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EXPERIMENTAL ARTICLES

Aerobic Degradation of 2,4,6-Trinitrotoluene by the Yeast Strain *Geotrichum candidum* AN-Z4

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Abstract—The yeast strain *Geotrichum candidum* AN-Z4 isolated from an anthropogenically polluted site was able to transform 2,4,6-trinitrotoluene (TNT) via the formation of unstable intermediate hydride Meisenheimer complexes with their subsequent destruction and accumulation of nitrite and nitrate ions as the end mineral forms of nitrogen. Aeration of the medium promoted more profound destruction of this xenobiotic by the strain *G. candidum* AN-Z4 than static conditions. The yeast strain was shown to produce citrate, succinate, and isocitrate, which sharply acidified the medium and influenced the TNT destruction. Two possible pathways of TNT biodegradation were confirmed experimentally: (1) via the destruction of the TNT-monohydride complex (3-H^--TNT) and (2) via the destruction of one protonated TNT-dihydride complex (3,5-$2H^-$-TNT · H^+). The strain *G. candidum* AN-Z4, due to its ability for TNT degradation, may be promising for bioremediation of TNT-contaminated soil and water.

Key words: 2,4,6-trinitrotoluene, hydride Meisenheimer complexes, *Geotrichum candidum*, nitrite ion, nitrate ion.

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The production and application of explosives lead to the extensive environmental contamination with stable xenobiotics that endanger health, such as 2,4,6-trinitrotoluene (TNT). This explosive and intermediates of its partial conversion, represented mainly by the products of transformation of one or, rarely, two nitro groups, are toxic and potentially mutagenic compounds [1–3]. Annual production of TNT is approximately 1 million kg [4]; it has an increasing impact on human health by penetrating into the organism through the digestive and respiratory systems [5].

Numerous attempts to degrade this highly stable compound by introducing various microorganisms into TNT-contaminated sites were unsuccessful, since bioconversion involved mostly the nitro groups while the destruction of the TNT aromatic ring that is necessary for the xenobiotic mineralization was insignificant [6–9].

However, a number of TNT-resistant microorganisms capable of reducing not only the nitro groups, but also the aromatic ring, were recently isolated [10–14]. The latter pathway leads to the destruction of the TNT molecule. Obviously, microorganisms capable of hydride-ion reduction of TNT and subsequent degradation of the intermediates are promising for bioremediation of TNT-contaminated sites.

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Apart from the environmental issues of TNT transformation, it is of interest as a model of the nitro arene behavior in the organisms of higher eukaryotes, since nitro aromatic compounds are found not only in the military industry, but are also components of many drugs and pesticides.

The aim of this work was to study the ability of the yeast strain *Geotrichum candidum* AN-Z4 to carry out profound destruction of TNT and evaluate a possible application of this strain for bioremediation of TNT-contaminated sites.

MATERIALS AND METHODS

Yeast isolation and identification. Identification of the yeast strain isolated from petrochemical wastes was performed according to Barnett et al. [15]. Species designation of the strain was confirmed by sequencing the of D2 region of the large subunit of ribosomal RNA performed in the MIDILABS laboratory (www.midilabs.com).

Yeast cultivation in the absence and presence TNT. The yeast strain *G. candidum* AN-Z4 was cultivated aerobically at 30°C for two days on Sabouraud agar containing the following (g/l): glucose, 10.0; peptone, 10.0; yeast extract, 5.0; NaCl, 0.25; and agar, 20.0. Transformation of TNT by *G. candidum* AN-Z4 was carried out in synthetic medium compounds the following composition (mM): glucose, 28; $(NH_4)_2SO_4$, 7.6; $MgSO_4$, 2; Na_2HPO_4, 1.94; and KH_2PO_4, 14.06

(pH 6.0). TNT (400 μM) was added as a solution in 95.6% ethanol (0.8 ml of ethanol per 50 ml of the medium). In the control variant (without TNT), ethanol was added into the medium in the same amount.

To study the TNT destruction, the yeasts were cultivated on Sabouraud agar, and then the harvested cells were washed twice with 16 mM K-Na phosphate buffer (pH 6.0), precipitated by centrifugation, and added into synthetic medium (50 ml). The cells were cultivated aerobically in shaken flasks (150 rpm) at 30°C. After inoculation, initial optical density of the suspension (A_{600}) was 1.0. Samples for physicochemical analyses were collected every 30–60 min.

Spectrophotometric measurements. The yeast biomass was assessed by measuring of the optical density at 600 nm on a Lambda 35 UV-visible spectrophotometer (Perkin Elmer, United States). The cell-free culture liquid was used as a control.

