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Simultaneous Utilization of Two Different Pathways in Degradation of 2,4,6-Trinitrotoluene by White Rot Fungus *Irpex lacteus*

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This study confirmed that white rot fungus *Irpex lacteus* was able to metabolize 2,4,6-trinitrotoluene (TNT) with two different initial transformations. In one metabolic pathway of TNT a nitro group was removed from the aromatic ring of TNT. Hydride-Meisenheimer complexes of TNT (H^--TNT), colored dark red, were confirmed as the intermediate in this transformation by comparison with the synthetic compounds. 2,4-Dinitrotoluene as a following metabolic product was detected, and nitrite produced by denitration of H^--TNT supported this transformation. In the other TNT pathway, nitro groups in TNT were successively reduced to amine groups via hydroxylamines. Hydroxylamino-dinitrotoluenes and amino-dinitrotoluenes were identified as the intermediates. The activity of a membrane-associated aromatic nitroreductase was detected in the cell-free extract of *I. lacteus*. This enzyme catalyzed the nitro group reduction of TNT with NADPH as a cofactor. Enzyme activity was not observed in the presence of molecular oxygen.

Key words: trinitrotoluene, *Irpex lacteus*, metabolic pathway, nitroreductase, hydride-Meisenheimer complexes

The biological degradation of explosives such as 2,4,6-trinitrotoluene is an interesting research area due to its widespread use and environmental contamination, and its toxicity and mutagenicity (13, 22, 23). Even though various bacteria and fungi can degrade TNT in aerobic and anaerobic conditions (2, 3, 4, 20), the initial reactions of degradative processes are reductive rather than oxidative owing to nitro groups which reduce the electron density of an aromatic ring, and inhibit oxidative attack by the electrophilic oxygenases (21). The initial reduction pathways of TNT are divided into two reactions. One pathway is a cascade reduction of nitro groups in an aromatic ring, which produces the reduced intermediates, such as hydroxylamino-dinitrotoluenes, amino-dinitrotoluenes and diamino-nitrotoluenes, and thereafter condensation of the reduced forms into biologically inert azoxy compounds occurs (6, 7, 8). The other metabolic pathway is the denitration of an aromatic ring, which is initiated by nucleophilic addition of a hydride ion at C-3 of TNT. A dark-red metabolite accumulating transiently in the culture fluid was characterized as hydride-Meisenheimer complex, and sub sequently converted to dinitrotoluenes and nitrite (6, 7, 21). Membrane-associated nitroreductases are involved in both reductive transformations (6, 21).

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To date most TNT degrading microorganisms have been known to have only one metabolic pathway (11, 17, 18, 19). Recently, Haïdour *et al.* (7) reported that *Pseudomonas* sp. was able to carry out two different initial reactions simultaneously in the TNT metabolism. However, none of the fungal strains have shown this kind of metabolism in TNT degradation (5, 15, 18). This study confirmed that white rot fungus *Irpex lacteus*, which was isolated in Korea and has already shown a high degradation activity for hydrocarbons and TNT (9, 10, 16) was able to metabolize TNT with two different initial transformation mechanisms. Also membrane-associated enzyme mediated the cascade reduction of the nitro group in the aromatic ring.

Materials and Methods

Fungus and chemicals

Irpex lacteus was obtained from the Mycology Laboratory, Kangnung National University. Stock cultures were grown on potato dextrose agar (Difco, USA) at 28°C, and stored at 4°C. Analytical-grade TNT, 2-hydroxylamino-4,6-dinitrotoluene (2-OHAmDNT), 4-hydroxylamino-2,6-dinitrotoluene (4-OHAmDNT), 2-amino-4,6-dinitrotoluene (2-AmDNT), 4- amino-2,6-dinitrotoluene (4-AmDNT), 2,4-dinitrotoluene (2,4-DNT) 2,6-dinitrotoluene (2,6-DNT) were purchased from Supelco Co. (Bellefonte, USA) and AccuStandard

Inc. (New Haven, USA). All solvents were HPLC grade purchased from J.T. Baker (Phillipsburg, USA).

