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Biodegradation of 2,4,6-Trinitrotoluene by White-Rot Fungus *Irpex lacteus*

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White-rot fungus *Irpex lacteus* degraded TNT significantly in proportion to the culture time. After 48 h incubation, about 95% of TNT was degraded. Two reduced metabolites were identified as 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT) which was further degraded.

KEYWORDS : ADNTs, Biodegradation, *Irpex lacteus*, TNT

The best known explosive compound 2,4,6-trinitrotoluene (TNT) generated as waste from the munitions and defense industries cause a significant environmental problems. The TNT is known as mutagenic and toxic to humans and other mammals and seven nitro-substituted explosives, including TNT have been listed as priority pollutants by the US Environmental Protection Agency (EPA) (Keith and Telliard, 1979). Therefore, there are considerable efforts to use microorganisms or plants for the biodegradation of TNT. Transformation of TNT typically involves a sequential reduction of nitro groups to the corresponding aminodinitrotoluenes (ADNTs), which are somewhat further transformed (Lenke *et al*., 2000; Nishino *et al*., 2000). White-rot fungi are the only microorganisms, which have been found to significantly mineralize TNT (Lenke *et al*., 2000). A number of reports on the mineralization of TNT by *Phanerochaete chrysosporium* and other fungi that mineralize TNT under ligninolytic conditions are available (Bumpus and Tatarko, 1994; Esteve-Núòez *et al*., 2001; Fernando *et al*., 1990; Hawari *et al*., 1999; Hodgson *et al*., 2000; Kim and Song, 2001). The ligninolytic white-rot fungi produce nonspecific oxidative enzymes, including lignin peroxidases (LiP) and manganese-dependent peroxidases (MnP) under nitrogen-limiting conditions (Bumpus and Tatarko, 1994; Fernando *et al*., 1990; Hawari *et al*., 1999). The ability of these organisms to degrade many persistent recalcitrants correlates with the activity of these enzymes.

In the present study the ability of another white-rot fungus *Irpex lacteus* was tested to transform TNT and identified its reaction intermediates using GC-MS.

Materials and Methods

Organism and culture conditions. The *I. lacteus* strain KR 35W was maintained on MGPY (1% malt extract, 1% glucose, 0.5% peptone, and 0.5% yeast extract) agar slants at 4°C. The fungal inocula were prepared in 250 *ml* Erlenmeyer flasks, containing 100 *ml* of MGPY medium, for 7 days. Four mycelial agar discs (0.9 cm) were obtained from a fresh MGPY agar culture for use as the inocula. The liquid inocula were gently homogenized, and used at a 10% (v/v) dilution. The stationary cultures were performed using 50 *ml* medium in 250 *ml* Erlenmeyer flasks, containing 100 ppm of TNT (Supelco, USA), at 28°C, as described in the literature (Shin, 2004).

Analyses of degradation products. To estimate the degradation rate of TNT, the contents of each flask were extracted three times with 30 *ml* of methylene chloride. The extracts were combined and concentrated by evaporation under a gentle stream of nitrogen. The resulting extracts were resuspended with 10 *ml* of methanol and analysed by reverse-phase HPLC (Shimadzu, Japan) with a Shim-pack CLC-ODS (M) column (4.6 × 250 mm). Elution was performed by a linear gradient of 20% acetonitrile containing 1% acetic acid, increased to 90% after 60 min at a flow rate of 1 *ml*/min. The retention time for TNT was determined by monitoring elution at 235 nm. GC-MS to identify and verify TNT metabolites was performed using GC-MS (HP5980 series GC-MSD; Hewlett Packard). The HP5-MS column (60 m, 0.25 mm I.D., 0.25 μm film thickness) was used for separation. The temperature program started at 50°C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 120°C at a rate of 12°C/min. The second temperature ramp was up to 200°C at a rate of 15°C/min and the final temperature ramp was up to 300°C at a rate of 20°C/min, this temperature being maintained for 15 min. Mass spectra were recorded at 1 scan s^{-1} under mass range of 50~500 amu.

