Chemosphere 79 (2010) 426–433

Contents lists available at ScienceDirect

Chemosphere

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Influence of pH on 2,4,6-trinitrotoluene degradation by *Yarrowia lipolytica*

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ARTICLE INFO

Article history:
Received 21 September 2009
Received in revised form 23 January 2010
Accepted 28 January 2010
Available online 24 February 2010

Keywords:
2,4,6-Trinitrotoluene
TNT–hydride complexes
Deep destruction
Nitrite
Nitrate
Yeast

ABSTRACT

The microbial reduction of the aromatic ring of 2,4,6-trinitrotoluene (TNT) can lead to its complete destruction. The acid-tolerant yeast *Yarrowia lipolytica* AN-L15 transformed TNT through hydride ion-mediated reduction of the aromatic ring (as the main pathway), resulting in the accumulation of nitrite and nitrate ions, as well as through nitro group reduction (as minor pathway), resulting in hydroxylamino- and aminoaromatics. TNT transformation depended on the yeasts' ability to acidify the culture medium through the production of organic acids. Aeration and a low medium buffer capacity favored yeast growth and resulted in rapid acidification of the medium, which influenced the rate and extent of TNT transformation. This is the first time that nitrate has been detected as a major product of microbial TNT degradation, and this work demonstrates the importance of pH on TNT biotransformation. The ability of *Y. lipolytica* AN-L15 to reduce the TNT aromatic ring to form TNT–hydride complexes, followed by their denitration, makes this strain a potential candidate for bioremediation of sites contaminated with explosives.

1. Introduction

Remediation of soils, surface water, and groundwater contaminated with TNT and related compounds can be achieved using physical, chemical, and biological methods (Rodgers and Bunce, 2001; Boparai et al., 2008; Fuller et al., 2009). Each of these strategies provides advantages and disadvantages regarding the fate and detoxification of TNT and its metabolites. Incineration, for example, is probably the most effective treatment method for TNT-polluted soils, but this technology can become very expensive due to its energy demand, the need for excavation, transport of the contaminated soil off site, and basically complete destruction of the treated soil (Esteve-Núñez et al., 2001; Rodgers and Bunce, 2001). In recent studies weathering and rainfall-driven dissolution were also shown to be effective for remediation of soils polluted with explosives (Furey et al., 2008; Taylor et al., 2009a,b), however treatment durations are significantly longer than for excavation and incineration. Irreversible covalent binding of explosives and their transformation products to organic materials in soil or groundwater is believed to be a major pathway for their elimination from contaminated environments (Achtnich et al., 1999, 2000; Yang et al., 2008).

The potential advantages of bioremediation are public acceptance, low cost, and ease of operation (Esteve-Núñez et al., 2001; Rodgers and Bunce, 2001). However, most TNT bioremediation processes result, at least temporarily, in the accumulation of nitro group reduction intermediates (Funk et al., 1993; Stahl and Aust, 1995; Nepovim et al., 2005), which may cause aplastic anemia, cataracts, liver damage, and tumors in urinary systems (Hathaway, 1985; Yinon, 1990; Leung et al., 1995). Hence, the development of improved TNT biotransformation methods that reduce the accumulation of such nitro group reduction products and increase the rate of the aromatic ring destruction could help to decrease the risk to humans and the environment.

TNT nitro group reduction by microbial enzymes can result in the production of nitroso- and hydroxylamino-dinitrotoluenes, which can covalently bind to proteins and nucleic acids (Fu, 1990; Leung et al., 1995). In contrast, the direct reduction of the TNT aromatic ring (also often referred to as TNT–hydride or Meisenheimer complex formation) can result in the denitration of TNT and destruction of the aromatic system (Pak et al., 2000; Jain et al., 2004; Ziganshin et al., 2007; Wittich et al., 2008). The principal possibility of the elimination of a nitro group from the TNT–monohydride complex (3-H^-–TNT) with simultaneous 2,4-dinitrotoluene (2,4-DNT) production has been described previously (Kim et al., 2002; Jain et al., 2004). 2,4-DNT production from TNT can be desirable, since 2,4-DNT mineralization has been demonstrated. This reaction can be initiated by a dioxygenase attack at the 4-nitro group position resulting in 4-methyl-5-nitrocatechol

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doi:10.1016/j.chemosphere.2010.01.051

formation accompanied with the release of nitrite (Nishino et al., 1999, 2000).

Researchers have also proposed dihydride complexes of TNT (3,5–$2H^-$–TNT·H^+ isomers and 3,5–$2H^-$–TNT) as the source of the nitrite liberation (Pak et al., 2000; Williams et al., 2004; Ziganshin et al., 2007; Wittich et al., 2008). Pak et al. (2000) suggested that the denitration is the result of dimerization reactions after TNT reduction by the flavoprotein xenobiotic-reductase B from *Pseudomonas fluorescens* I-C. Williams et al. (2004) proposed that transformation of one of the 3,5–$2H^-$–TNT·H^+ isomers by members of the old yellow enzyme family resulted in the release of nitrite. Wittich et al. (2008) reported nitrite release from 3,5–$2H^-$–TNT, with concomitant 2-hydroxylamino-6-nitrotoluene accumulation, and during condensation of 3,5–$2H^-$–TNT with hydroxylamino-dinitrotoluenes, producing secondary diarylamines. In our recent work (Ziganshin et al., 2007), we demonstrated the ability of the yeast *Yarrowia lipolytica* AN-L15 to produce eight distinct hydride complexes and release nitrite from TNT under static cultivation conditions, with one of the 3,5–$2H^-$–TNT·H^+ isomers being the most likely source of nitrite. Another mechanism of nitrite release from TNT has also been reported (Tront and Hughes, 2005). This pathway of microbial TNT degradation resulted in 3-methyl-4,6-dinitrocatechol accumulation which was identified using stable isotope and tandem mass spectrometry.