High performance liquid chromatography. TNT and the products of its metabolism were analyzed on an Agilent Series 1100 HPLC chromatograph equipped with an autosampler, an injector, a fraction collector, a diode array detector, a Supelcosil LC-8 precolumn, and a Supelcosil octyl C-8 column (150 × 4.6 mm, particle size, 5 μm) [16].

Mass spectrometry of TNT-hydride complexes was performed as described earlier [16].

Ion chromatography. Nitrite and nitrate ions in the culture liquid were analyzed by using a Dionex ion chromatograph (United States) equipped with a GP40 gradient pump, a CD20 conductivity detector, an AS40 autosampler, an IonPac AG9-HC precolumn (4 × 50 mm), and an IonPac AS9-HC analytical column (4 × 250 mm). Elution was performed with 9 mM Na_2CO_3 solution at a rate of 1.0 ml/min. $NaNO_2$ and $NaNO_3$ were used as standards.

Organic acids excreted by the yeast were analyzed with an IonPac AS11 analytical column (4 mm). Gradient elution was performed at a rate of 1 ml/min by using a solvent system of bidistilled water, 1 mM NaOH, and 100 mM NaOH. An initial mobile phase consisted of 90% bidistilled water and 10% 1 mM NaOH and was maintained for 2 min; in the next 3 min, the amount of 1 mM NaOH in the elution system was increased to 100%; in the following 10 min, 1 mM NaOH was decreased to 65%, while the content of 100 mM NaOH increased to 35%; at the end, the composition of the mobile phase was returned to the initial level over 1 min and maintained for 6 min.

Chemical reagents. TNT and 2,4-dinitrotoluene (2,4-DNT) were purchased from Chem Service (West Chester, United States); 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT) were received from AccuStandard (New Haven, United States).

RESULTS AND DISCUSSION

The mechanisms of TNT transformation by evolutionarily different organisms have been studied for a long time; however, the issue of TNT biotransformation is far from being well understood.

Transformation of TNT by *G. candidum* AN-Z4 performed under intense aeration at an initial pH 6.0 included the formation of hydride Meisenheimer complexes and the products of TNT mononitroreduction; it was accompanied by decreasing pH. The strain studied was capable of the synthesis of all eight TNT mono- and dihydride complexes, which were earlier characterized in our experiments with *Yarrowia lipolytica* AN-L15 [16].

The inoculation of the medium containing TNT (400 μM) with the cells of *G. candidum* AN-Z4 up to the optical density (A_{600}) of 1.0 resulted initially in the accumulation of the predominant metabolite, C-3 TNT-monohydride complex (3-H^--TNT), and minor compounds, C-1 TNT-monohydride complex (1-H^--TNT), 2-HADNT, and 4-HADNT (Fig. 1a). After 1 h, 3-H^--TNT was partially converted to a number of other TNT-hydride complexes, although their concentrations remained low at this stage (Fig. 1a). At this stage, nitrite accumulation commenced that was reliably confirmed by ion chromatography (Fig. 1b).

At the second stage of yeast cultivation, an increase in HADNT concentration was accompanied by a decrease in the content of 3-H^--TNT and by active synthesis of the other TNT-mono- and dihydride complexes. Moreover, the diminution in 3-H^--TNT concentration was associated with accumulation of 2,4-DNT and nitrate ion (Fig. 1).

It should be noted that the formation of 2,4-DNT started only at the stage of the 3-H^--TNT declining, continued up to its complete loss, and reached a maximum at pH below 4.2. Concurrently, accumulation of nitrate ion occurred, whereas the nitrite ion concentration remained low (Fig. 1). In the control variant (without TNT), neither NO_2^- or NO_3^- were detected, which is indicative on direct involvement of TNT in their formation.

At the same time, such possible metabolites of 2,4-DNT transformation as mononitrotoluenes and their derivatives were not found.

The maximal detected concentrations of TNT metabolites were as follows (μM): 3-H^--TNT, 215; 2-HADNT, 32; 4-HADNT, 97; and 2,4-DNT, 46. The final concentrations of nitrite and nitrate ions were 12 and 52 μM, respectively. The amount of 3-H^--TNT was assessed as described earlier [16].

