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Removal of Nitro- and Amino- Aromatic Energetic Compounds by Fungal Enzymes

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by

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ABSTRACT OF THE THESIS

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Professor Shaily Mahendra, Chair

Energetic compounds, such as nitro- and amino-substituted aromatics, are widespread and persistent in industrial and military sites, which serve as non-point sources for contamination of groundwater and surface waters. Many of these compounds are carcinogenic, mutagenic, hepatotoxic, and cause other deleterious effects on human health and ecology. The application of microorganisms could provide cost effective solutions to manage large contaminated areas where other ex-situ (pump & treat or excavation) or in-situ (physical/chemical/thermal) technologies are prohibitively expensive. While both bacterial and fungal communities are known to degrade energetic compounds such as 2,4-dinitrotoluene (DNT) and 2-amino-4-nitrotoluene (ANT), fungi can catalyze the degradation of pollutants by secreting non-specific extracellular enzymes and also decrease pollutant mobility via biosorption. Thus, ligninolytic fungal enzymes are attractive candidates for in situ remediation of explosives but have limited stability in certain environmental conditions. Various methods have been previously proposed for improving enzymatic activity and longevity to achieve high biodegradation rates without the need for frequent replenishment.

This research investigated biodegradation of DNT, ANT, and 2,4-diaminotoluene (DAT) by live cultures of the wood-decaying fungus, *Phanerochaete chrysosporium*, and its purified enzyme, manganese peroxidase (MnP). A novel approach for increasing MnP stability in reactive conditions, by producing recombinant enzymes and packaging into ribonucleoprotein nanoparticle cages, called vaults, was also tested. In laboratory studies, *P. chrysosporium* removed over 50% of DNT and ANT by biosorption and another 25-30% by biodegradation within 120 hours. The stability and activity of natural MnP (nMnP) and vault-packaged MnP (vMnP) were monitored over time. While initial degradation rates by nMnP were slightly faster, overall substrate removals were similar, indicating vault packaging did not limit substrate diffusion. As vault structure is dynamic and can hold up to 78 copies of macromolecules, the packaged enzymes resist inactivation and maintain activities longer even at low doses. The rates of degradation of ANT by nMnP as well as vMnP in-vitro were 44% and 49% faster, respectively than those catalyzed by *P. chrysosporium* cultures. Furthermore, vMnP, even at the lower activity, was able to degrade ANT and DAT, whereas no significant removal was observed with the nMnP enzyme under tested conditions. This study is the first report of DAT degradation by MnP, which adds new insights to the previously published pathway for DNT degradation.

Collectively, these results indicate that fungal enzymes packaged in vault nanoparticles are more stable, concentrated, and active, and would be effective in biodegradation of energetic compounds in industrial waste treatment systems and contaminated environments.

The thesis of Anjali Ghanshyam Lothe is approved.

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Acronyms

ABTS 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

ANT 2-Amino-4-nitrotoluene.

ATSDR Agency for Toxic Substances and Disease Registry.

DAT 2,4-Diaminotoulene.

DNT 2,4-Dinitrotoluene.

MVP Major Vault Protein.

PCR Polymerase chain reaction.

SDS-PAGE Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis.

Sf9 *Spodoptera frugiperda*.

TRI Toxics Release Inventory.

USEPA United States Environmental Protection Agency.

YPD Yeast Peptone Dextrose.

Introduction

Nitro- and amino-aromatic compounds such as 2,4-dinitrotoluene (DNT), 2-amino-4-nitrotoluene (ANT) and 2,4-diaminotoluene (DAT), are precursors to the explosive materials and are grouped under the category of secondary high explosives. [1] DNT, ANT, and DAT are not naturally occurring compounds. Report on the toxicological profile of dinitrotoluenes by ATSDR (2016) states that the frequency of 2,4-dinitrotoluene detection in the environment is low. Typical concentration range of DNT in water is 0.04-48.6 mg/L and in soil is <0.001to 84 mg/kg. [2] Concentrations of ANT and DAT in the environment are rarely reported, but their harmful effects on the environment and human health are extensively studied. [3–5] DNT, ANT, and DAT are used as ingredients for the production of polyurethane foam. DNT is also a precursor to trinitrotoluene and an ingredient for toluene diisocyanate synthesis. DAT is also extensively used for manufacturing toluene diisocyanate and dyes. These compounds can find their way into soil and groundwater from the military training sites [6] or the abandoned factories. [7–9] DNT occurrence near the coastal tidal zone has also been reported [10], and it can be detected in the environment because of wastewater discharge or improper waste disposal. DNT tends to persist in the environment for a long time because of its low vapor pressure and Henry's Law constant, which prevents its volatilization. [11,12] Wastewater generated during the trinitrotoluene purification may contain mixtures of DNT and ANT. [13] Another source of nitro and amino aromatics in the soil and groundwater are landmines residues. [14] Studies have also verified the distribution of nitroaromatics and their amino-derivatives via the food chain. [15] DNT is identified as a Class B carcinogen; ANT is a confirmed Class A3 carcinogen whereas, DAT is a known mutagen and hepatocarcinogen. [16] Adsorption of DNT, DAT, and ANT on skin causes methemoglobinemia, skin irritation, and dermatitis. Other diseases associated with

the exposure of these compounds are anemia, cyanosis, and liver damage. [17–20] ANT and DAT have also been listed on TRI list of toxic chemicals 2016 by USEPA. [21–23]

Physical and chemical treatment processes for degradation of these contaminants include advanced oxidation, chemical reduction, and separation methods like resin adsorption, liquid-liquid extraction, ultra-filtration and reverse osmosis, advanced adsorption using granular activated carbon. [24] A major disadvantage of separation processes is that the pollutant remains unchanged whereas other technologies are uneconomical as well as energy intensive. Many recent studies on biodegradation of DNT have shown that this compound can be degraded under aerobic and anaerobic conditions. [25] Under aerobic conditions, the degradation is initiated by dioxygenase, forming 4-methyl-5-nitrocatechol, but under anaerobic or limiting oxygen conditions, as in the case of many groundwaters, terminal reductases reduce DNT to DAT via the formation of ANT. It has been observed that the fungus *Phanerochaete chrysosporium* can mineralize DNT within 24 days. [26] *P. chrysosporium* is a wood-rotting fungus capable of breaking lignin to CO_2 and H_2O. The first step of degradation of DNT by *P. chrysosporium* includes reduction of DNT to ANT, which is an intracellular process. After this reduction, if nitrogen limiting conditions are provided, the fungus produces extracellular manganese peroxidase (MnP) and lignin peroxidase, ligninolytic enzymes which, subsequently mineralize ANT. [27, 28] Apart from *P. chrysosporium* many other organisms have been studied for degradation of DNT and are summarized in Table 1. Studies show that reduction of DNT to its amino-derivatives is a common pathway under anaerobic conditions. [25]

Although degradation by fungi seems promising, these fungi require specific substrates for growth and favorable environmental conditions, such as pH, temperature, and oxygen concentration, which limit their applicability for in-situ applications. Cell-free extracts and purified enzymes also have limited stability in the environment. Indeed, most ligninolytic enzymes are active only in

acidic or alkaline pH and are sensitive to temperature changes. [29,30] Immobilization of enzymes on the solid matrices can enhance enzymatic stability and longevity but, at the cost of diffusion limitation. [31] Most immobilization techniques involve toxic precursors and harsh reaction conditions. [32]

Vault nanoparticles can provide a solution to this problem by increasing enzyme longevity and stability without substrate diffusion limitations. Vaults are naturally-occurring nanoparticles found in most eukaryotes, including humans. [33] These are the capsule shaped hollow ribonucleic protein structures, capable of storing other proteins inside them. This property of vault nanoparticles is well studied and explored in the field of medical science because vaults are believed to have great potential for target specific drug delivery. [34, 35] A vault particle, is made up of 78 copies of major vault protein (MVP), with approximately 39 MVP in each half. Previously, manganese peroxidase derived from the *P. chrysosporium* was packaged in the vault nanoparticle by Wang and coworkers. [36] Phenol was used as the model compound to study the vault-encapsulated MnP (vMnP) application for environmental remediation. The study compared the catalytic efficiency of natural MnP (nMnP), INT fused recombinant MnP (MnP-INT), and vMnP for phenol degradation. vMnP was able to degrade 98% phenol within 24 hours at starting enzyme activity of 55.7 U/L. The study also showed that vault encapsulation of MnP increased its tolerance towards pH and temperature variance. [36]

Prime objective of this research was to compare the degradation by vMnP with nMnP and *P. chrysosporium* using DNT, ANT, and DAT as model compounds. This study builds upon the previously published pathway for DNT degradation and aims to compare the rate of degradation of DNT and ANT by *P. chrysosporium* with the rate of free enzyme mediated degradation. vMnP and nMnP catalyzed degradation reactions at different starting enzyme activities will be used to evaluate

the change in the rate of degradation with respect to enzyme activity. Stability of these enzymes will also be analyzed using ANT and DAT degradation experiments at low enzyme activities.