The main goal of this research was to better understand the process of TNT transformation by *Y. lipolytica* AN-L15 and contribute towards the identification of optimal environmental conditions for TNT destruction.

2. Experimental methods

2.1. Chemicals

TNT, 2,4-DNT were purchased from ChemService (West Chester, PA); 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT) were obtained from Supelco (Bellefonte, PA); 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT) were received from AccuStandard (New Haven, CT).

2.2. Yeast strain and culturing methods

Experiments were carried out with the yeast strain *Y. lipolytica* AN-L15 (Ziganshin et al., 2007). For whole-cell assays, the yeast was grown aerobically for 20 h at 30 °C on Sabouraud agar containing (g L^{-1}) glucose, 10; peptone, 10; yeast extract, 5; NaCl, 0.25; and agar, 20. Cells were harvested, washed with 16 mM phosphate buffer (pH 6.0 or 7.0), and transferred into 250 mL Erlenmeyer flasks containing 50 mL of synthetic medium of the following composition (mM): glucose, 28; $(NH_4)_2SO_4$, 7.6; $MgSO_4$, 2, buffered using 16 mM K–Na–phosphate buffer to pH 6.0 or 7.0. The initial cell concentration was adjusted to an optical density of 0.2, and growth was measured at 600 nm (A_{600}) using cell-free culture medium as reference. Cell suspensions were diluted before measurements to achieve a final A_{600} of less than 0.5 for the measurements. TNT was added to a final concentration of 440 μM from an ethanolic stock solution (0.8 mL of 95.6% ethanol into 50 mL of medium), and the flasks were incubated at 30 °C. Control flasks contained ethanol in the same concentration as in the presence of TNT. Three different cultivation methods were used: (i) "aerobic" by shaking the flasks at 150 rpm; (ii) "static" (without shaking); and (iii) "anaerobic" by statically incubating flasks in an anaerobic glovebag containing an atmosphere of 90% N_2, 5% CO_2, and 5% H_2. Samples for nitroaromatics, nitrite, nitrate, yeast growth, and pH analyses were taken every 6 h for the first 24 h and every 12 h for the remainder of each experiment. Anaerobic treatments were set up, incubated, and sampled inside an anaerobic glovebag. All treatments were set up in triplicate and treatments were swirled before sampling.

The ability of *Y. lipolytica* AN-L15 to use nitrite and nitrate as the sole source of nitrogen was studied using a synthetic medium containing (mM): glucose, 28; $MgSO_4$, 2; Na_2HPO_4, 1.94; KH_2PO_4, 14.06; and either $NaNO_2$, 3.8 or $NaNO_3$, 7.8 (pH 6.0). These cultures were incubated aerobically on the shaker at 150 rpm at 30 °C with an initial A_{600} of 0.1.

The ability of the strains to oxidize nitrite during their growth was investigated in synthetic medium including (mM): glucose, 28; $NaNO_2$, 0.24; $(NH_4)_2SO_4$, 7.6; $MgSO_4$, 2; Na_2HPO_4, 1.94; KH_2PO_4, 14.06 (pH 6.0). The initial A_{600} was adjusted to 1.0, and cells were cultivated aerobically on the shaker at 150 rpm at 30 °C.

2.3. High performance liquid chromatography and mass spectrometry (HPLC–MS)

HPLC–MS analyses for the identification and quantification of TNT and its metabolites were carried out as described previously (Borch and Gerlach, 2004; Ziganshin et al., 2007). In brief, a Supelcosil LC-8 guard column and a Supelcosil octyl (C-8) column (150 × 4.6 mm; 5 μm particle size) were used, and the flow rate of the mobile phase was 1 mL min^{-1}. The mobile phase initially consisted of 99% sodium phosphate buffer (25 mM, pH 7.0) and 1% methanol. The gradient was changed to 30% methanol over 2 min, then to 43% methanol over the next 13 min, finally increased to 100% methanol over 12.5 min, and held constant for 0.5 min. The solvent ratio was returned to the initial level over 1 min and held for an additional 5 min before injection of the next sample.

2.4. Gas chromatography–mass spectrometry (GC–MS)

The presence of 2,4-DNT was confirmed by GC–MS using an Agilent 6890 GC, equipped with an Agilent 5973 inert mass selective detector and a 7683 series autosampler. An Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm) was used with a helium carrier gas flow rate of 1 mL min^{-1}. Temperatures of injector and GC–MS interface were 240 °C and 250 °C, respectively. The GC oven temperature was raised from 120 °C to 240 °C at 6 °C min^{-1}. Samples were analyzed after extraction of 10 mL culture fluid with ethyl ether. The obtained extract was vacuum-dried, resuspended in 0.1 mL of acetone, diluted to 1 mL with hexane, and 1 μL of this sample was injected into the GC–MS.

2.5. Ion chromatography

Nitrite and nitrate concentrations were determined using a Dionex ion chromatograph equipped with a conductivity detector, an IonPac AG9-HC guard column, and an IonPac AS9-HC analytical column. The mobile phase consisted of 9 mM Na_2CO_3 at a flow rate of 1 mL min^{-1}; $NaNO_2$ and $NaNO_3$ were used for standardization.