Fungal degradation of TNT

Fungal cultures were started by inoculation with 5 agar plugs (5 × 5 mm) of active *I. lacteus* mycelium and were grown in YMG medium (4 g yeast extract, 10 g malt extract, 4 g glucose per 1 L distilled water, pH 6.0) on a rotary incubator (130 rpm) for 5 days at 28°C. The resulting fungal suspension was blended with a homogenizer. Culture flasks containing 20 ml of YMG medium were inoculated with 0.5 ml of the blended fungal inoculum and preincubated (130 rpm) for 5 days. Subsequently, 200 µl of TNT stock solution in methanol was added to make final concentrations of 100 mg L^{-1} and fungal cultures were incubated at 28°C. The analysis of residual TNT and its reduction products during further incubation was described elsewhere (9). For analysis of [H^-]-Meisenheimer complexes of TNT, 10 ml of culture supernatant was extracted with an equal volume of ethyl acetate. The extract was dried over anhydrous sodium sulfate, redissolved in 10 ml ethyl acetate, and H^--TNT were analyzed. As the standard compounds, H^--TNT were prepared by chemical reduction of TNT (6, 21). Sodium borohydride 2.8 g was added to 1 ml of 10 mM TNT in acetonitrile. The reaction mixture instantly turned a dark red color and then was filtrated with PTEF membrane filter (Gelman, USA).

During the biodegradation of TNT, nitrite formed by denitration was determined. One ml of culture supernatant was added to 0.4 ml *N*-(1-naphthyl)-ethylenediamine-sulfanilamide solution containing 0.3 M of sulfanilic acid and 0.5% of *N*-(1-naphthyl)-ethylenediamine-sulfanilamide dichloride. The reaction mixture was incubated for 20 min at room temperature and OD at 543 nm was determined. Sodium nitrite was used as the standard.

Preparation of cell free extract

Fungal cultures pre-grown for 5 days were harvested by filtration and centrifugation. Mycelial mats were washed successively with ice-cold water, 0.5% NaCl solution, and 50 mM sodium phosphate (pH 7.0). The mycelial mats (15 g) were resuspended in 80 ml of buffer A (50 mM sodium phosphate, 330 mM sucrose, pH 7.0), and homogenized 3 times for 30 sec with a blender and ultrasonicator. Unbroken cells and other debris were removed by centrifugation (4,000 g, 20 min). The resulting supernatant was concentrated with a freeze dryer and resuspended in 50 mM of sodium phosphate. Sucrose and low molecular weight proteins were removed by dialysis and resuspended in buffer A without sucrose to adjust 1 mg/L of final protein concentration.

Emzymatic conversion of TNT

Nitroreductase assays were carried out in glass scintillation vials containing buffer A without sucrose, protein fraction, 0.5 mM NADPH, and 0.2 mM substrate in 1 ml of total volume. Reactions started with flushing the head space of the vials with N_2 for 15 seconds and incubating the vials at 45°C. Reactions were stopped by the addition of 0.5 ml each of methylene chloride and ethylacetate.

Analytical methods

Residual TNT and its metabolites were analyzed by HPLC (Millipore, USA), and analytical methods were described elsewhere (9). For analysis of negatively charged metabolites elution was by an isocratic elution for 5 minutes with 45% acetonitrile and then linear gradient during 25 minutes to 100% acetonitrile. Both solvents contained 10 mM tetrabutylammonium phosphate. Peak detection was made at 475 nm.

Results and Discussion

Cascade reduction of TNT metabolism

Fig. 1 shows the typical profiles of TNT transformation at 0 and 6 hours of incubation with *I. lacteus*. TNT completely disappeared within 10 hours of incubation (Fig. 1A, 2A) and a variety of its metabolites were produced. 2-OHAmDNT and 4-OHAmDNT appeared as the first detectable products of TNT metabolism. These metabolites accumulated during 6 hours, and completely transformed to the next reduction metabolite between 24 and 40 hours of incubation (Fig. 1B, 2B). 2-AmDNT and 4-AmDNT were also detected as a result of the transformations of two OHAmDNT isomers (Fig. 1B, 2C).