Materials and Methods

Growth of *Phanerochaete chrysosporium* and Manganese Peroxidase Production

The fungus *P. chrysosporium* (ATCC: 24725) was cultivated for 5-7 days on agar plates containing 20 g/L agar, 10 g/L malt extract, 10 g/L glucose, 2 g/L peptone, 2 g/L yeast extract, 1 g/L asparagine, 2 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, and 1 mg/L thiamine at 37 °C. [37] Fungal spore suspension was made by scraping *P. chrysosporium* mycelium from the agar plates using a sterile inoculation loop and suspending it in the appropriate volume of liquid culture medium. The spore count was performed on hemocytometer by diluting the spore solution ten times. A coverslip was placed over hemocytometer, and 10 μL of the diluted spore solution was added in the provided slot. The cytometer was then placed under a microscope to count the spores and number of spores were calculated using equation 1.

$$\text{Number of spore (Spores/mL)} = \text{Average spore count per square} \times \text{Dilution factor} \times 10^4 \quad (1)$$

To induce the production of manganese peroxidase (MnP), the nitrogen limiting conditions were provided by growing *P. chrysosporium* in the modified Tien and Kirk liquid culture media containing, 10 g/L glucose, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2$, 0.03 g/L $MnSO_4$, 0.06 g/L NaCl, 6 mg/L $FeSO_4.7H_2O$, 6 mg/L $CoCl_2$, 6 mg/L $ZnSO_4.7H_2O$, 6 mg/L $CuSO_4$, 0.6 mg/L $AlK(SO_4)_2.12H_2O$, 0.6 mg/L H_3BO_3, 0.6 mg/L $Na_2MoO_4.2H_2O$, 0.012 g/L yeast extract,

0.2 g/L di-ammonium tartarate ($C_4H_{12}N_2O_6$), 1 mg/L thiamin, 0.07 g/L veratryl alcohol, and 0.5 g/L tween 80, in 20 mM sodium acetate buffer (pH 4.5). [38] Fungus was cultivated in 125 mL baffled culture flasks, equipped with 0.2 μm filter for sterile air exchange, by inoculating 50 ml of the above mentioned liquid media with 10^4 spores/mL. The culture flasks were shaken continuously at 150 rpm, 37°C. On the seventh day post-inoculation when MnP activity reached maximum, MnP was harvested by centrifuging the culture supernatant at 3000g for 10 min to remove the fungal biomass. [39] The crude MnP extract was then passed through 30 kDa Amicon ultra-centrifugal filters, and the concentrated natural MnP enzyme was collected after centrifuging at 4000g for 45 min.

Enzyme Activity Assay

To measure MnP (ϵ=11590 L/(mol*cm)) activity, using the malonate assay, 0.100 mL enzyme sample was mixed with 0.050 mL, 10 mM $MnCl_2$ and 0.80 mL, 50 mM malonate buffer (pH 4.5). The reaction was initiated by adding 0.050 mL, 2mM H_2O_2. [40,41] An enzyme free control was used to set the baseline absorbance of the substrate. The absorbance change was measured for 3 min at 270 nm. Similarly, ABTS assay was also used to evaluate the activity of MnP (ϵ=36000 L/(mol*cm)); 0.015 mL enzyme was mixed with 0.150 mL, 50 mM malonate buffer (pH 4.5) containing 0.015 mL, 1.4 mM ABTS, 0.015 mL, 20 mM $MnCl_2$ and reaction was initiated by adding 0.015 mL, 4 mM H_2O_2. Change in absorbance was measured for a minute at 420 nm. One unit of enzyme activity was defined as the amount of enzyme needed to oxidize 1 μmol of the substrate in 1 min. Enzyme activity was calculated using the equation 2,

$$\text{Activity}(U/L) = \frac{\Delta A \times V_T \times 10^6}{\xi \times b \times V_{en}} \tag{2}$$

where, U/L is units of enzyme activity per liter, ΔA is rate of absorbance change (min^{-1}), V_T is total volume (L), ξ is molar extinction coefficient (L $mol^{-1}cm^{-1}$), V_{en} is enzyme volume (L) and, b is path length (cm). Activity for vault packaged MnP was estimated using the microplate assay, described elsewhere. [36] First, the activity of the natural enzyme was measured using the ABTS assay described above. Then the nMnP was serially diluted into the subsequent microplate wells, and undiluted vMnP was also added to the microplate wells. All the reactions were initiated by adding H_2O_2 simultaneously, using a multichannel pipetter. The reactions were incubated for 5 min at room temperature, and development of green color due to $ABTS^+$ formation was compared. The activity for vault packaged enzyme was then calculated using the appropriate dilution factor.

Production of Recombinant INT-fused Manganese Peroxidase (MnP-INT)

Plasmid construct described by Wang et al. [36] was used for transformation of DH10Bac *E.coli* enabling the formation of bacmid, which was extracted from the positive colonies, screened and selected using ampicillin resistance, and blue-white screening. Bacmid extraction was performed using plasmid extraction kit supplied by New England Bio Labs. The extracted bacmid was verified for successful recombination of the gene of interest by colony PCR using M13 primers. *Spodoptera frugiperda* (Sf9) cells were then transfected by positive bacmids using instructions from Bac-to-Bac manual supplied by Invitrogen to produce recombinant viruses which were then amplified once. The amplified viral stock was then used to infect healthy Sf9 cells to express the MnP-INT protein. Sf9 cells at final concentration of 2 x 10^6 cells/mL in 50 mL SF900II SFM media were infected using 10 μL of the amplified baculovirus stock and incubated at 27°C, 150 RPM for 96 h. Extracellular expression was verified by SDS-PAGE and Western blot analysis using anti-INT

antibody (Figure. 3). On the fourth day post-infection, the culture supernatant was collected by pelleting the cells at 500g for 15 min and centrifuged again for an hour at 100,000g to remove the baculovirus. MVP protein was obtained from recombinant *Pichia pastoris*, modified to produce the major vault protein intracellularly. Recombinant yeast was grown on the YPD agar plates containing 100 μg/L zeocin for long-term storage. For large scale expression yeast was grown overnight at 30 °C, 225 RPM in 3 mL of YPD liquid media with 100 μg/L zeocin. The overnight grown culture was then used to inoculate 100ml of YPD liquid media without zeocin to a final OD_{600} of 0.03. The culture was incubated at 30 °C, 225 RPM for 32 h after which the yeast cells were harvested by centrifuging at 3000g for 5 min.

A gram of yeast cell pellet was resuspended in 6 volumes of lysis buffer (50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5% glycerol, 1 mM Dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), 1X protease inhibitor sigma 8849 (PI)) and lysed using 4 volumes of 0.5 mm glass beads by bead beater for 5 min. Lysate was then centrifuged at 20,000g for 20 mins at 4°C and the resultant supernatant was ultracentrifuged at 100,000g for an hour at 4°C. The pellet obtained was then homogenized using a douncer and resuspended in 3.5 volumes of buffer contain 50 mM Tris-HCl (pH 7.4), 75 mM NaCl, 0.5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 1 mM PMSF and 1X PI. Samples were further treated to obtain the MVP protein as described elsewhere. [42] The purified vault samples were then incubated with the MnP-INT solution, continuously shaking at low rpm for an hour at 4°C. The resultant vault-MnP pellet obtained after ultracentrifuging at 100,000g, was then resuspended in buffer A described elsewhere. [36]

Degradation of 2,4-Dinitrotoluene, 2-Amino-4-nitrotoluene and 2,4-Diaminotoluene by *P. chrysosporium*

The fungal degradation reactions were performed in 125 ml flasks, equipped with 0.2 μm filter, containing 25 ml of modified Tien and Kirk medium with inoculation of fungal spores to a final concentration of 10^4 spore/mL, as described above. Abiotic and killed controls were also maintained similar to the degradation reactions. In case of killed controls, the four day old fungal cultures were autoclaved at 121°C for 45 minutes, and the substrates were added after the culture returned to the room temperature. All cultures were kept in triplicates to obtain statistically significant results. Substrates DNT, ANT, and DAT, to a final concentration of 10 mg/L were added to the flasks after 4 days of fungal growth. Samples were collected after every 24 h for 96 h as 200 μL aliquots, and an equal volume of methanol was added to inactivate the enzyme. This mixture was filtered using 0.2 μm nylon filters and stored at -20 °C till needed for analysis.

Natural MnP and vault packaged MnP Catalyzed Degradation of 2,4-Dinitrotoluene, 2-Amino-4-nitrotoluene, and 2,4-Diaminotoluene

To evaluate the efficiency of nMnP and vMnP for removal of nitroaromatic explosives, 200 μL enzymatic degradation reactions were performed in sterile 2 mL, 96 well polypropylene plate sealed with a silicon mat. nMnP was purified as mentioned above. To rule out losses due to abiotic factors and H_2O_2, enzyme free controls, and H_2O_2 controls, were also kept in identical conditions. For nMnP and vMnP catalyzed reactions the reaction mixture consisted of appropriate enzyme activity in 50 mM malonate buffer (pH 4.5), 0.1 mM $MnCl_2$, 10 mg/L substrate and the reaction was initiated by adding 0.2 mM H_2O_2. 2,4-dinitrotoluene (97%), 2-amino-4-nitrotoluene (2-Methyl-5-

nitroaniline) (98%) and 2,4-diaminotoluene (98%) were obtained from Sigma Aldrich, Ark Pharma Inc., and Arcos Organics, respectively. Stocks for DNT and ANT were made by dissolving appropriate amounts in methanol whereas DAT stock was generated in the deionized water. All reactions were incubated in shaking incubators at 150 rpm at 30°C. The reactions were stopped by adding 1 volume of methanol [43] every 24 h and the sample was filtered using 0.2 μm nylon filter and stored at -20 °C till needed for analysis on HPLC-UV. All controls and reactions were kept in triplicates to obtain statistically significant results.

To ensure that MnP was active when added at lower concentrations, the positive control reactions were performed using 10 mg/L phenol as the substrate under similar reaction conditions as described above. These phenol reactions were stopped by addition of iron-ferricyanide, and 4-aminoantipyrine supplied in the K-8012 Phenols CHEMets Kit.

Analytical Methods

The concentrations of DNT, ANT, and DAT were measured on HP 1050 HPLC using the method described by Thomas et al. using a ZORBAX Eclipse XDB C18, 5 μm, 4.6 x 150 mm HPLC column. [44] According to the previously published method, DNT, ANT, and DAT were detected at 296 nm and were eluted after 7 min, 3 min, 1.8 min, respectively at 1 mL/min using 40% H_2O and 60% CH_3OH . [44] Analysis of phenol was performed using the spectrophotometric method. Phenolic compounds are known to react with 4-aminoantipyrine in the presence of iron-ferricyanide to form a reddish-brown color dye, which absorbs the 510 nm wavelength. [45,46] Standards from K-8012 Phenols CHEMets Kit were used for making a standard curve, all the degradation reactions were stopped by adding iron-ferricyanide and 4-aminoantipyrine, and the absorbance was measured

on spectrophotometer immediately.

Results

Estimation of nMnP and vMnP Activity and Production of Recombinant Enzyme.