Organic acids excreted by the yeast during its growth were analyzed using the same ion chromatograph equipped with a Dionex IonPac AS11 column. The flow rate of the mobile phase was 1 mL min^{-1}. The mobile phase consisted initially of 90% distilled water and 10% 1 mM NaOH. After 2 min, the gradient was changed to 100% 1 mM NaOH over 3 min. After 5 min, the solvent ratio was changed to 65% 1 mM NaOH and 35% 100 mM NaOH over 10 min. Finally, the eluent ratio was returned to the initial level over 1 min and held for 14 min before injection of the next sample.

3. Results

The goal of this research was to better understand the process of TNT transformation by *Y. lipolytica* AN-L15 and identify the optimal culture conditions for TNT biodegradation. In order to achieve this goal, the effects of pH and aeration on TNT transformation during *Y. lipolytica* AN-L15 growth were investigated.

3.1. TNT transformation during Y. lipolytica AN-L15 growth under aerobic conditions (initial pH 6.0)

Fig. 1a shows the differences in pH values and A_{600} measurements (as a proxy for *Y. lipolytica* growth) between TNT-containing and TNT-free systems. Cultivation of *Y. lipolytica* in the absence of TNT resulted in yeast growth within the first 6 h, whereas the presence of TNT appeared to inhibit growth. In the absence of TNT, *Y. lipolytica* reached stationary phase after about 48 h, while it took approximately 72 h in TNT-containing medium. The earlier onset of growth in the TNT-free systems coincided with an earlier observed decrease in pH. Yeast species have been shown to produce and excrete organic acids during the utilization of sugars, which can result in a decreased pH in their surrounding environment (Anastassiadis et al., 2002; Finogenova et al., 2005). Citrate and pyruvate were observed during *Y. lipolytica* growth and the detection of these organic acids coincided with a decrease of the medium pH. The presence of TNT inhibited yeast growth and delayed the observed decrease in pH.

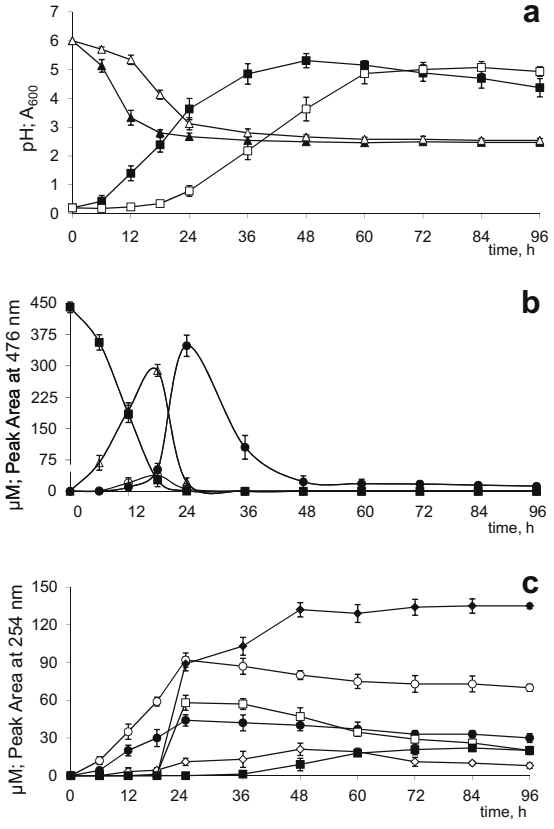

Fig. 1. (a) Change of pH and optical density (A_{600}) during aerobic growth of *Y. lipolytica* AN-L15 (initial medium pH 6.0). Symbols: ■, growth (A_{600}) and ▲, pH change in the absence of TNT; □, growth (A_{600}) and △, pH change in the presence of TNT. (b) Accumulation of metabolites during TNT transformation. Symbols: ■, TNT (μM); △, 3-H$^-$–TNT (μM); ○, 1-H$^-$–TNT (peak area); ●, sum of TNT–mono and dihydride complexes related to 3-H$^-$–TNT (i.e. compounds 1–6, which are defined in our recent publication (Ziganshin et al., 2007)) expressed as sum of peak area at 476 nm. (c) ●, 2-HADNT (μM); ○, 4-HADNT (μM); ◇, NO_2^- (μM); ♦, NO_3^- (μM); □, 2,4-DNT (μM); ■, HAMNT (peak area). After 96 h, 34% of the initially added TNT was accounted for in the form of 2-HADNT, 4-HADNT, 2,4-DNT, NO_2^-, and NO_3^-. Error bars indicate one standard deviation of the triplicate experiments.

TNT transformation by strain AN-L15 at an initial pH of 6.0 resulted in the accumulation of TNT–monohydride complexes as well as nitro group reduction metabolites, such as HADNTs (Fig. 1b and c). The major metabolite observed after 18 h was a TNT–monohydride complex protonated at the C-3 position (3-H$^-$–TNT, see Fig. 2 for structure). A TNT–monohydride complex protonated at the C-1 position (1-H$^-$–TNT), 2-HADNT, 4-HADNT, nitrite, and small amounts of "other" TNT–hydride complexes were also observed (Fig. 1b and c; see Fig. 2 for molecular structures of these compounds). The highest concentration of 3-H$^-$–TNT (282 μM) was observed after 18 h, when a total of 406 μM TNT had been transformed (Fig. 1b). The amount of 3-H$^-$–TNT was estimated as described previously (Ziganshin et al., 2007). Continued cultivation resulted in the complete disappearance of 3-H$^-$–TNT, 1-H$^-$–TNT, and the production of six TNT–monohydride and dihydride complexes (Fig. 1b). These complexes were characterized by us previously using UV–visible diode array detection (DAD) combined with negative-mode atmospheric pressure chemical ionization mass spectrometry (APCI-MS) (Ziganshin et al., 2007) and are isomers of 3-H$^-$–TNT, a C-3,C-5 dihydride complex (3,5–2H$^-$–TNT), and protonated C-3,C-5 dihydride complexes (3,5–2H$^-$–TNT·H$^+$). HPLC retention times, major mass fragments observed during APCI-MS, and UV–visible absorbance spectra for the hydride complexes as well as TNT nitro group reduction products can be found in Ziganshin et al. (2007).

