the presence of peroxidase activity in *P. chrysosporium* grown in low nitrogen medium only. This assay confirms that peroxidase enzymes are not secreted by this fungus in malt extract medium under non-ligninolytic conditions. In order to confirm that peroxidase activity was not present in the ME medium, the medium was inoculated with pregrown *P. chrysosporium* mycelia pellets. No activity was detected in ME medium whereas enzyme activity was detected in cultures grown in LN medium (ligninolytic conditions). It is well known that peroxidase can be detected in ligninolytic culture conditions when LN medium is used. In non-ligninolytic culture conditions in which ME (or high nitrogen) medium was used, peroxidase enzymes were not present. It has been previously described that other investigators have used the method of Tien and Kirk (1988) to assay for lignin peroxidase (LiP) activity and the method of manganese peroxidase (MnP) activity. We have used a simple assay to determine the absence and presence of total peroxidases in LN and ME medium. High density cultures of the fungus grown in LN medium contained peroxidase enzymes, whereas cultures grown in ME had little, if any, peroxidase activity.

Nitrite release assay was used to determine if any nitrite release could be detected by the disappearance of nitroaromatic compounds by the white rot fungus. When the

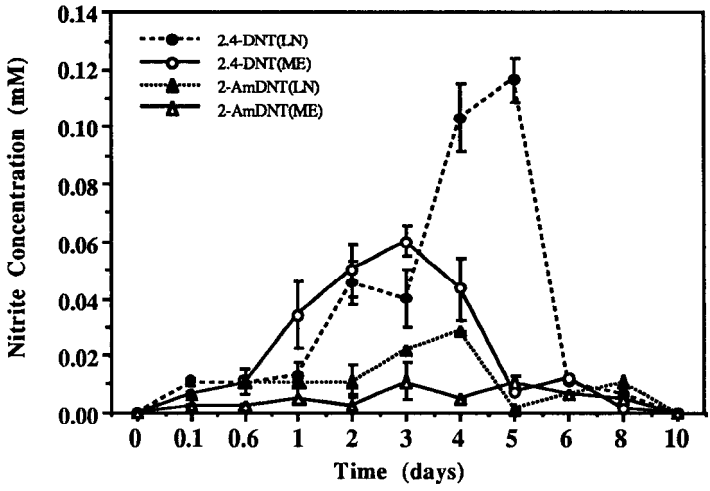

Figure 2. Level of nitrite release into the culture medium during growth of *P. chrysosporium* in the presence of 2,4-DNT or 2-Am-4,6-DNT. Cultures were treated as described in Figure 1. Aliquots of culture medium were removed for nitrite release assay daily for 12 days after addition of chemicals. The chemicals were added to two day old cultures in ME and six day old cultures in LN medium. ---▲---, 2-Am-4,6-DNT in LN; ---●---, 2,4-DNT in LN; ---Δ---, 2-Am-4,6-DNT in ME; ---O---, 2,4-DNT in ME.

fungus was grown in ME and LN media in the presence of 2,4-DNT an increase in nitrite production occurred on day one of incubation. An increase of nitrite production was seen in fungal cultures grown in both ME and LN medium in the presence of 2-Am-4,6-DNT on day three. Figure 2 presents data as mM nitrite release in the culture media. The amount of nitrite present is a reflection of the nitrite formed by the breakdown of the nitroaromatic compounds less any nitrite that is neutralized as a nitrogen source by the fungus. The formation of nitrite in controls (medium alone with or without fungus) and also in medium with only the test compounds in the absence of fungus was measured and the control values for nitrite concentration was near zero in every case.

When *P. chrysosporium* was grown in either ME or LN medium containing 0.14 mM 2,4-DNT as substrate, complete disappearance of 2,4-DNT occurred by day six (Figure 3A and 3B). When *P. chrysosporium* was grown in LN medium there was a gradual disappearance of the metabolite, 2-Am-4,6-DNT (Figure 3B). A similar gradual disappearance of 2-Am-4,6DNT occurred in ME medium (Figure 3A). These results clearly indicate that biotransformation of nitroaromatic compounds by this fungus occurs under both ligninolytic and non-ligninolytic growth conditions, although rates differ significantly for 2,4- DNT and 2-Am-4,6-DNT. Most of the previous work on *P. chrysosporium* with respect to peroxidase formation was done with TNT under LN conditions in the presence of peroxidase enzymes. In ME medium inoculated with two-day pregrown mycelia, nitrite release occurred in the first few days of incubation after the addition of TNT shown in