Production of MnP was monitored using the malonate assay over a period of 18 days, from the start of the fungal cultivation in the modified Kirk medium as described above. The cultures were kept in quadruplicates to ensure the statistically significant results. It was verified that the MnP activity in the batch reactions started on day 4 and peaked on the 7th day post-inoculation and gradually decreased to the baseline activity. The maximum average activity of 5200 U/L was obtained (Figure 1). Microplate ABTS assay was used to estimate the activity of vMnP. Figure 2 shows the development of green color due to oxidation of ABTS. Western blot analysis by the anti-INT antibody (Figure 3) verifies that recombinant protein expressed by Sf9 insect cells is MnP-INT (60 kDa). [36]

Degradation of 2,4-Dinitrotoluene and 2-Amino-4-nitrotoluene by *P. chrysosporium*

It was observed that in the presence of live fungi, 84.2% of DNT was removed within 120 h, and later dropped below detection limits. The killed controls showed the removal of 51.7% due to biosorption, while the abiotic controls remained significantly constant (Figure 4).

In a separate batch reaction, 57.7% of ANT was removed by *P. chrysosporium* within 96 h,

after which the rate of removal was negligible. Biosorption in the killed control reactions showed 32.3% decrease in the ANT concentration (Figure 5), whereas insignificant change was observed in the abiotic controls. Significant difference between biosorption and degradation was observed after 48 h of incubation with fungi.

Degradation of 2,4-Dinitrotoluene, 2-Amino-4-nitrotoluene and 2,4-Diaminotoluene by nMnP and vMnP.

All the substrates were treated with different starting enzyme activities (15 U/L, 34 U/L, 40 U/L and 150 U/L). It was observed that DNT concentration remained constant over the period of 96 h. For the starting enzyme activity of 34 U/L vMnP, 36.3% degradation of ANT was observed within 96 h, no significant change was observed in ANT concentration in the presence of 34 U/L nMnP (Figure 6). The abiotic control reactions performed to account for non-enzymatic losses also showed an insignificant difference in the concentration of ANT. Positive control reactions (Figure 7) showed, 84% and 90% of phenol removal by 34 U/L and 150 U/L vMnP, respectively. However, 34 U/L and 150 U/L nMnP removed only 50% and 68.8% of phenol, respectively. With higher nMnP and vMnP activity of 150 U/L, 72.7% of ANT was degraded. Final concentration of ANT after 96 h was similar for both vMnP and nMnP catalyzed reactions (Figure 8).

It was also observed that the rate of substrate removal for the initial 48 h in case of vMnP was 0.10 mg/L.h whereas for nMnP was 0.15 mg/L.h but the rate of removal for vMnP in next 48 h reduced to 0.06 mg/L.h and for nMnP was 0.02 mg/L.h, an average rate of removal for both the enzymes was similar (Figure 8). The similar set of reactions were also repeated for 15 U/L and 40 U/L of vMnp to analyze the change in degradation rate for ANT. 38% removal in ANT concentra-

tion was noticed after 96 h of treatment in the case of both the starting enzyme activities. There was no significant removal observed in the case of enzyme free controls, performed to account for losses due to the presence of the empty vault control and hydrogen peroxide (Figure 9).

DAT degradation experiments were performed in the presence of 15 and 40 U/L free enzymes. 51% and 59% DAT oxidation were observed respectively, for 15 and 40 U/L of vMnP, whereas, 15 U/L nMnP catalyzed DAT reactions showed negligible removal and only 25% DAT was removed by 40 U/L nMnP (Figure 10).

Discussion

The use of enzymes as biocatalysts have been around in pharmaceutical industry but have not received much attention for bioremediation because of the need to use high amounts of the enzymes, as they can be denatured in the environmental conditions. [43] To reduce the amount of enzyme required, increasing its longevity is an attractive option. It has been shown that vault nanoparticle can be a plausible candidate to achieve this objective. [36] The benefit of using the vault packaged enzyme is that it is a non-hazardous, biodegradable natural protein. Wang et al. have provided experimental data in support of the stability of vMnP in varying pH and temperature conditions. ANT and DAT degradation by vMnP at low starting enzyme activities reiterates the fact that vault packaged enzymes can be used in smaller quantities and for longer duration without the requirement of replenishment.

P. chrysosporium has been extensively used for biodegradation of a wide range of compounds, and has also been tested for DNT degradation, but the experimental proofs describing the rates of DNT degradation are limited. Degradation of DNT (Figure 4) and ANT (Figure 5) show that

effectively 30% DNT and 25 % ANT were degraded by *P. chrysosporium*, a significant amount of these compounds was lost to biosorption. A wide range of contaminants including heavy metals, inorganic mercury, 2,4-dichlorophenol, and reactive dyes have been treated through biosorption by *P. chrysosporium*. [47–50] Studies show that the biosorption kinetics for *P. chrysosporium* varies with pH and temperature [49], maximum biosorption for reactive dyes was obtained at a temperature range of 30-40 °C, [48]. The fungal degradation reactions in this study were also performed at 37 degrees, which along with the specific nutrient requirements for the growth of the fungus, could be resource intensive and energy as well as cost inefficient, and lead to biosorption.

Use of free enzymes is believed to have a high potential for in-situ applications but, it can be an ineffective method because of their inactivation in environmental conditions. Uses of immobilized or protected enzymes have been researched to enable applications of biocatalysts for bioremediation purposes. [51] Enzyme immobilization techniques like adsorption, [52, 53] covalent binding, [54, 55] entrapment, [52, 56] etc., seem promising with respect to environmental applications but are limited by diffusion of contaminants to the enzymes' active sites. [31] This study takes a step forward with the use of vault nanoparticles as enzyme protection cages. Degradation experiments in the presence of varying active nMnP and vMnP concentrations (15 U/L, 34 U/L, 40 U/L and 150 U/L) were carried out, to evaluate the effects of vault packaging. It can be noted that when ANT was treated in the presence of 150 U/L of nMnP and vMnP, 72.7% degradation was detected for both the enzymes. 150 U/L nMnP and vMnP were essentially the same for ANT removal outcomes, but when similar sets of reactions were repeated at a lower activity, nMnP showed an insignificant decrease in the ANT concentration (Figure 11) while vMnP showed significant ANT removal. Similar trends were observed for nMnP, and vMnP catalyzed DAT degradation. Statistically significant removal of DAT was observed at lower (15 U/L) vMnP concentration while the

removal by nMnP concentrations lower than 40 U/L was insignificant.

The idea that vMnP works effectively even at smaller concentrations compared to nMnP can be associated with the lower inactivation rates. Wang et al. have demonstrated that vault packaged MnP maintains its activity even after incubation at variable temperatures for a longer duration. This study has also performed experiments demonstrating the k_m value for the vault packaged enzyme being slightly higher than that of the recombinant protein. [36] Vault nanoparticle has also been proved structurally stable at variable pH and temperatures. [57]

The fact that one vault nanoparticle can package multiple copies of the enzymes could also explain the trends for biodegradation observed at lower enzyme activities. In a previous study Kickhoefer et al. encapsulated the largest non-vault, INT fused, firefly luciferase protein (61kDa) and were able to demonstrate fluorescence in the recombinant vaults. This study mentions that one vault particle has 96 copies of major vault protein with internal volume of 5×10^7 Å^3 which is sufficient for binding nearly hundreds of proteins per vault particle. [35] Ability of vault to encapsulate more than one copy of protein helps in concentrating the MnP enzyme, which may increase the reaction efficiency as observed for ANT and DAT degradation. Also, the studies show that locally concentrated enzymes show effective results compared to spatially separated enzymes. [58]

The rate of degradation of ANT changed in minimal proportions on increasing the enzyme activity for vMnP (Figure 9 and 12). Similar observations were made for phenol and DAT degradation rates by vMnP (Figure 7 and 10). We suggest that nearly constant rate of degradation at variable enzyme activity is observed because the rate of reaction for vaults packaged enzymes may not be entirely substrate or enzyme dependent but may also depend on the factors that affect the interaction of the enzyme with the substrate. Studies prove vault cage is a dynamic structure rather than a rigid mass. Poderycki et al. and Querol-Audi et al. have suggested that the forces binding two

halves of the vaults may be less stable than the forces binding 39 monomers of major vault protein (MVP), forming the half vault particle. [59, 60] Another study by Yang et al. concluded that vaults interact with each other by exchanging their halves at a very high rate, this mechanism is common vault behavior irrespective of its environment. [61] All these studies help strengthen the idea that nearly similar rates of degradation for the substrates are not only governed by minimum enzyme activity but also by vault-vault interactions.

In this study we built upon the previously predicted pathway for DNT degradation by Valli et al. (Figure 13). [62] The study concluded that DNT intracellularly reduces to either ANT or DAT, then under ligninolytic conditions, these fungal metabolites are mineralized by manganese peroxidase or lignin peroxidase. Removal of 84.2% of DNT by *P. chrysosporium* within 96 h in our study, is in confirmation with the previous study. Valli et al. have determined isomers of ANT and DAT as the metabolites for *P. chrysosporium* and have shown degradation of 2-amino-4-nitrotoluene by purified MnP for development of the pathway and has hypothesized that DAT would pursue the similar pathway as ANT. [62] Our study has provided experimental proof for DAT degradation (Figure 10). There are limited studies for degradation of DAT, and it has been shown to degrade under aerobic conditions by *Pseudomonas aeruginosa*, after ten days of incubation period. [63] Degradation of DAT by 40 U/L vMnP provides the experimental confirmation for improved biodegradation efficiency of vMNP compared to the live organism or nMnP.