The next stage of TNT transformation (between 18 and 24 h) did not only result in the production of the "other" TNT–hydride complexes and HADNTs, but also in the accumulation of 2,4-DNT, nitrite, and nitrate (Fig. 1b and c). The production of 2,4-DNT was confirmed by HPLC and GC–MS analyses through comparison with a commercially available standard. The HPLC (analysis at 50 °C) and GC retention times were 15.1 and 7.5 min, respectively. The HPLC–DAD–MS analysis revealed an absorption maximum (λ_{max}) at 250 nm and a major ion of m/z 181, which indicates a proton loss during APCI-MS (Fig. S1, Supplementary material).

Accumulation of 2,4-DNT by *Y. lipolytica* AN-L15 occurred only during the phase of 3-H$^-$–TNT disappearance, between 18 and 24 h after the pH had dropped below 4.2. After 24 h, 58 μM of 2,4-DNT had accumulated. The production of nitrate was also observed during this time and reached 135 μM after 48 h. Nitrite concentration remained below 21 μM. This higher than stoichiometric accumulation of nitrite and nitrate compared to 2,4-DNT production, indicates the production of additional metabolites, which, however, could not be detected in this research. Nitrate continued to accumulate until the TNT–hydride and TNT–dihydride complexes had decreased to very low concentrations (peak areas) after 48 h (Fig. 1). Control experiments, in the absence of TNT, did not show any production of nitrite or nitrate by *Y. lipolytica* AN-L15 (data not shown), confirming that TNT denitration is their source and not the nitrification of the ammonium available in the culture medium.

Prolonged growth of AN-L15 was accompanied by a decrease in 2,4-DNT concentration and the appearance of two HPLC peaks with retention times of 10.9 and 11.1 min, when HPLC was performed at 36 °C. Negative mode APCI-MS of these metabolites resulted in main ions at m/z 167, which indicates a proton loss from 2-hydroxylamino-4-mononitrotoluene (2-HAMNT) and 4-hydroxylamino-2-mononitrotoluene (4-HAMNT) during APCI-MS (Fig. S1, Supplementary material). HAMNTs can be the result of 2,4-DNT nitro group reduction, and their production from 2,4-DNT was confirmed in separate experiments in which 220 μM of 2,4-DNT were added to *Y. lipolytica* AN-L15 cultures. After 2 d 40 μM of 2,4-DNT had been converted to HAMNTs; no other 2,4-DNT transformation products were detected.

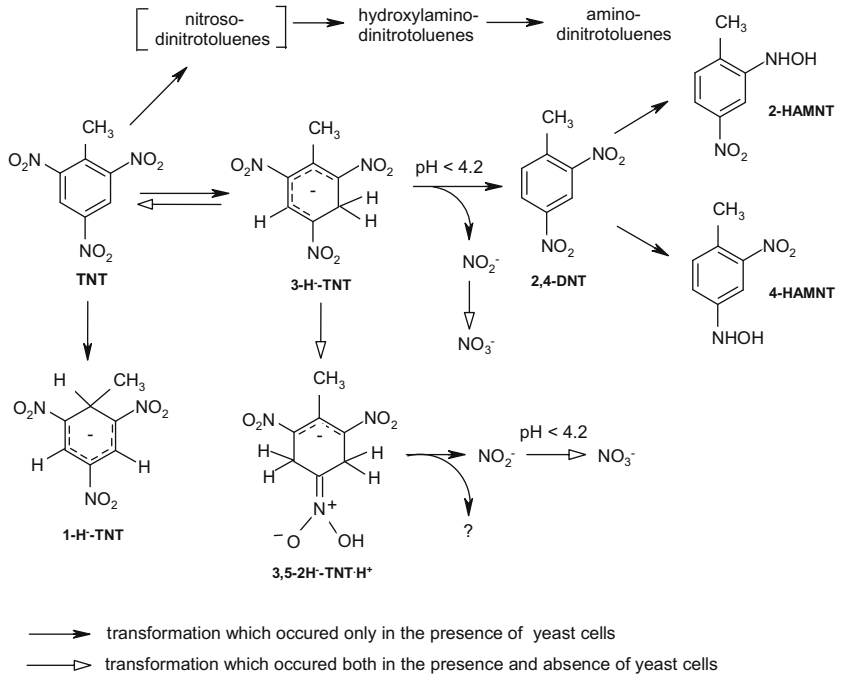

Fig. 2. Proposed biotic and abiotic pathways of TNT transformation by *Y. lipolytica* AN-L15 under different environmental conditions. Structures of three representative hydride complexes are illustrated; all eight hydride complexes detected in this research as well as their biotic and abiotic conversion pathways are depicted in our previous work (Ziganshin et al., 2007). Nitroso-dinitrotoluenes are shown as possible intermediates during the transformation of TNT to HADNTs and ADNTs, although nitroso-dinitrotoluenes were not detected in this research.