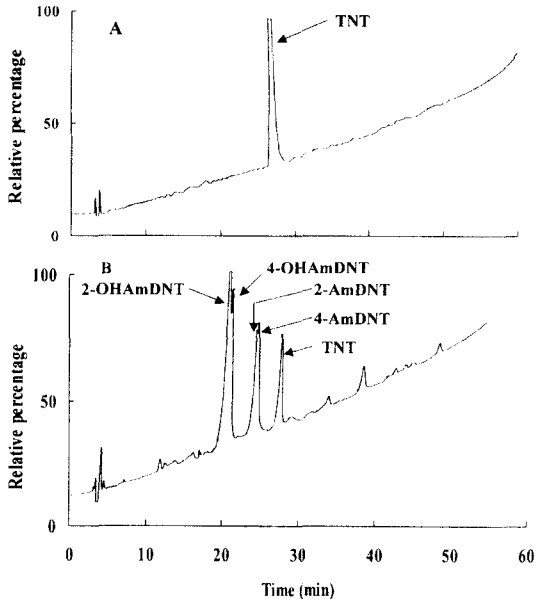

Fig. 1. Time course profile (A, 0 h, B, 6 h of incubation) of removal of TNT (100 mg/L) and formation of its metabolites by *Irpex lacteus*.

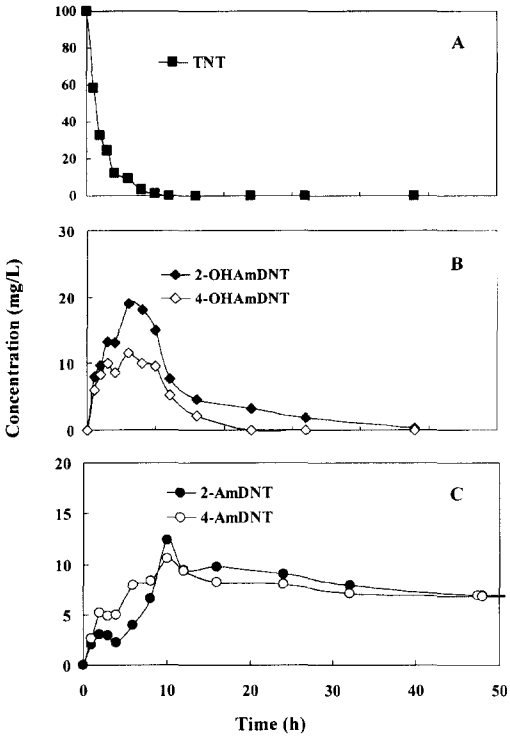

Fig. 2. Degradation of TNT and its metabolites by *Irpex lacteus* (A, TNT, B, hydroxylaminodinitrotoluene isomers, C, aminodinitrotoluene isomers).

Although these metabolites could be detected for a prolonged period, most of these metabolites were removed after 20 days of incubation (data not shown). As already mentioned in the previous reports, these results indicate that *I. lacteus* carries out a cascade reduction process in the TNT metabolism (9, 10).

Denitration process of TNT metabolism

Vorbeck *et al.* (21) reported that hydride-Meisenheimer

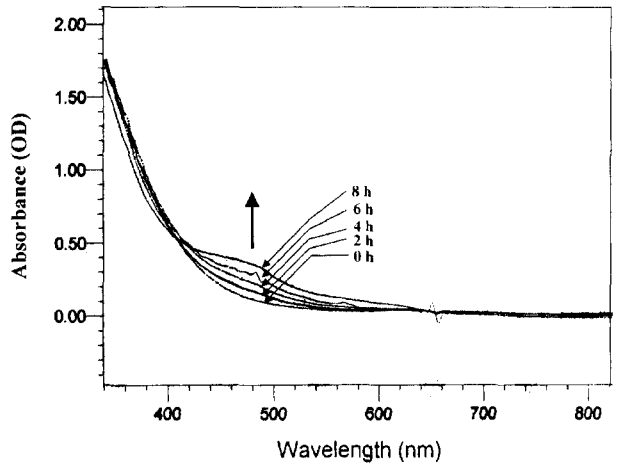

Fig. 3. Change in the absorption spectrum of the culture supernatant of *Irpex lacteus* in TNT degradation.