There is much literary evidence supporting the intracellular reduction of DNT to its amino derivatives and are summarized under Table 1. [25, 64–68] These studies support intracellular conversion of DNT to either ANT or DAT by different microorganisms. [25] USEPA has also published in the DNT technical fact sheet that DNT gets converted to ANT naturally, within 70 days. [69]. Our study is in agreement with the previous results as, DNT removal was observed only by *P.*

chrysosporium but when s treated by purified MnP no significant removal of was observed. Apart from the microorganisms and plants, zero valent iron has also been used for reduction of DNT. DNT has been shown to reduce to DAT by zero valent iron within 20 min. Other studies have also performed kinetics evaluation at varying pH conditions. [44, 70]

For the application of the vault technology towards DNT degradation, future work will be required to develop sequential techniques which start with the abiotic/biotic reduction of DNT followed by vault-encapsulated enzyme-catalyzed mineralization of the reduced products. The findings in this research imply that vault packaged enzymes would be an efficient technology for industrial applications due to their ability to degrade a wide variety of compounds like nitro- and amino-aromatic explosive and phenolic compounds at low active enzyme concentrations. Increasing the enzyme activity of vMnP did not significantly change the rate of degradation hence, for field applications the only necessity would be to determine the minimum amount of active enzyme required for degradation. Since vault packaged enzymes are more stable, the amount of active enzymes available for degradation per gram of the product applied would be significantly higher and will be available for a longer time due to slower inactivation, as compared to the natural enzymes, thereby reducing the cost of treatment. To strengthen the conclusion that vault-encapsulated enzymes catalyzed degradation is primarily controlled by the vault-vault interaction rather than higher enzyme activity, similar experimental studies could be repeated using different contaminants as well as different enzymes packaged in the vault nanoparticles. These observations could also be used to engineer the factors affecting vault-vault interaction hence, increasing the substrate degradation rates. Vaults, therefore, could be identified as enzyme concentrating dynamic protective cages.

Figures

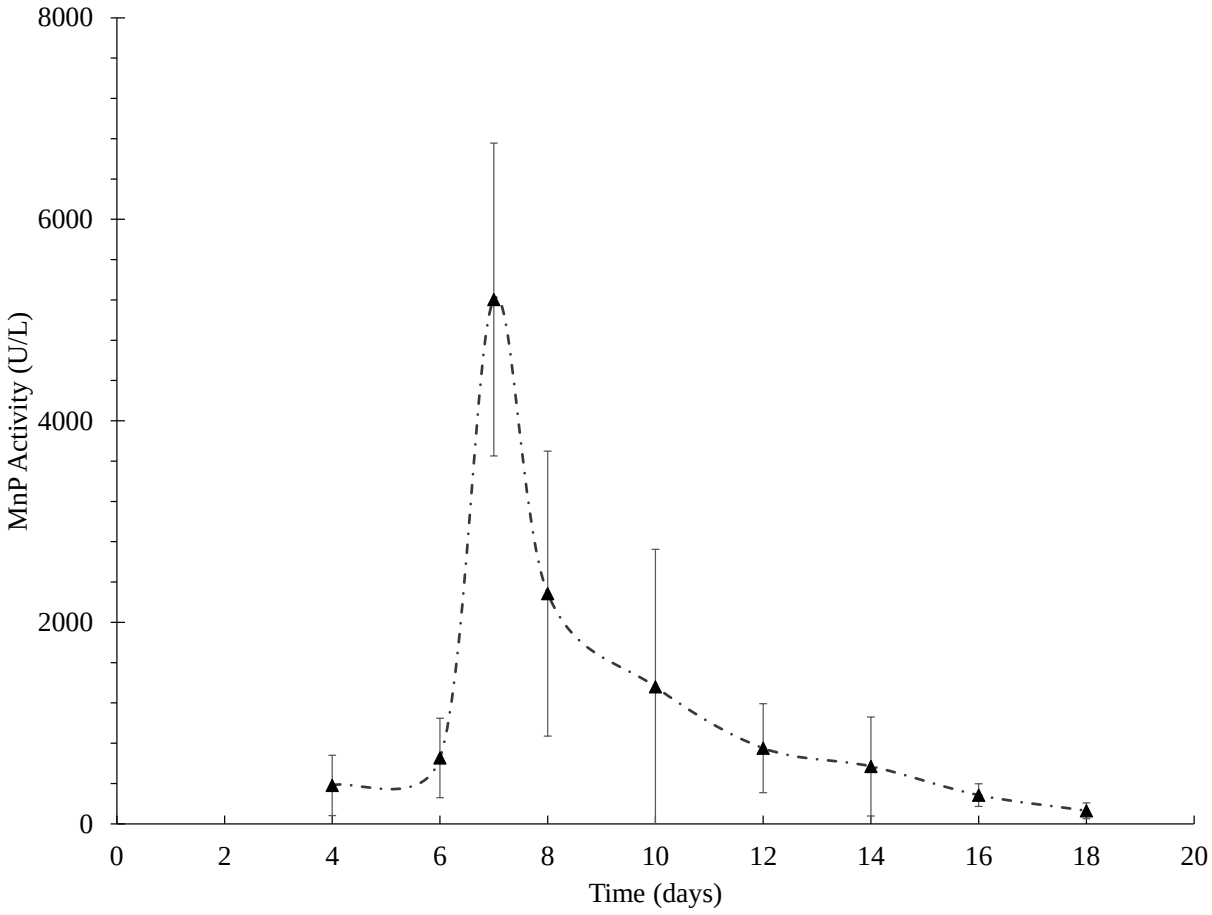

Figure 1: Activity of manganese peroxidase in the *P. chrysosporium* cultures. On the fourth day postincubation, at 37°C, activity quantification for MnP enzyme was initiated. The activity (U/L) was determined using the maloante assay, by measuring the change in absorbance at 270 nm for 3 mins. Activity estimation was done in quadruplicates and is represented as the mean activity ± standard deviation.

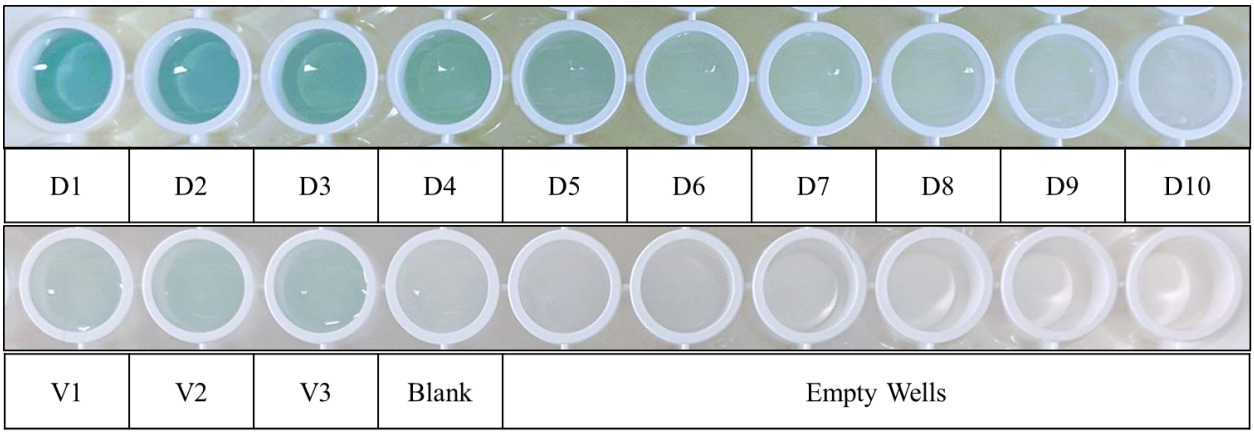

Figure 2: Vault-encapsulated manganese peroxidase activity estimation. Wells D1-D10 show the ABTS oxidation results in presence of serial dilutions of nMnP. Each well contained 10 times diluted enzyme (e.g., nMnP in D7 well was 10^7 time diluted from D1) and intensity green color correlates to activity. Wells V1, V2, and V3 represent the triplicates for the vMnP enzyme. Empty wells account for any background coloration and blank represents the enzyme free control with absent ABTS to $ABTS^+$ conversion. Here, activity of vMnP was equivalent to activity of 10^6 times diluted nMnP stock.

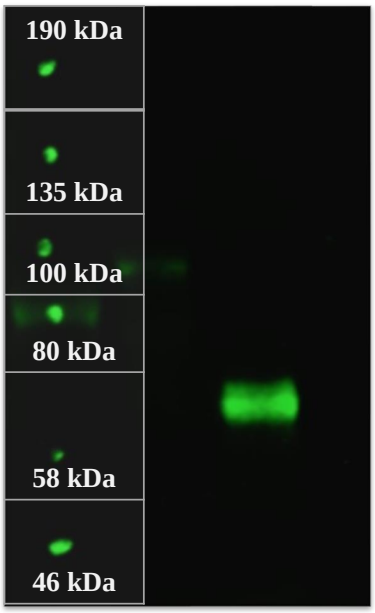

Figure 3: Verification of recombinant MnP-INT expression. The MnP-INT band obtained after Western blot analysis on 7.5% SDS-PAGE gel by anti-INT antibody provided the evidence of MnP-INT expression by Sf9 insect cells