Interestingly, during the conversion of 3-H^-–TNT into its protonated dihydride complexes and 2,4-DNT, transformation of the remaining TNT into 3-H^-–TNT was slower than transformation into HADNTs, resulting mostly in accumulation of nitro group reduction products. Remarkably, the increase in the concentrations of HADNTs between 18 and 24 h (from 89 μM to 136 μM), when TNT and 3-H^-–TNT concentrations declined, was greater than the amount of residual TNT present in the medium at 18 h (about 30 μM). As demonstrated previously (Ziganshin et al., 2007), it is possible for 3-H^-–TNT to convert back into TNT via a disproportionation reaction. At least a part of the TNT produced from this reaction appeared to have undergone nitro group reduction to produce HADNTs. After 24 h, 44 μM of 2-HADNT and 92 μM of 4-HADNT had accumulated and their concentrations began to decrease (Fig. 1c). Other potential conversion products of TNT such as amino-dinitrotoluenes, diarylamines, tetranitro-azoxytoluenes, tetranitro-azotoluenes, amino-dimethyl-tetranitrobiphenyls, or products resulting from potential Bamberger rearrangements were not detected. Hence, it is possible that the disappearance of HADNTs in our experiments was a result of covalent binding with biological macromolecules, such as proteins, (phospho-)lipids, or nucleic acids as described previously (Leung et al., 1995). Such products would have been unlikely to form in the experiments by Wittich et al. (2008) since these were conducted with purified enzymes and thus in the absence of other biological macromolecules. After 96 h, 34% of the added TNT could be accounted for, based on the analysis of metabolites quantifiable with the methods used in this research.

3.2. TNT transformation during Y. lipolytica AN-L15 growth under aerobic conditions (initial pH 7.0)

Yeast growth in TNT-containing medium at an initial pH 7.0 was also delayed and began during the phase of 3-H^-–TNT disappearance and "other" TNT–hydride complex production (Fig. 3). The final concentration of biomass was approximately the same as in the absence of TNT, although the maximum A_{600} was reached with a delay of approximately 36 h. The decrease in pH was also delayed, and a pH below 4.2 was reached between 36 and 48 h (Fig. 3a) compared to between 18 and 24 h for the pH 6.0 systems described above (Fig. 1).

As observed in the pH 6.0 systems, TNT conversion in the pH 7.0 treatments resulted in the production of 3-H^-–TNT, "other" TNT–monohydride and dihydride forms, 1-H^-–TNT, 2-HADNT, 4-HADNT, 2,4-DNT, nitrite, and nitrate (Fig. 3b and c).

The production of 3-H^-–TNT was as fast in the pH 7.0 systems (Fig. 3) as in the pH 6.0 systems (Fig. 1); however, the disappearance of 3-H^-–TNT and production of the "other" TNT–hydride complexes in the pH 7.0 systems was significantly slower, indicating the influence of pH on 3-H^-–TNT transformation. The disappearance of 3-H^-–TNT and production of "other" TNT–hydride complexes related to 3-H^-–TNT occurred between 18 and 48 h and was correlated with a decrease in pH from approximately 6.5 to 4.

As observed in the pH 6.0 treatments, more TNT was converted into its nitro group reduction metabolites during this phase (18–48 h) than theoretically possible from the residual TNT, indicating 3-H^-–TNT disproportionation. Furthermore, in contrast to the pH 6.0 systems significantly less 2,4-DNT accumulated, and no HAMNT production was observed. Instead, 4-ADNT was detected in the culture medium, indicating that the nitro group reduction pathway was slightly promoted at the higher pH (Fig. 3). Ultimately, the following maximum concentrations of nitro group reduction products were observed: 57 μM of 2-HADNT, 84 μM of 4-HADNT (after 48 h), and 7.1 μM of 4-ADNT (after 96 h) (Fig. 3c). After 96 h, 28% of the added TNT could be accounted for, based on the analysis of metabolites quantifiable with the methods used in this research.

While less 2,4-DNT was observed in the pH 7.0 systems than in the pH 6.0 systems, more nitrite accumulation was observed. After 36 h, 12 μM of 2,4-DNT and 84 μM of nitrite had accumulated in the cultures with initially neutral pH compared to almost 60 μM