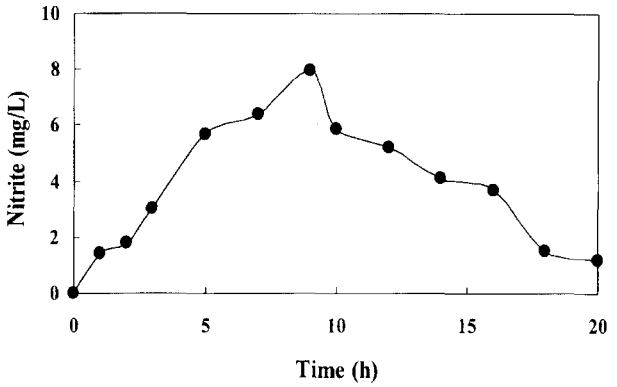

Fig. 4. Change in the nitrite concentration in the culture supernatant of *Irpex lacteus* in TNT degradation.

complex of TNT, a dark red-brown metabolite, accumulated transiently during TNT degradation. This metabolite is charged negatively and is converted to yellow dihydride-Meisenheimer complex. Nitrite and dinitrotoluenes are formed as the results of this pathway (6, 7, 21).

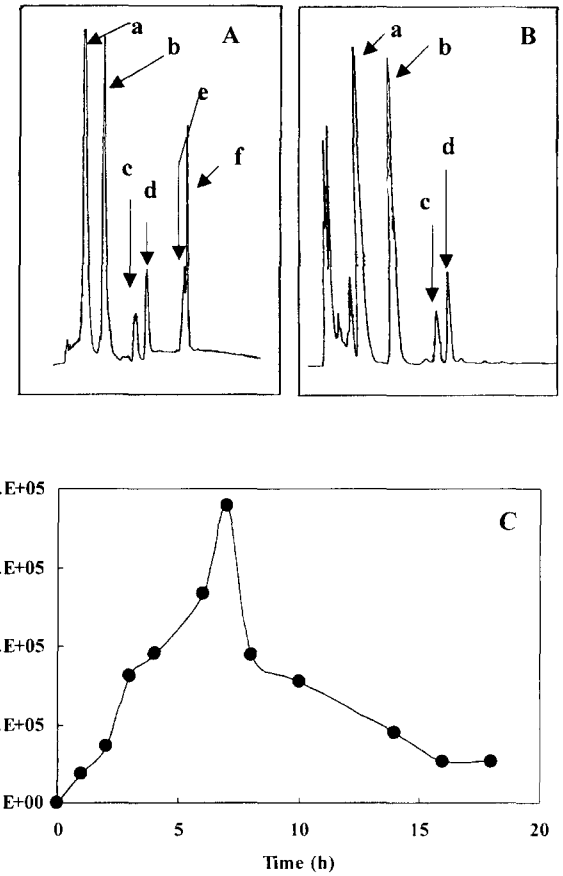

Fig. 5. Chromatogram of TNT and its metabolites by ion-pair HPLC with (A) and without (B) tetrabutylammonium counterion, and change of peak f (C) in TNT degradation by *Irpex lacteus*. Peak a; ethyl acetate, peak b; unknown, peak c; unknown, peak d; TNT, peakse and f; H^--TNT suspected).

To investigate the denitration process of TNT metabolism in *I. lacteus*, H^--TNT and nitrite were monitored in culture supernatant. During the degradation of TNT by *I. lacteus*, the culture supernatant turned dark red and darkened 8 hours of incubation, but faded during further incubation. When the absorption spectrum of the culture supernatant was monitored, OD_{475} which might be responsible for H^--TNT increased according to the time and increase of the dark red color (Fig. 3). Nitrite from denitration of H^--TNT was released, and reached 8.5 mg/l after 9 h (Fig. 4). French *et al.* (6) reported that H^--TNT migrated to the solvent front in standard HPLC but was retarded within the column of ion-pair HPLC in the presence of tetrabutylammonium counterion due to its negative charge. To examine whether the dark red metabolite in our culture was charged negatively or not, culture supernatant was analyzed by ion-pair HPLC. In ion-pair HPLC analysis six main peaks were detected (Fig. 5A). When the samples were reanalyzed in the same mobile phase but without tetrabutylammonium counterion, peaks e and f were eluted at the solvent front and did not appear in Fig. 5B. This result indicated that these peaks represented negatively charged molecules, and were assumed to be H^--TNT. The peaks identical to peaks e and f were also detected in synthetic H^--TNT mixtures. When peak f was monitored during the incubation (Fig. 5C), the change pattern was quite similar to those of OD_{475} (Fig. 3) and nitrite (Fig. 4). Although more precise confirmation of H^--TNT by LC/MS is necessary, these results indicated that *I. lacteus* could carry out denitration pathway in the TNT metabolism.