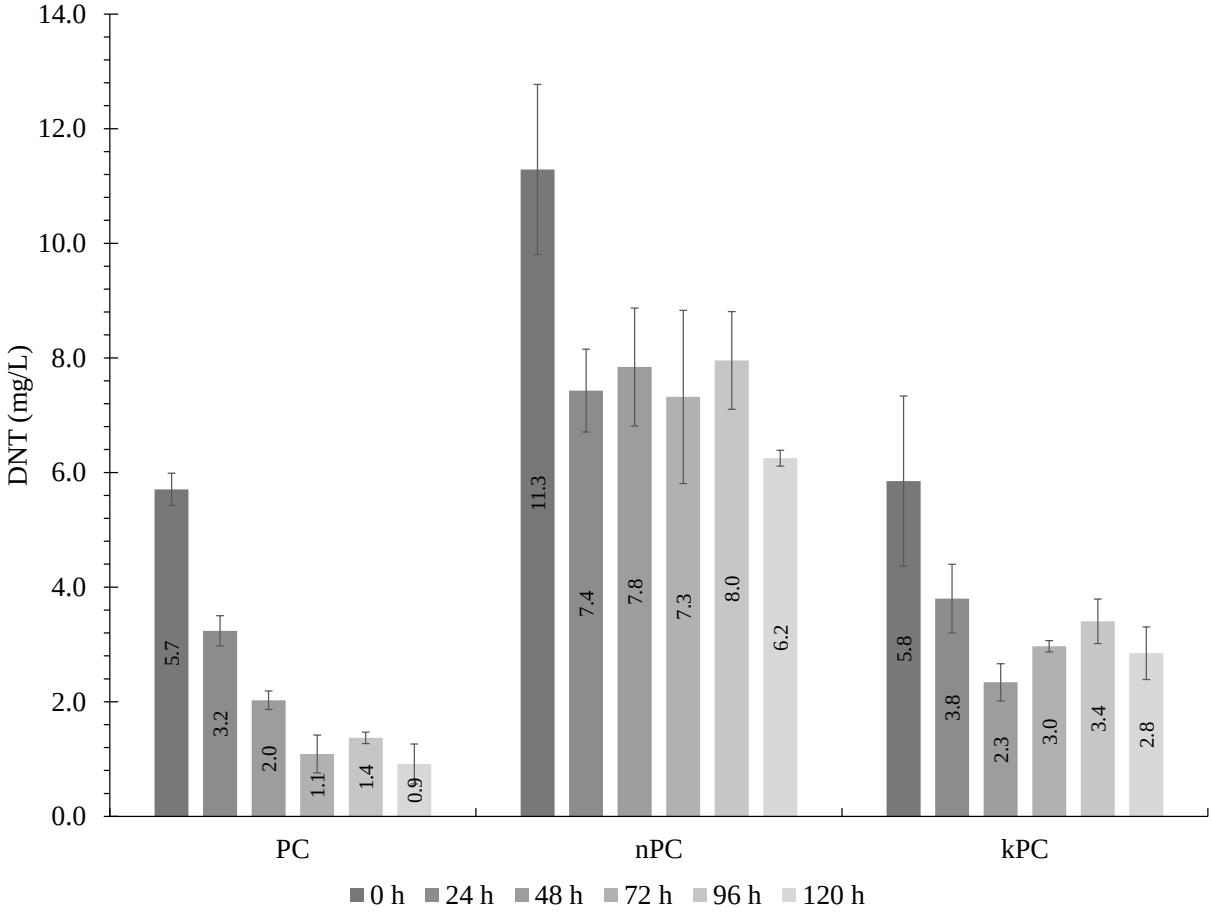

Figure 4: Degradation of 2,4-dinitrotoluene by *P. chrysosporium*. **PC** - DNT degradation by live *P. chrysosporium*. **kPC** - killed control samples containing the dead fungal biomass. **nPC** - abiotic controls. All reactions were performed in triplicates and treated similarly. DNT removal within initial 24 h in abiotic controls, could be observed due to the matrix effect. Samples from the batch reactor were collected immediately after addition of the substrate, in an interval of 24 h for 96 h. Decrease in the DNT concentration at 0 h in killed control and degradation reaction can be associated with the biosorption on the fungal mycelia. 84.2% DNT was removed by *P. chrysosporium* including 51.7% removal due biosorption. Data are representation of the mean value of triplicates ± standard deviation. (p-value <0.05)

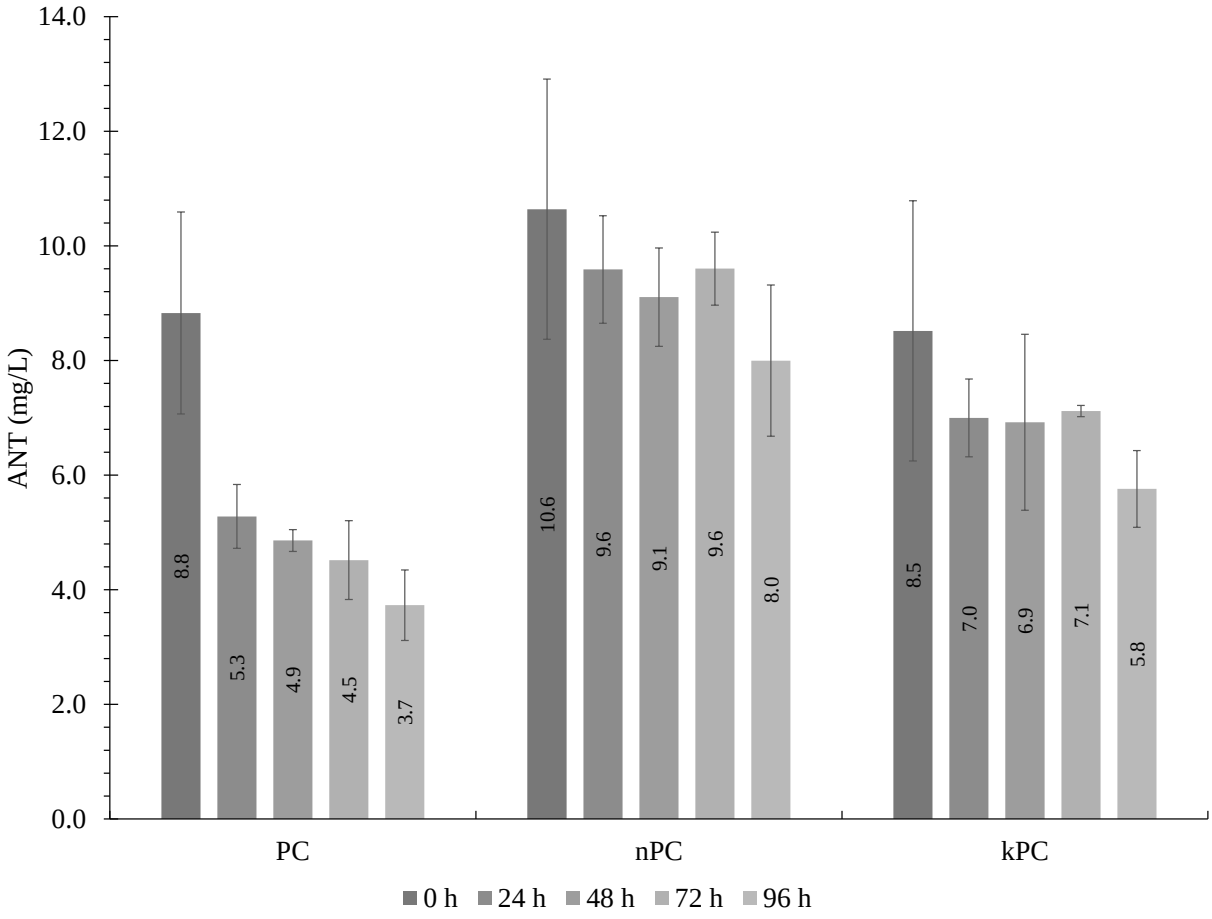

Figure 5: Degradation of 2-amino-4-nitrotoluene by *P. chrysosporium*. **PC** - ANT degradation by live *P. chrysosporium*. **kPC** - killed control samples containing the dead fungal biomass. **nPC** - abiotic controls. All reactions were performed in triplicates and treated similarly. ANT removal within initial 24 h in abiotic controls, is attributed to the matrix effect. Samples from the batch reactor were collected immediately after addition of the substrate, in an interval of 24 h for 96 h. Decrease in the ANT concentration at 0 h in killed control and degradation reaction can be associated with the biosorption on the fungal mycelium. 57.7% ANT was removed by *P. chrysosporium* including 32.3% removal due biosorption. Data are representation of the mean value of triplicates ± standard deviation. (p-value <0.05)

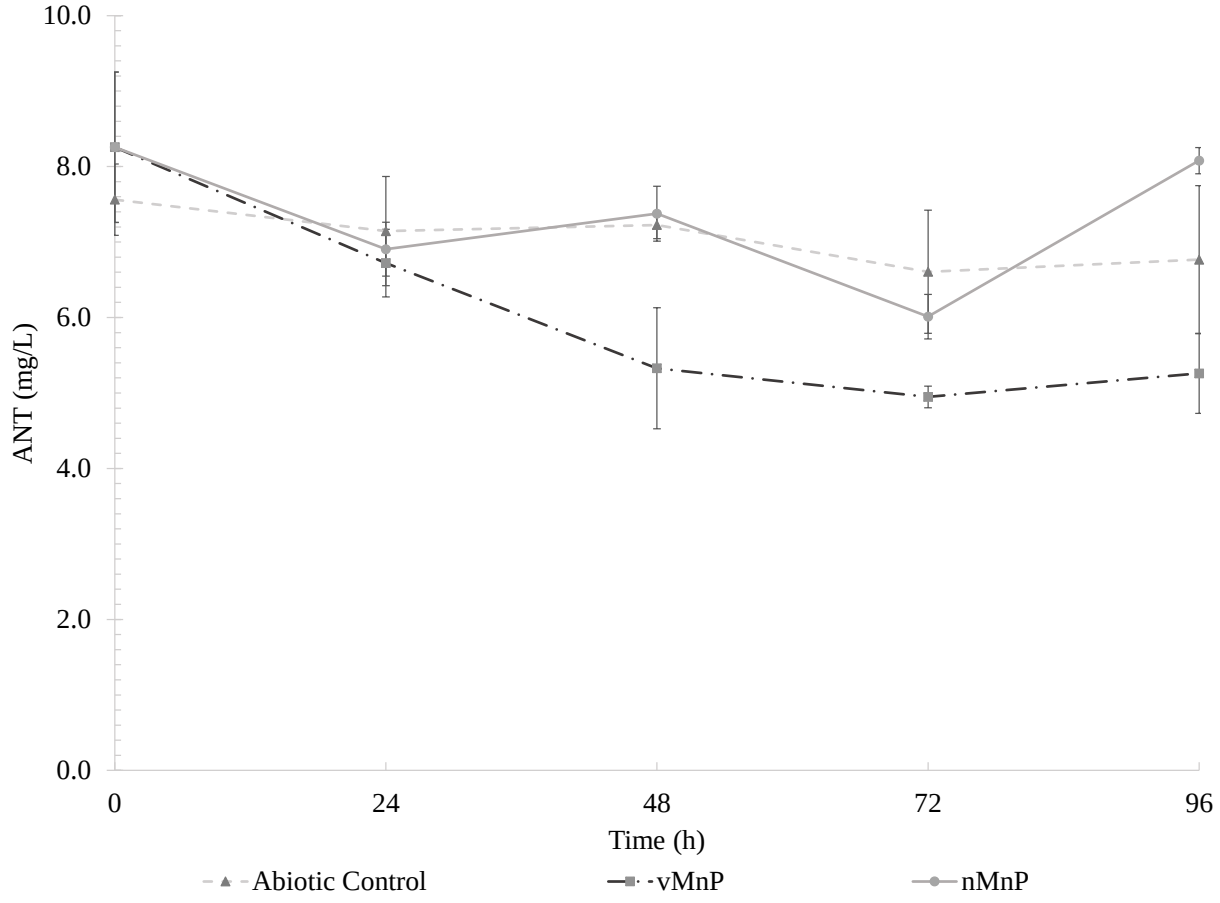

Figure 6: 2-Amino-4-nitrotoluene degradation catalyzed by 34 U/L free enzymes. **Abiotic control**- Enzyme free control. **vMnP**- 34 U/L vault-encapsulated manganese peroxidase catalyzed degradation. **nMnP**- 34 U/L natural manganese peroxidase catalyzed degradation. ANT was treated with 34 U/L of nMnP and vMNP for 96 h. Sacrificial reactions were performed in triplicate for each time point and stopped by adding the equal volume of methanol. nMnP was ineffective at low active concentration whereas, vMnP showed some degradation.