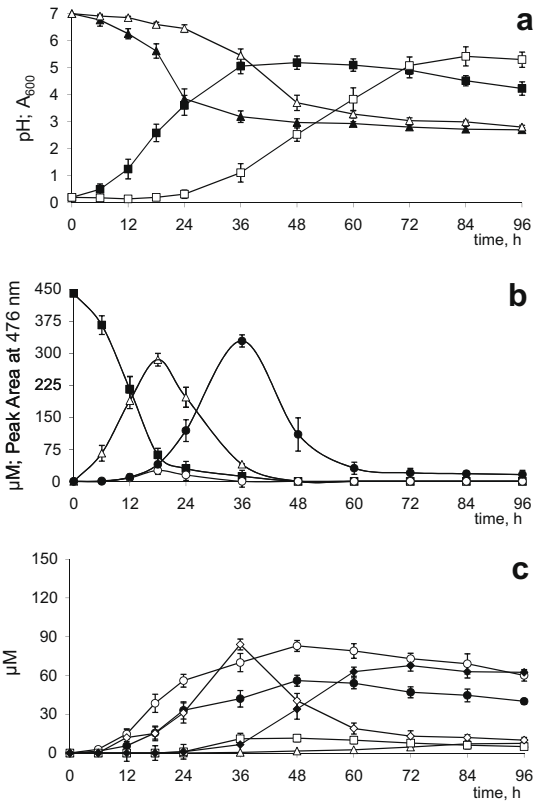

Fig. 3. (a) Change of pH and optical density (A_{600}) during aerobic growth of *Y. lipolytica* AN-L15 (initial medium pH 7.0). Symbols: ■, growth (A_{600}) and ▲, pH change in the absence of TNT; □, growth (A_{600}) and △, pH change in the presence of TNT. (b) Accumulation of metabolites during TNT transformation by strain AN-L15. Symbols: ■, TNT (μM); △, 3-H⁻–TNT (μM); ○, 1-H⁻–TNT (peak area); ●, sum of TNT–mono and dihydride complexes related to 3-H⁻–TNT (i.e. compounds 1–6, which are defined in our recent publication (Ziganshin et al., 2007)) expressed as sum of peak area at 476 nm. (c) ●, 2-HADNT (μM); ○, 4-HADNT (μM); ◇, NO_2^- (μM);♦, NO_3^- (μM); □, 2,4-DNT (μM); △, 4-ADNT (μM). After 96 h, 28% of the initially added TNT was accounted for in the form of 2-HADNT, 4-HADNT, 4-ADNT, 2,4-DNT, NO_2^-, and NO_3^-. Error bars indicate one standard deviation of the triplicate experiments.

2,4-DNT and approximately 20 μM nitrite in the pH 6.0 systems. Nitrite concentration rapidly decreased between 36 and 60 h, coinciding with a decrease in pH to below 4. A concurrent increase in nitrate concentration was observed (68 μM after 72 h).

3.3. TNT transformation during Y. lipolytica AN-L15 growth under static conditions

Fig. S2 in Supplementary material shows the dynamics of growth, pH change, and TNT conversion during *Y. lipolytica* AN-L15 growth under static conditions with an initial medium pH of 6.0. The limited availability of O_2 in these static systems, as well as gradient-dependent distribution of nutrients and metabolites, TNT and its degradation products, resulted in slower growth of *Y. lipolytica* AN-L15 cells, a delayed decrease in pH, and slower TNT transformation. Formation of small amounts of 2,4-DNT (13 μM) was observed. Nitrite was first detected during the phase of 3-H⁻–TNT production and reached a maximum concentration of 35 μM after 48 h. Nitrite to nitrate oxidation rates increased once the pH had decreased to below 4.5 between 48 and 60 h, and nitrate accumulated to approximately 78 μM after 96 h. Maximum concentrations of TNT nitro group reduction products were observed after 60 h for the HADNTs (45 μM for 2-HADNT, 75 μM for 4-HADNT) and 96 h for 4-ADNT (10 μM).

Cultivation of yeast cells under static conditions in the medium with TNT at an initial pH 7.0 was described in detail in our recent publication (Ziganshin et al., 2007). These experiments showed the production of HADNTs, 4-ADNT, and small amounts of 2-amino-4,6-dinitrotoluene (2-ADNT), but they were not accompanied by 2,4-DNT and nitrate production. The absence of the latter two compounds correlates with the only slight decrease in pH in statically cultivated pH 7.0 systems. The pH in these systems decreased to only 6.2 after 60 h (during the phase of 3-H⁻–TNT disappearance) in the presence of TNT. It appears that a low pH value (below approximately pH 4.2) is necessary in order to produce considerable amounts of 2,4-DNT and nitrate from the produced 3-H⁻–TNT.

Anaerobic experiments demonstrated the inability of *Y. lipolytica* AN-L15 to grow or transform TNT in the absence of O_2 (data not shown).

3.4. Stability of 3-H⁻–TNT at different pH values

Since the pH of the culture medium decreased during *Y. lipolytica* AN-L15 growth, it is possible that changes in proton concentration influenced the transformation of TNT and its metabolites. Since 3-H⁻–TNT appeared to be one of the key intermediates, the stability of biologically produced 3-H⁻–TNT was evaluated at different pH values in the absence of yeast cells. HPLC-purified and concentrated 3-H⁻–TNT was vacuum-dried and re-dissolved in phosphate buffer (pH 7.0). No conversion of 3-H⁻–TNT was observed during the purification as confirmed by HPLC analysis. Aliquots of 3-H⁻–TNT in phosphate buffer were transferred into glass vials and acidified to pH values from 6.0 to 2.5 using 0.25 M H_2SO_4. After 30 min, the vials were sampled and analyzed. Fig. 4 clearly shows that 3-H⁻–TNT was stable at pH values between 7.0 and 6.0, but that a decrease to below pH 5.0 resulted in the production of TNT, as well as two 3-H⁻–TNT-related monohydride complexes (compounds 1 and 5 described in Ziganshin et al. (2007)) and the TNT–dihydride complex (3,5–2H⁻–TNT). The rate of conversion of 3-H⁻–TNT increased with increasing proton concentrations (i.e. with decreasing pH). Accumulation of 2,4-DNT, nitrite, and nitrate was not observed in the absence of yeast cells, indicating that conversion of 3-H⁻–TNT to its isomers and 3,5–2H⁻–TNT can proceed abiotically, while the release of nitrite and 2,4-DNT production appear to be biologically mediated.