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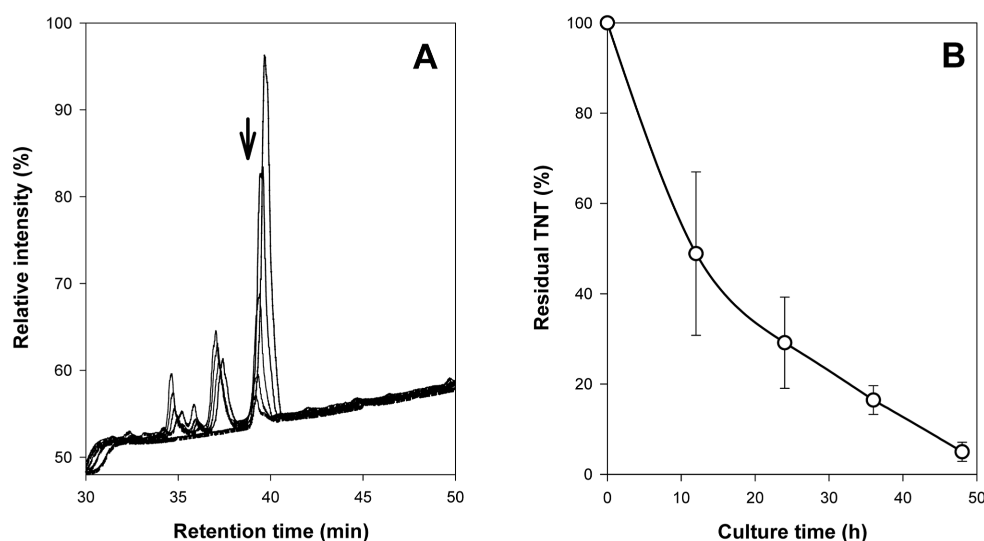

Fig. 1. Degradation of TNT by *Irpex lacteus*. HPLC chromatogram (A) and degradation rate of TNT (B) according to the culture time.

Results

A HPLC-chromatogram of the solution containing TNT at the beginning of the experiment showed a single peak, which eluted after 39.7 min (Fig. 1A). After incubation with *I. lacteus*, the intensity of TNT was decreased according to the incubation time and another two peaks were appeared. The time course of TNT degradation by *I. lacteus* was shown in Fig. 1B. TNT concentration decreased with a significant rate and about 95% of TNT disappeared after 48 h. During the degradation of TNT by *I. lacteus*, the residual TNT and degradation products were monitored through GC-MS analysis. As shown in Fig. 2, TNT was converted to about six degradation products over 24 h that eluted after 16.9, 17.6, 17.7, 17.8, 18.1, and 18.6 min respectively. The abundances of TNT and other degradations products were decreased to time-dependent manner, suggesting that *I. lacteus* may have an ability to degrade TNT and intermediates completely. Two intermediates in Fig. 2 were identified as 4-amino-2,6-dinitrotoluene (4-ADNT, peak 2) and 2-amino-4,6-dinitrotoluene (2-ADNT, peak 3) by comparison with their corresponding standards using retention times and molecular mass ions (Fig. 3).

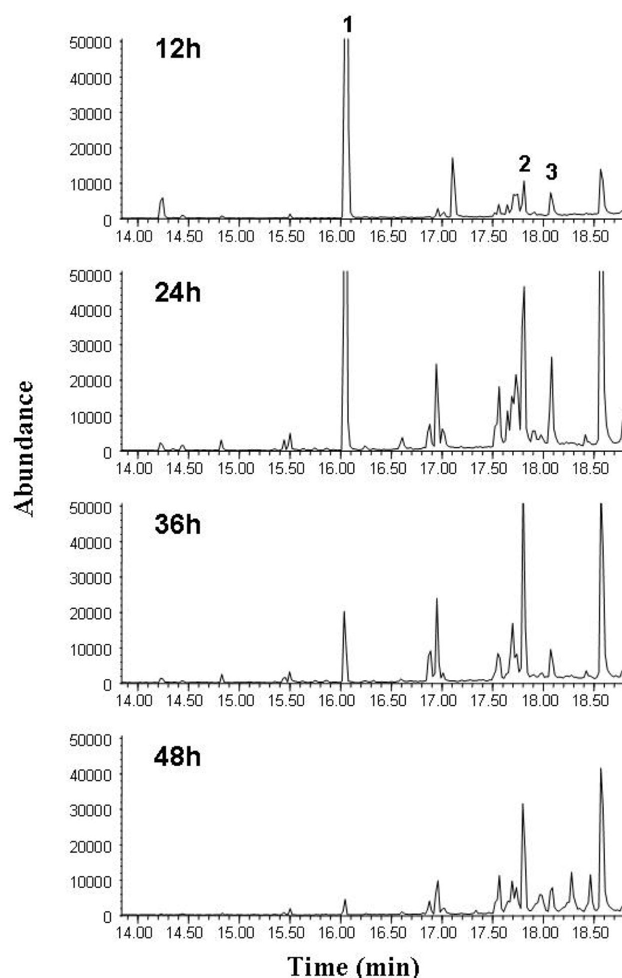

Fig. 2. GC chromatograms demonstrating the degradation of TNT after incubation with *I. lacteus*.