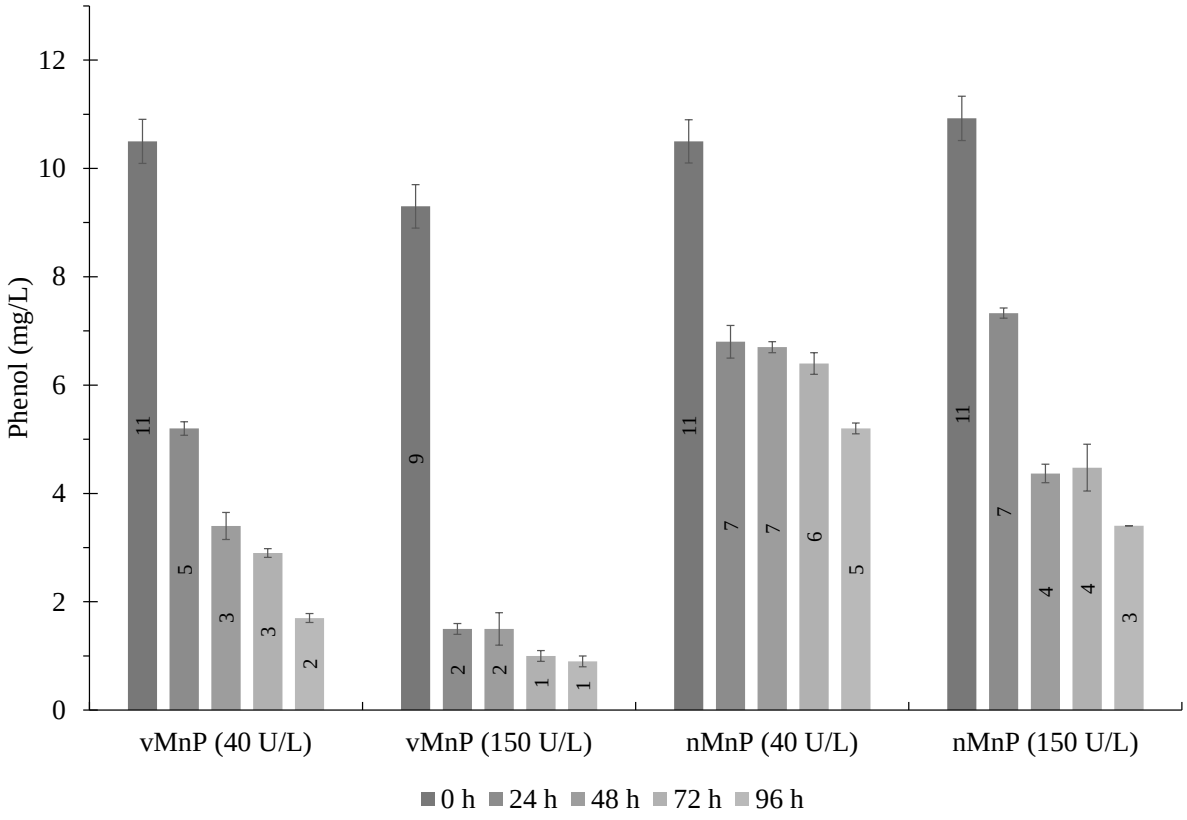

Figure 7: Degradation of phenol catalyzed by free enzymes. **vMnP (40 U/L)**- 40 U/L vault-encapsulated manganese peroxidase catalyzed degradation. **vMnP (150 U/L)**- 150 U/L vault-encapsulated manganese peroxidase catalyzed degradation. **nMnP (40 U/L)**- 40 U/L natural manganese peroxidase catalyzed degradation. **nMnP (150 U/L)**- 150 U/L natural manganese peroxidase catalyzed degradation. Phenol degradation reactions were performed to serve as positive controls for enzyme catalyzed reactions. Detection limit for spectrophotometrical analysis was 1 mg/L. The reactions were performed at varying enzyme activities for the duration of 96 h under similar conditions as explained before for ANT reactions. Highest rate of phenol removal was observed for 150 U/L vMnP.

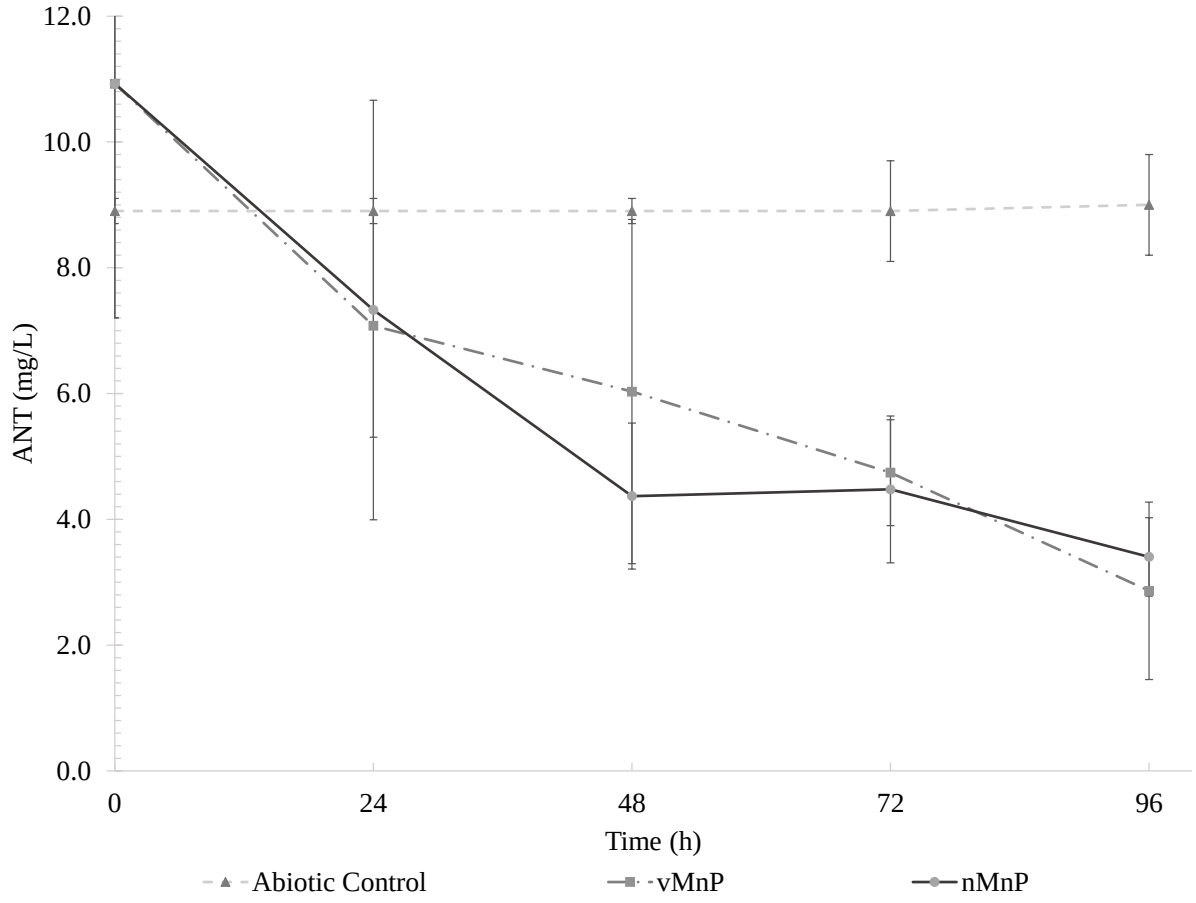

Figure 8: Degradation of 2-amino-4-nitrotoluene by 150 U/L natural manganese peroxidase and vault packaged manganese peroxidase. **Abiotic control**- Enzyme free control. **vMnP**- 150 U/L vault-encapsulated manganese peroxidase catalyzed degradation. **nMnP**- 150 U/L natural manganese peroxidase catalyzed degradation. 200 μL of sacrificial reactions were kept for every time point in triplicate. Both nMnP and vMnP showed the equal removal of ANT (72.7%).