3.5. Nitrite ion oxidation in the presence and absence of yeast cells

Since TNT aromatic ring reduction by *Y. lipolytica* AN-L15 resulted in the production of nitrite and nitrate, the yeasts' ability

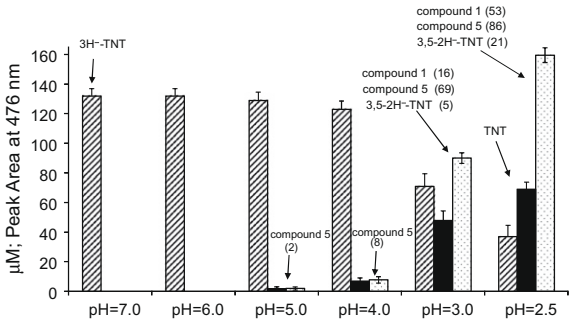

Fig. 4. Abiotic conversion of HPLC-purified 3-H⁻–TNT at different pH (after 30 min). The synthetic medium (initial pH 7.0) was acidified using 0.25 M H_2SO_4. Concentrations of 3-H⁻–TNT and TNT are expressed in μM, whereas the concentrations of TNT–monohydride complexes related to 3-H⁻–TNT (compounds 1 and 5 characterized in Ziganshin et al. (2007)) and 3,5–2H⁻–TNT are expressed as the sum of their peak areas at 476 nm. The average peak area of each complex is shown in parentheses.

to grow and utilize them as potential nitrogen sources for growth was tested: *Y. lipolytica* was unable to grow in the presence of nitrite or nitrate as the sole source of nitrogen or oxidize ammonium to nitrite or nitrate (data not shown).

Furthermore, the addition of nitrate (240 μM) to pH 6.0 medium containing ammonium as the source of nitrogen resulted in growth of *Y. lipolytica*, but no decrease in nitrate concentrations was observed (data not shown). The addition of nitrite (240 μM) to pH 6.0 growth medium containing ammonium as the source of nitrogen, resulted in rapid nitrite oxidation to nitrate after the pH of the culture medium had decreased to below pH 4 (at around 4 h). More than 95% of the nitrite added to the culture medium disappeared within 8 h, with approximately 80% of the nitrite being converted to nitrate (Fig. S3a, Supplementary material).

Since there appeared to be a relationship between the pH of the medium and the onset of nitrite to nitrate conversion, the stability of nitrite was assessed in the absence of yeast cells under aerobic conditions (150 rpm) at four different pH values: 4.5, 4.0, 3.5, and 3.0. The synthetic medium was acidified using 0.25 M H_2SO_4, although other acids such as HCl and citric acid were also tested with equivalent results. Nitrite addition (240 μM) to pH 3.0 medium clearly demonstrated the possibility of abiotic conversion of this anion with non-stoichiometric accumulation of nitrate (Fig. S3b, Supplementary material). After 9.5 h, approximately 70% of nitrite added had been converted abiotically and approximately 20% of the added nitrite had accumulated as nitrate. With increasing pH (from 3.0 to 4.0), the rates of nitrite to nitrate conversion decreased. At pH 4.5 no statistically significant conversion of nitrite to nitrate was observed (Fig. S3b, Supplementary material), clearly indicating a strong dependence of abiotic nitrite conversion on pH. No change in pH was observed in these experiments.

A comparison of nitrite conversion in the presence and absence of yeast cells indicates that nitrite is more readily and more completely converted to nitrate in the presence of yeast cells once the pH has decreased to below pH 4. The pH of the biotic treatments after 8 h of incubation was approximately pH 3; a comparison of the amount of nitrite converted (>95% after 8 h) and nitrate produced in the treatments (80%) containing yeast cells (Fig. S3a) with the abiotic treatments at pH 3 (70% of nitrite converted and 20% of nitrate produced, Fig. S3b), strongly indicates the involvement of yeast cells in the oxidation of nitrite.

4. Discussion

Y. lipolytica AN-L15 transformed TNT via two different pathways: (i) direct reduction of the aromatic ring and (ii) nitro group reduction. Aromatic ring reduction led to the formation of eight distinct mono and dihydride complexes, which were transformed further over time, depending on the environmental conditions, to 2,4-DNT, nitrite, nitrate, and other unidentified products. Nitro group reduction of TNT was also observed resulting in the production of HADNTs and ADNTs and was slightly promoted at an initial pH 7.0. Aerobic conditions stimulated strain AN-L15 to reduce TNT nitro groups to mostly HADNTs, while static conditions increased the yield of ADNTs. However, the concentrations of TNT nitro group reduction products (about 30%) were much lower than those of the TNT–hydride complexes (about 70%), indicating that aromatic ring reduction is the main pathway for TNT transformation by *Y. lipolytica* AN-L15.

The research described herein clearly shows that pH has a significant influence on the transformation of the TNT–hydride complexes. While transformation of TNT to 3-H^-–TNT occurs at similar rates in treatments with initial pH values of 6 and 7, this reaction is inhibited at pH values below 4. In contrast, the formation of other hydride complexes from 3-H^-–TNT is promoted at pH values of 4 and below. The decrease in pH is induced by the production of organic acids during the growth of *Y. lipolytica*.

The yeast cells modulated the pH of the medium through the production of organic acids during their growth. Citric and pyruvic acids were detected during growth of *Y. lipolytica*, and the production of organic acids during growth of *Candida* species (as which *Y. lipolytica* was formerly classified) is a known process (Anastassiadis et al., 2002; Finogenova et al., 2005). The strong pH dependency demonstrated here might explain the lack of 2,4-DNT production after 3-H^-–TNT formation from TNT by *Mycobacterium* sp. strain HL 4-NT-1, *Rhodococcus opacus* HL PM-1 (Vorbeck et al., 1998), *Enterobacter cloacae* PB (French et al., 1998), and *Pseudomonas fluorescens* I-C (Pak et al., 2000). These organisms appeared to be unable to acidify their growth media under the conditions tested.