Since the production of organic acids by yeasts is well known [17], we determined the pH of the medium in the course of TNT transformation by *G. candidum* AN-Z4 (Fig. 1b). The excretion of citrate, succinate, and isocitrate that was revealed by ion chromatogra-

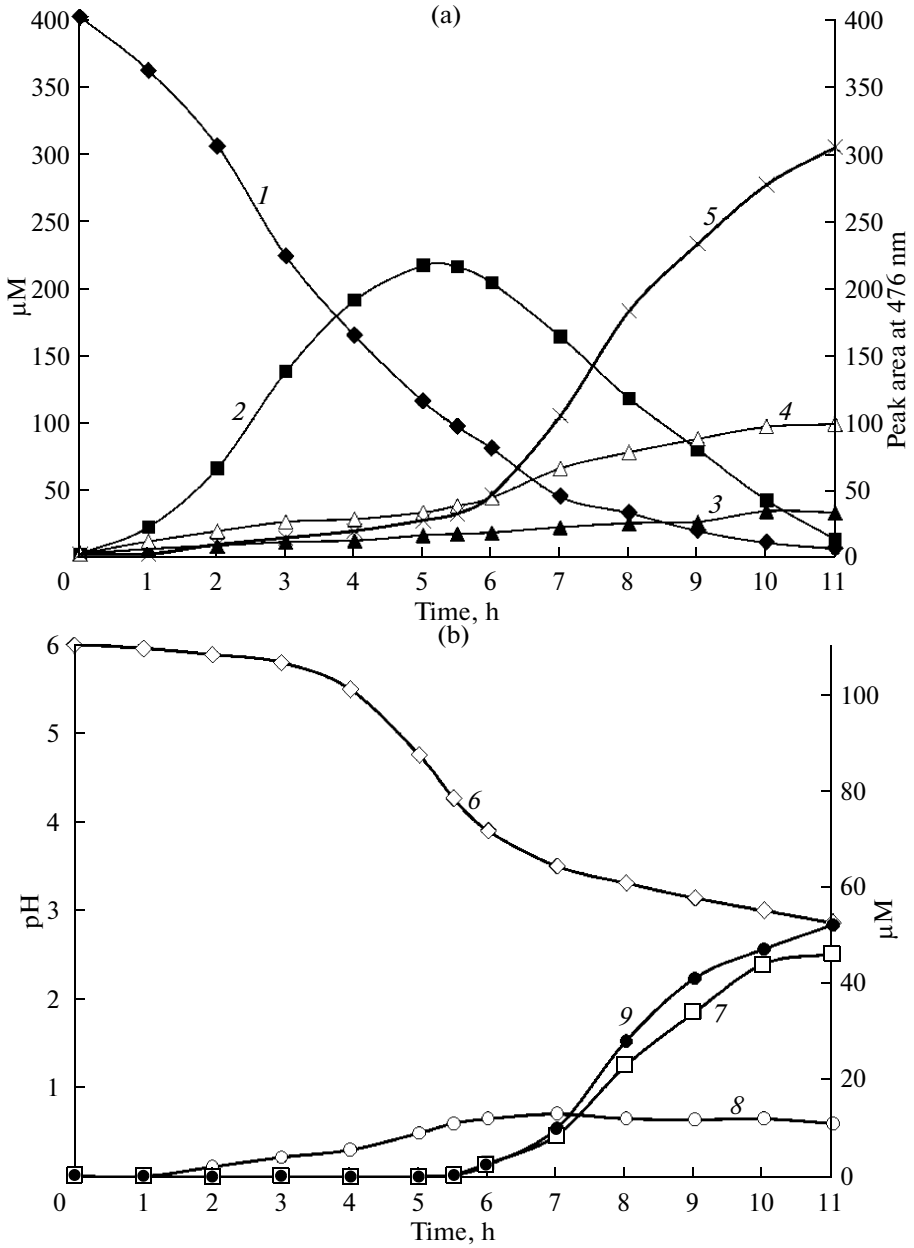

Fig. 1. Production of metabolites in the course of TNT transformation by *G. candidum* AN-Z4 under aerobic conditions at pH 6.0. Curve designations (a): TNT (*1*); 3-H$^-$-TNT (*2*); 2-HADNT (*3*); 4-HADNT (μM) (*4*); the sum of the other TNT-hydride complexes expressed as peak areas, HPLC (*5*); (b): pH (*6*); 2,4-DNT (*7*); nitrite ions (*8*); nitrate ions (*9*).

phy resulted in drastic acidification of the medium. In particular, cultivation of the yeast strain for 11 h in the presence of TNT under aerobic conditions was accompanied by a decrease in pH from 6.0 to 2.85.