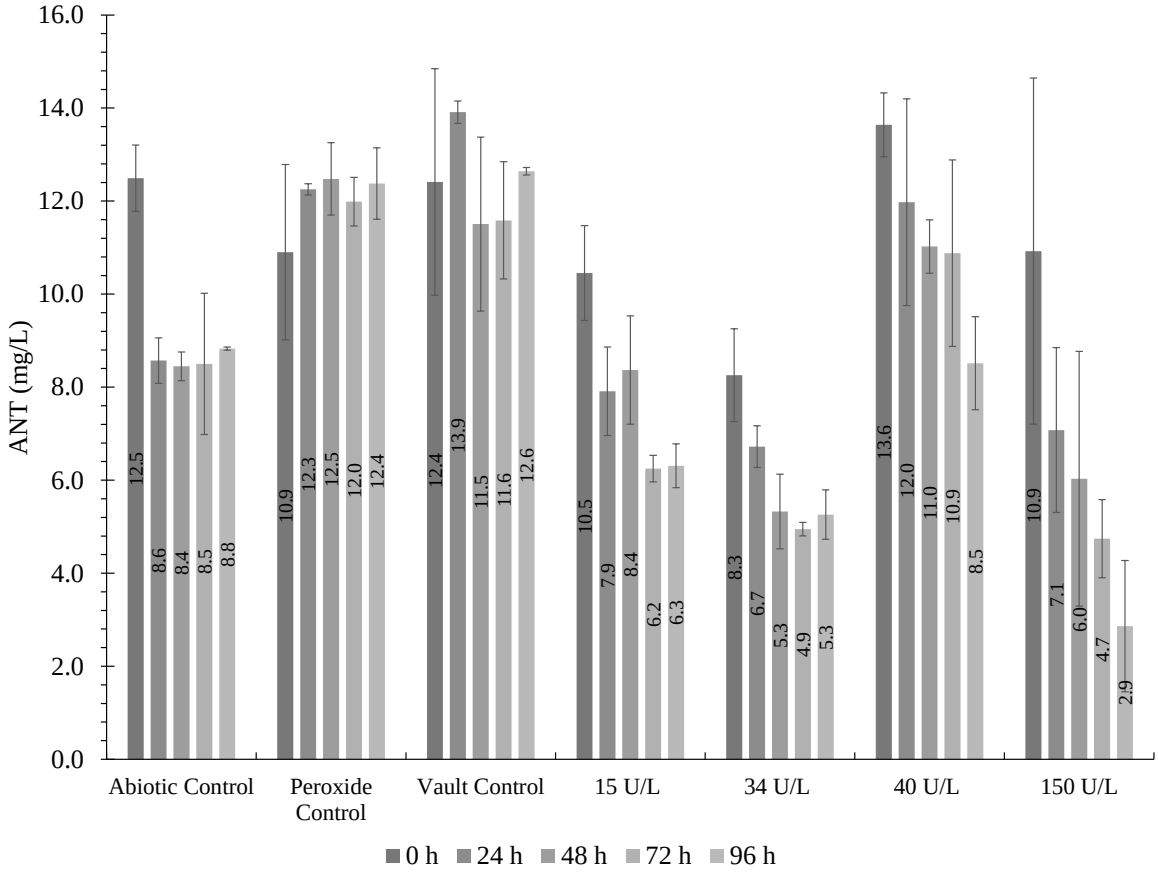

Figure 9: Rate of degradation of 2-amino-4-nitrotoluene at varying vault-encapsulated manganese peroxidase activity. **Abiotic Control**- Enzyme and hydrogen peroxide free control. **Peroxide Control**- Enzyme free control. **Vault Control**- Enzyme free control reactions containing empty recombinant major vault protein. **15 U/L, 34 U/L, 40 U/L, 150 U/L**- Activities of vMnP. Similar percentage removal was observed at all enzyme activities. There was no significant removal observed in the case of enzyme free controls, performed to account for losses due to the presence of the empty vault control and hydrogen peroxide. All degradation experiments were performed in triplicates under similar conditions. The difference in starting ANT concentration is observed because of the variable response obtained on HPLC due to analysis at different times and change in mobile phase composition over time. (p-value <0.05)

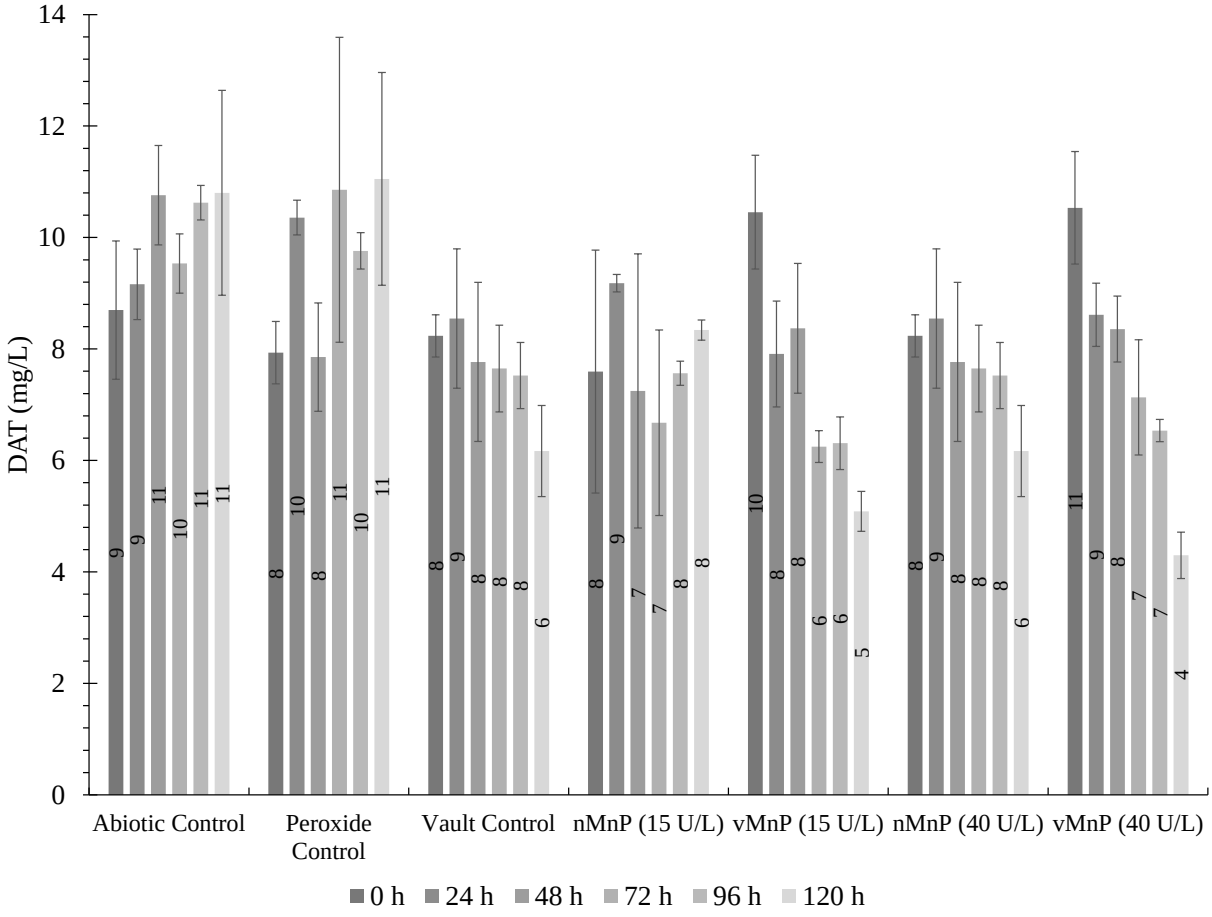

Figure 10: Degradation of 2,4-diaminotoluene by free enzymes. **Abiotic Control**- Enzyme and hydrogen peroxide free control. **Peroxide Control**- Enzyme free control. **Vault Control**- Enzyme free control reactions containing empty recombinant major vault protein. **nMnP (15 U/L)**- 15 U/L nMnP catalyzed degradation, **vMnP (15 U/L)**- 15 U/L vMnP catalyzed degradation, **nMnP (40 U/L)**- 40 U/L nMnP catalyzed degradation, **vMnP (40 U/L)**- 40 U/L vMnP catalyzed degradation. vMnP demonstrated more than 50% degradation at both 15 U/L and 40 U/L activity whereas, nMnP catalyzed degradation was insignificant in both the cases. All degradation reactions were performed in triplicates under same the conditions as ANT experimental setup.