Here, we report that nitrite eliminated from the protonated TNT–dihydride complex is very quickly converted to nitrate by strain AN-L15 at a pH lower than approximately 4. The amount of nitrite detected in the aerobic pH 7.0 treatments was higher than in the aerobic pH 6.0 treatments, since the biologically-induced pH decrease in the pH 7.0 treatments was slower than in the pH 6.0 treatments. Once the pH had decreased to below 4.2, nitrite was converted to nitrate. This conversion of nitrite to nitrate was incomplete in the presence of yeast cells, which can be explained by a combination of biotic and abiotic nitrate transformation processes. In addition to an assumed stoichiometric conversion of nitrite to nitrate by yeast cells, a fraction of the produced nitrite was converted abiotically to nitric oxide (NO) and nitric acid (nitrate) via the following disproportionation reaction (van Cleemput and Baert, 1984; Cai et al., 2001).

$$3HNO_2 \rightleftarrows HNO_3 + 2NO\uparrow + H_2O \quad (1)$$

The nitric oxide produced was likely lost from the systems through volatilization.

It is also possible that a fraction of the produced nitrite reacted with amino group-containing cell components under acidic conditions to form nitrosoamines or related compounds (Brown, 1999). Such reactions would have reduced the total amount of nitrite and nitrate accumulated in the experimental systems.

The heterotrophic nitrification by fungi and yeasts is a well known process (Lang and Jagnow, 1986; Kuenen and Robertson, 1994). Heterotrophic nitrifiers oxidize a wide range of reduced nitrogen-containing compounds; however, their inability to gain energy from the nitrification requires the presence of an alternative (often organic) electron donor (Kuenen and Robertson, 1994). Lang and Jagnow (1986) showed the ability of *Verticillium lecanii* to produce NO_3^- during NH_4^+ oxidation, and the highest activity was observed at pH 3.5 in the presence of glucose as the sole energy source. However, the body of available literature describing the heterotrophic oxidation of nitrite by fungi is small (Kuenen and Robertson, 1994).

Biodegradation of 3-H^-–TNT was previously proposed to occur with 2,4-DNT and nitrite accumulation (Kim et al., 2002; Jain et al., 2004). Our recent work showed that nitrite can also be released from 3,5–2H^-–TNT·H^+ in the presence of AN-L15 strain (Ziganshin et al., 2007). The research presented here demonstrates that, in addition to biologically catalyzed transformation reactions, numerous pH dependent abiotic disproportionation and equilibrium reactions are involved in TNT transformation via aromatic ring reduction. The data presented here indicates that 3-H^-–TNT is the hydride complex responsible for 2,4-DNT production accompanied by nitrite release. In the work presented here, a fraction of the produced 2,4-DNT was converted to HAMNTs, which was confirmed in separate experiments, where 2,4-DNT transformation by strain AN-L15 was tested. In addition, a fraction of the produced 3-H^-–TNT can be transformed into 3,5–2H^-–TNT and 3,5–2H^-–

$TNT{\cdot}H^+$, which undergo further transformation to produce NO_2^-, NO_3^-, and hitherto unidentified metabolites. Our results are seemingly in slight disagreement with the recent work by Wittich et al. (2009), in which the authors reported 4-HAMNT as a direct product of 3,5–$2H^-$–TNT transformation by purified enzymes.

Since the production of 2,4-DNT from TNT was incomplete even when all nitro group reduction products were incorporated into the mass balance, it might be that other mechanisms of TNT transformation by *Y. lipolytica* AN-L15 occurred as well. However, although likely to be detectable with the chromatography methods employed in this research, other aromatic products of TNT transformation were not detected. Dimethyl-tetranitrobiphenyl isomers described as possible transformation products by Pak et al. (2000) or secondary diarylamines proposed by Wittich et al. (2008) and van Dillewijn et al. (2008) were not observed. Thus, it is possible that protonated dihydride complexes were completely biodegraded in our research.

Since *Y. lipolytica* is a respiring yeast, unable to grow fermentatively, the production of organic acids and TNT transformation were dependent on the aeration of the growth medium. Static incubation, i.e. severely oxygen limited conditions, resulted in decreased growth, only slight medium acidification, and slower TNT transformation.

Despite the fact that yeasts grew better under acidic conditions, separate experiments at initial pH values ranging from 2.5 to 4.0 did not lead to the efficient destruction of TNT. These experiments revealed the production of some nitro group reduction products but no significant production of 3-H^-–TNT (data not shown). This might be explained by the inhibition of enzymes responsible for the transformation of TNT to 3-H^-–TNT, the first step in the conversion of TNT via hydride complexes. Based on these observations, it appears that intensive aeration of neutral or slightly acidic medium (e.g. medium with an initial pH of 6.0), which is being acidified over time by the yeasts to a pH below 4.2, are the optimal conditions for TNT degradation by *Y. lipolytica* AN-L15.

Y. lipolytica AN-L15, isolated from oil-polluted soil, appears to be well-adapted to contaminated environments. The ability to survive under such conditions, in combination with its ability to effectively degrade TNT, make this microorganism attractive for bioremediation of soils, surface water, and groundwater polluted with explosives and other contaminants. Future investigations such as the identification of genes and proteins involved in TNT metabolism as well as radiolabeled TNT studies identifying the hitherto unidentified TNT metabolites shall clarify the exact mechanism of TNT transformation by this organism.

Acknowledgments

This work was supported by a Fulbright graduate student scholarship to Ayrat Ziganshin (Institute of International Education Grantee 15061570). Partial financial support was provided by the US Department of Defense, Army Research Office, Grant No. DAAD19-03-C-0103. The authors acknowledge funding for the establishment of the Environmental and Biofilm Mass Spectrometry Facility through the Defense University Research Instrumentation Program (DURIP) Contract No. W911NF0510255. John Neuman's indispensable help with the analytical procedures is gratefully acknowledged. Lastly, the insightful comments received during the review process are gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.01.051.

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