Discussion

Numerous authors have demonstrated the biodegradation of TNT by the white-rot fungi (Fernando *et al*., 1990; Michels and Gottschalk, 1995; Van Aken *et al*., 1999). The initial steps in the degradation of TNT involved the reduction of nitro groups (Parrish, 1977; Rieble *et al*., 1994). *P. chrysosporium* reduced TNT to a mixture of 4-ADNT, 2-ADNT, and 4-hydroxyamino-2,6-dinitrotoluene. Further degradation of these compounds and mineralization occurred under ligninolytic conditions, suggesting the involvement of ligninolytic enzymes (Parrish, 1977). Among

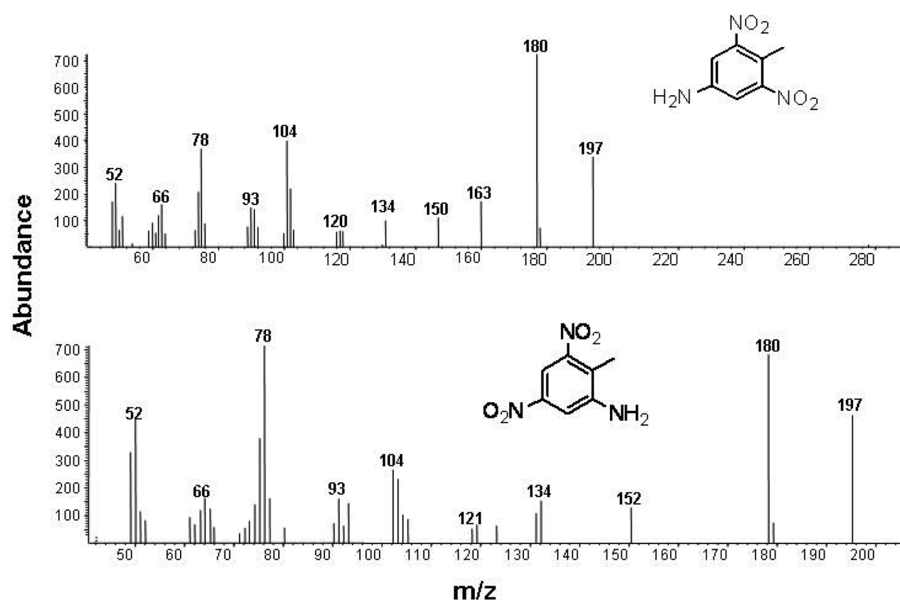

Fig. 3. The mass spectra of TNT reduced intermediates 4-ADNT (upper) and 2-ADNT (lower).

these enzyme systems, MnP may play an important role in TNT degradation. The preparations of MnP from white-rot fungi, *Nematoloma frowardii* and *Phlebia radiata*, were able to mineralize TNT and a mixture of reduction products from TNT (Scheibner *et al*., 1997; Scheibner and Hofrichter, 1998; Van Aken *et al*., 1999). However, LiP catalyzed oxidation of early TNT metabolites leading to the corresponding nitroso-dinitrotoluenes, but not capable of oxidizing the 4-ADNT and 2-ADNT (Michels and Gottschalk, 1995). Although we could identify only two metabolites, 4-ADNT and 2-ADNT in this experiment, the degradation process of TNT by *I. lacteus* was seemed to be similar to that of other white-rot fungi. Previously, we have reported that MnP of *I. lacteus* played a major role in the decolorization of textile industry wastewaters (Shin, 2004) and purified MnP (53 kDa) catalyzed oxidation of various dyes (Shin *et al*., 2005). In conclusion, white-rot fungus *I. lacteus* is a good candidate for the biodegradation of TNT as well as its reduction metabolites. Furthermore the MnP of this fungus together with LiP may involve in the mineralization of TNT.

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