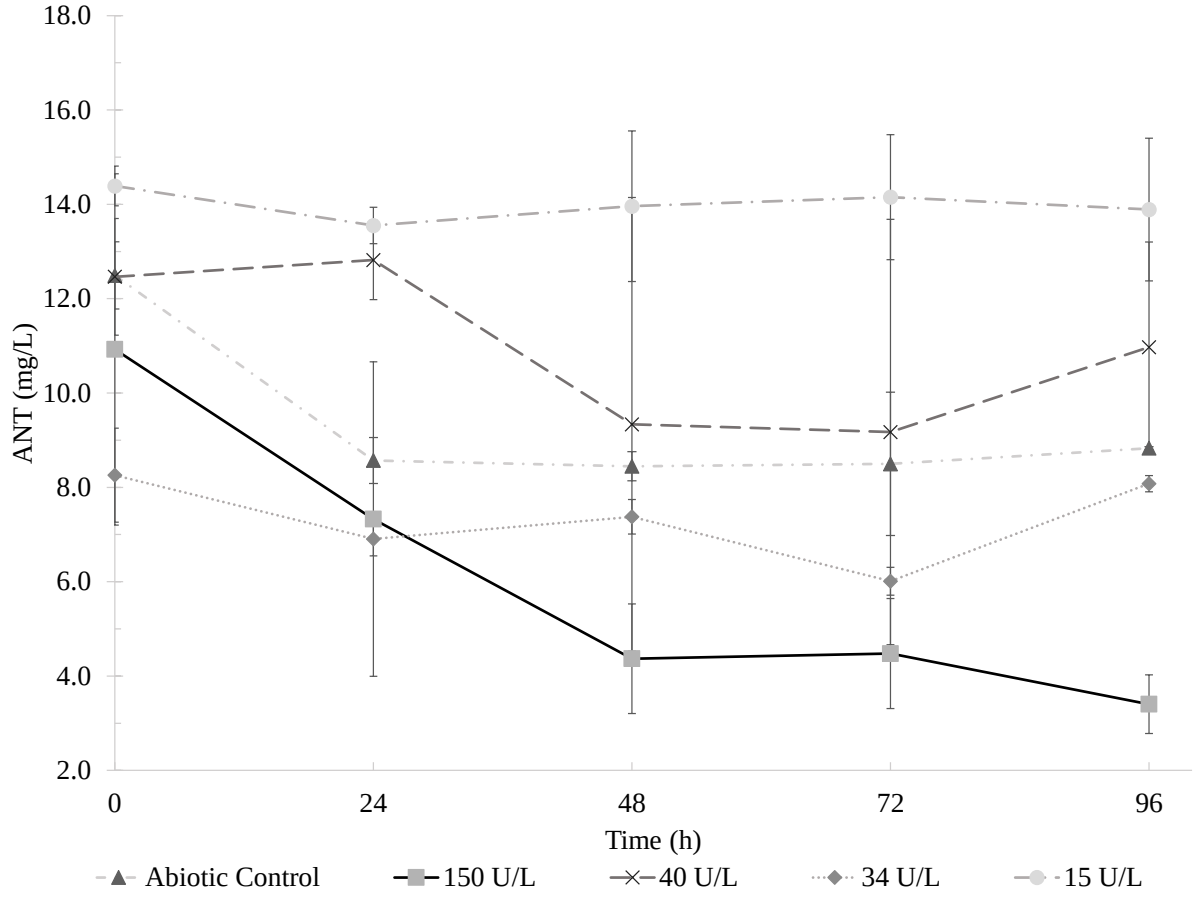

Figure 11: Degradation of 2-amino-4-nitrotoluene by varying activities of natural manganese peroxidase. **Abiotic control**- Enzyme free control. **150 U/L, 40 U/L, 34 U/L, 15 U/L**- Activities of nMnP. Only 150 U/L nMnP showed significant ANT removal. This figure shows the comparison of ANT degradation rates at varying active nMnP concentrations. As each data set represents an independent experiment, variation in starting concentration of ANT correlates to different analysis time and variable detector response.

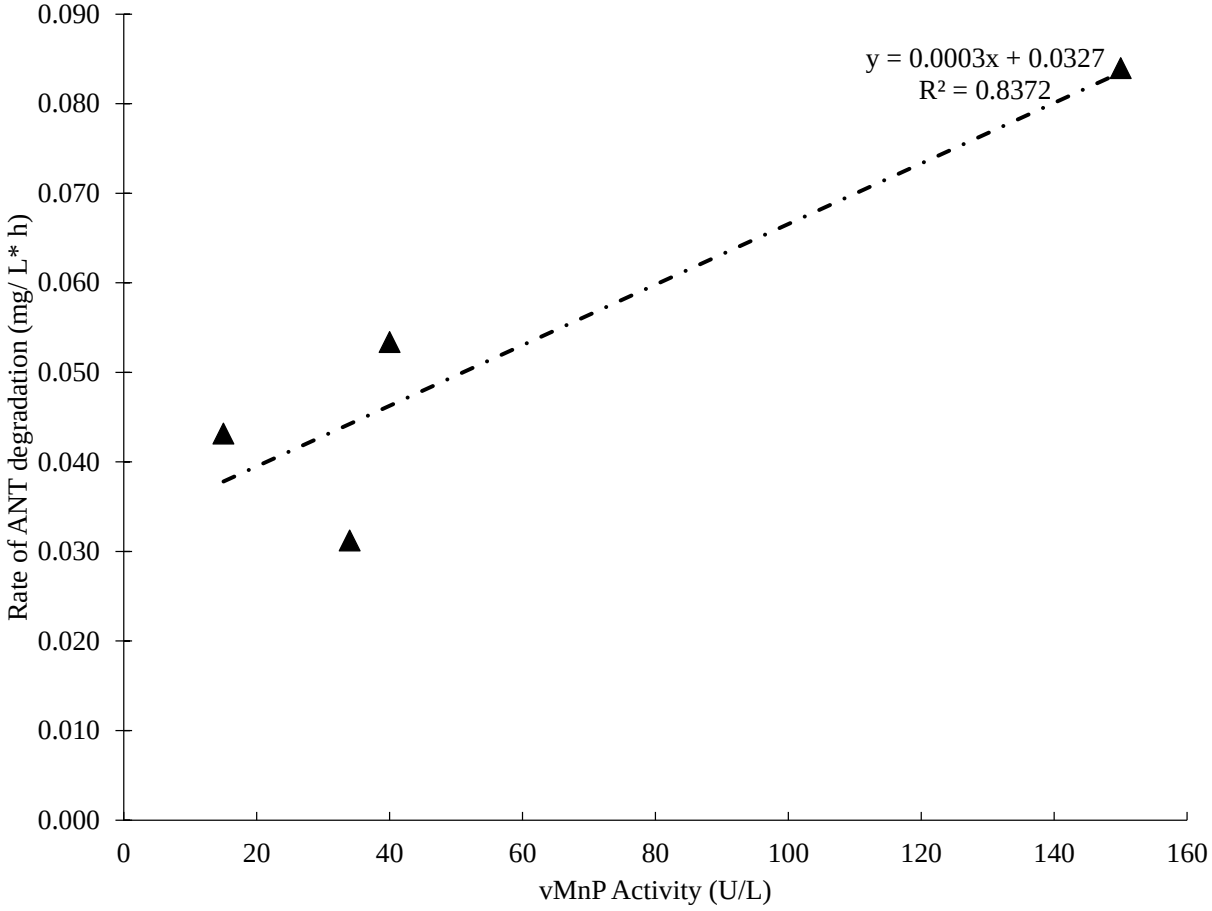

Figure 12: Relation of the rate of 2-amino-4-nitrotoluene degradation to vMnP activity. Rate of degradation was calculated as [Final ANT concentration - Initial ANT concentration / Total reaction time]. Although the R-squared value is not statistically acceptable, the scheme presents the idea of minimal change in the reaction rate over enzyme activity. Enzyme activity is presented in U/L, and unit of degradation rates are (mg/L . h)

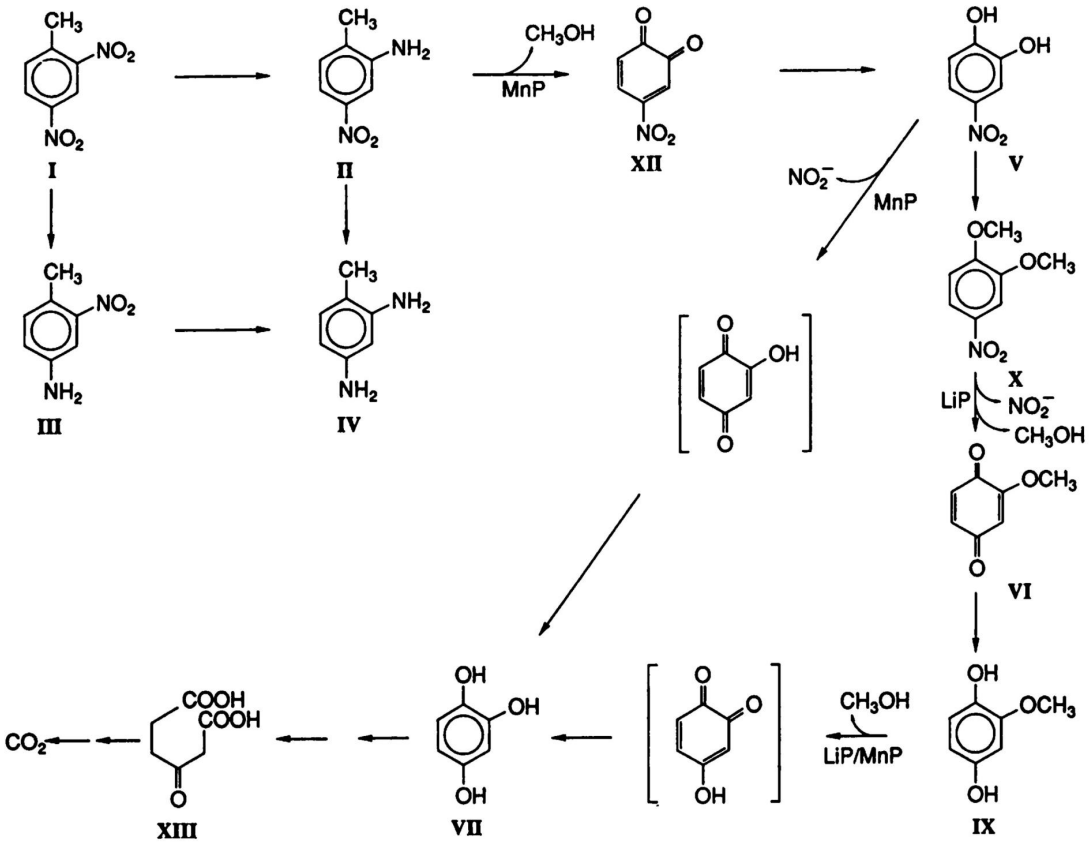

Figure 13: Pathway for degradation of 2,4-dinitrotoluene by *P. chryosoporium* and natural manganese peroxidase. [62] Valli et al. have proposed the intracellular reduction of 2,4-dinitrotoluene (I) to 2-amino-4-nitrotoluene (II) or 4-amino-2-nitrotoluene (III) or 2,4-diaminotoluene (IV). 2-amino-4-nitrotoluene out of all three fungal metabolites was degraded in the presence of purified manganese peroxidase or lignin peroxidase enzyme. Experimental proof for compound (I) and (II) was published whereas, MnP catalyzed the oxidation of compound (III) and (IV) was hypothesized.

Tables

Table 1: Review of degradation of 2,4-Dinitrotoluene by various living organisms

Mode of Degradation	**Degradation Product**	**Reaction Duration**	**References**
Denitrifying conditions bed reactor- activated sludge	DAT, ANT isomers	-	[68]
Clostridium acetobutylicum	DAT	30 h	[65]
Pseudomonas aeruginosa	ANT isomers and small quantities of DAT	4-15 days	[71]
Lactococcus lactis	DAT	2 days	[67]
Shewanella marisflavi EP1	DAT via temporary formation ANT isomers	24 h	[64]
Hemp, Flax, Sunflower, Mustard	ANT isomers and small quantities of DAT	30 days	[66]

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