Enzymatic conversion of TNT

It has been reported that *Phanerochaete chrysoporium* was able to mineralize nitroaromatic compounds under ligninolytic conditions and both fungal mycelium and extracellular proteins had important roles in 2,4,6-trichlorophenol degradation (1, 12). Although both mycelium and extracellular proteins were also required for degradation of TNT and its metabolites such as AmDNTs in our previous study with *I. lacteus*, intact mycelium had a more crucial role than extracellular proteins (9). In addition, membrane-associated nitroreductase was required for the first steps of TNT reduction in two different metabolic pathways (14, 21). To confirm the roles of membrane-associated proteins and extracellular proteins of *I. lactues* in degradation of TNT, the enzymatic transformations of TNT by cell-free extracts and the culture supernatant were done separately (Fig. 6). Cell-free extracts showed nitroreductase activity and this activity required NADPH as a co-factor. Removal of oxygen by flushing the head space of the incubation vial with N_2 was necessary for this enzyme activity (Fig. 6D). The enzyme activity was increased according to the increase of reaction time and protein concentration, and showed a temperature optimum of 50°C at pH 7.0 (Fig. 6A-C). TNT was reduced to 2-hydroxylamino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-di-

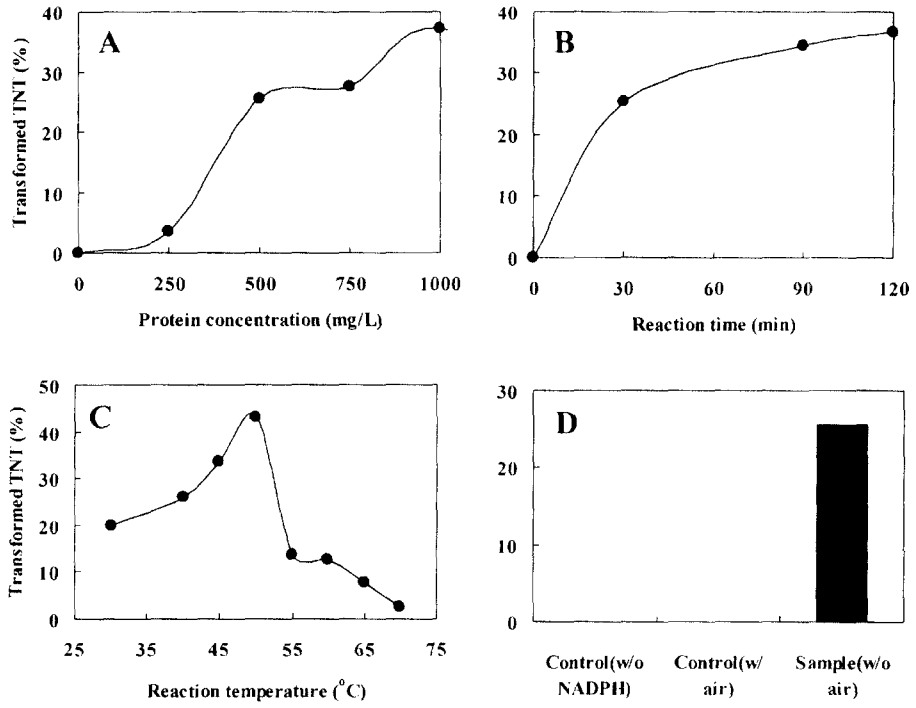

Fig. 6. Effect of protein concentration (A), reaction time (B), temperature (C), and co-factor (D) on the activity of nitroreductase of *Irpex lacteus* in TNT transformation.

nitrotoluene, tetra-azo and azoxy compounds, and negatively charged molecules representing peak f in Fig. 5A were detected in this enzymatic reaction but diaminonitrotoluene isomers were not detected. Amino-dinitrotoluene isomers were the most abundant products (data not shown). In contrast to the results with cell free extracts, nitroreductase activity was not detected in the culture supernatant. These results indicated that membrane-associated proteins of *I. lacteus* are responsible for both of the initial reactions of cascade reduction of the nitro group and denitration of TNT. Whether one enzyme system is responsible for these two different reductive pathways of TNT metabolism is the subject of further investigations, and purification and characterization of membrane associated proteins is also required. As far as azoxy derivatives are concerned, it would be difficult to determine whether these compounds are formed via enzymatic or chemical reactions, since both reactions have been reported (7, 8). Therefore, this metabolism also should be elucidated.

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