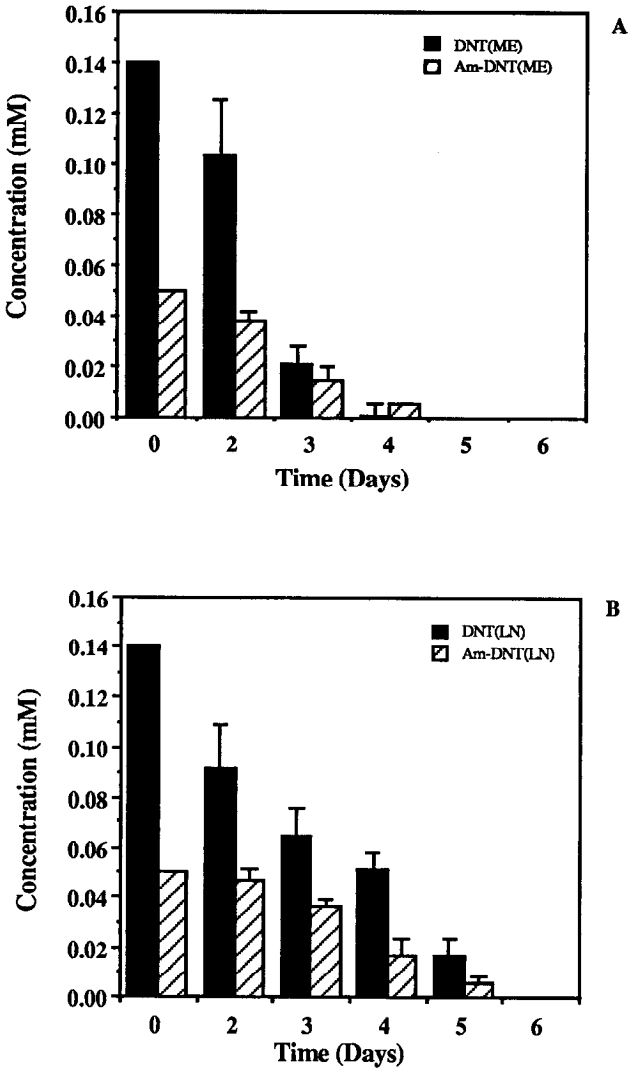

Figure 3. Biotransformation of 2,4-DNT (0.14 mM) and 2-Am-4,6-DNT (0.045 mM) in ME and LN medium by *P. chrysosporium* after 14 days of incubation. A, ME culture medium; B, LN culture medium.

Figure 2. This indicated that peroxidase enzymes were not involved in the degradation of nitroaromatic compounds because ligninolytic activity commences on the fifth or sixth day of fungal growth. Likewise, Yadav and Reddy (1993) have shown that *P. chrysosporium* was able to effectively degrade benzene, toluene, ethyl benzene, and xylenes (BTEX) and that this degradation was favored under non-ligninolytic culture conditions when no lignin-degrading enzymes were produced. Yadav and Reddy (1992) have also demonstrated that this fungus was

able to mineralize 2,4,5-richlorophenoxyacetic acid in malt extract medium in which lignin peroxidases were not produced. Dhawale et al. (1992) have also conducted similar studies using polycyclic aromatic hydrocarbons, in which they have shown that under non-ligninolytic conditions this fungus can degrade hazardous pollutants without the presence of peroxidase enzymes. We have shown that mineralization of the parent compound TNT occurs in the absence of peroxidase enzymes using radiolabeled ^{14}C-TNT (Dutta et al. 1998). It is important to mention that knowledge about degradation of TNT cannot be used indiscriminately to draw inferences on 2,4-DNT and 2-Am-4,6-DNT degradation by microorganisms. The toxicity of each compound towards microorganisms appears to differ and the degradation of TNT involve different pathways which exclude either 2,4-DNT or 2-Am-4,6DNT. The formation of 2-Am-4,6-DNT involve a nitroreductase which will not result in nitrite release. It is of great interest to study the degradation of 2,4-DNT and 2-Am-4,6-DNT individually because of the importance of these chemicals in dyes and the chemical industry. Our studies show that there are other fungal enzyme systems beside lignin degrading enzymes involved in the biotransformation of nitroaromatic compounds.

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REFERENCES

Banerjee H, Dutta SK (1997) 2-Amino,4,6dinitrotoluene exposure to mammalian cells causes p53 gene mutation and induces apoptotic changes. The FASEB J 11(9):A1239

Bumpus JA, Brock BJ (1988) Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium.* Appl Environ Microbiol 54: 1143-1150

Bumpus JA, Tatarko M (1994) Biodegradation of 2,4,6-trinitrotoluene by *Phunerochaete chrysosporium* identification of initial degradation products and the discovery of a TNT metabolite that inhibits lignin peroxidases. Curr Microbiol 28:185-190

Cripps C, Bumpus JA, Aust SD (1990) Biodegradation of azo and hetero-cyclic dyes by *Phanerochaete chrysosporium.* Appl Environ Microbiol 56: 1114-1118

Dass SB, Reddy CA (1998) Characterization of extracellular peroxidases produced by acetate buffered cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium.* FEMS Micro Letters 69:221-228

Dhawale SW, Dhawale SS, Dean-Ross D (1992) Degradation of phenanthrene by *Phanerochaete chrysosporium* occurs under ligninolytic as well as nonligninolytic conditions. Appl Environ Microbiol 58: 3000-3006

Dutta SK, Jackson MM, Hou L-H, Powell D, Tatem HE (1998) Non-ligninolytic TNT mineralization in contaminated soil by *Phanerochaete chrysosporium.* Bioremed J 2:97-103

Fernando T, Bumpus JA, Aust SD (1990) Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium.* Appl Environ Microbiol 56: 1666-1671

Gold MH, Warishi H, Valli K (1989) Extracellular peroxidases involved in lignin-degradation by the white rot basidiomycetes *Phanerochaete chrysosporium.* ACS Symp Series 389:127-140

Hartter DR (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In Rickert DE (ed) Toxicity of Nitroaromatic Compounds: Chemical industry institute of toxicology series. Hemisphere Publishing Corp, Washington DC, pp 1-13

Hewitt EJ (1964) In modem methods of plant analysis. Vol 7. Edtd. By Linskens HF, Samual BD, and Tracey MV. Springer-Verlaz, Berlin, p 66
Huttermann A, Trojanowski J, Loske D (1989) Process for the decomposition of complex aromatic substances in contaminated soils/refuse matter with microorganisms. German Patent No. DE3,731,816
Kalafut T, Wales ME, Rastogi VK, Nanmova RP, Zaripora SK, Wild JR (1998) Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr Microbiol 36:45-54
Kirk TK, Farrell RL (1997) Enzymatic "combustion": the microbial degradation of lignin. Ann Rev Microbiol 41:465-505
Kohler A, Jager H, Willershausen H, Graf H (1988) Extracellular ligninase of *Phanerochaete chrysosporium* Burdsall has no role in the degradation of DDT. Appl Microbiol Biotech 29:618-620
Michels J, Gottschalk G (1995) Pathway of *TNT* degradation by *Phanerochaete chrysosporium.* In Spain JC (ed) Biodegradation of nitroaromatic compounds. Plenum Press, New York, pp 321-325
Mileski GJ, Bumpus JA, Jurek MA, Aust SD (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium.* Appl Envirn Microbiol 45:2885-2889
Payne SM (ed) (1998) Strategies for accelerating cleanup of toxic waste sites. Lewis Publishers, CRC Press LLC, Boca Raton, FL pp 364-370
Rickert DE, Butterworth BE, Popp JA (1983) Dinitrotoluene acute toxicity, oncogenicity, genotoxicity and metabolism. Crit Rev Toxicol 13:217-234
Rieble S, Joshi DK, Gol MH (1994) Aromatic nitroreductase from the basidiomtcete *Phanerochaete chrysosporium.* Biochem Biophy Res Comm 205:298-304
Spiker JK, Crawford DL, Crawford RL (1992) Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white-rot fungus *Phanerchaete chrysosporium.* Appl Environ Microbiol 58:3199-3202
Stahl JD, Aust SD (1993) Metabolism and detoxification of TNT by *Phanerchaete chrysosporium.* Biochem Biophys Res Comm 192:477-482
Toze S (1996) TNT busters: microbes that degrade munition compounds in groundwater. Search 27:58-61
Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium.* Meth Enzymol 161:238-249
Valli K, Brock BJ, Joshi DK, Tien M (1992) Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium.* Appl Environ Microbiol 58:221-228
Yadav JS, Reddy CA (1992) Non-involvement of lignin peroxidases and manganese peroxidases in 2,4,5-ichlorophenyoxyacetic acid degradation by *Phanerochaete chrysosporium.* Biotech Lett 14:1089-1092
Yadav JS, Reddy CA (1993) Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycetes *Phanerochaete chrysosporium.* Appl Environ Microbiol 59:756-762