The acidification of the medium caused by acid production promoted both the destruction of 3-H$^-$-TNT with the formation of 2,4-DNT and the oxidation of nitrite into nitrate. However, the elimination of the nitro group from TNT directly in the form of nitrate ions rather than nitrite ions cannot be ruled out. It is possible that one enzyme is involved both in the oxidation of NO_2^- into NO_3^- and in the conversion of 3-H$^-$-TNT into 2,4-DNT and NO_3^-, which uses NO_2^- and 3-H$^-$-TNT as substrates. This assumption is supported by the fact that the addition of $NaNO_2$ (100 μM) into the medium during the 3-H$^-$-TNT transformation and accumulation of 2,4-DNT resulted in a sharply decreased yield of dinitrotoluene (from 46 to 14 μM) and promoted subsequent conversion of the monohydride complex via the formation of its dihydride forms. At the same time, the additional

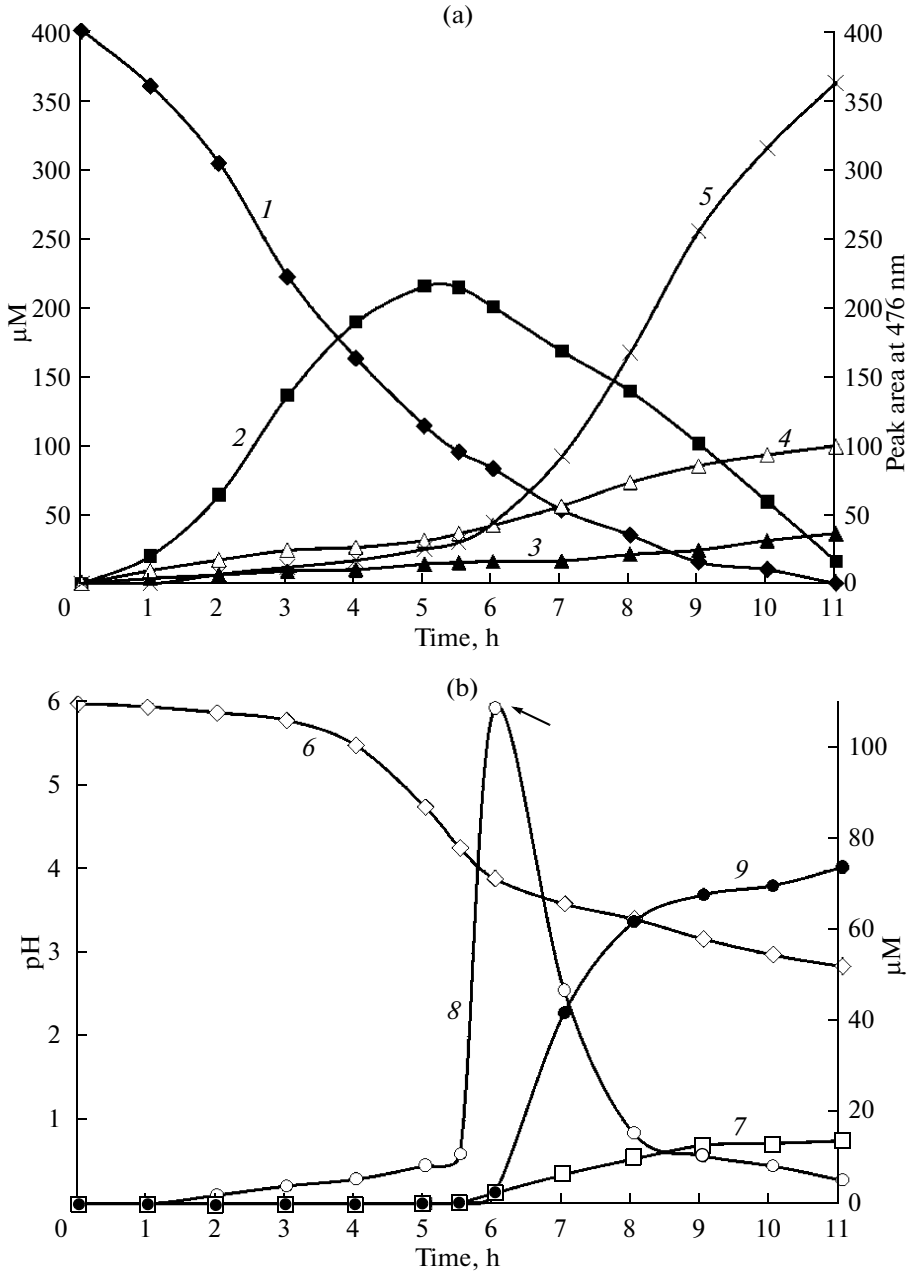

Fig. 2. Production of metabolites in the course of TNT transformation by *G. candidum* AN-Z4 under aerobic conditions at pH 6.0. At the stage of 2,4-DNT formation, $NaNO_2$ (100 μM) was added into the medium as an additional source of nitrite ions (shown by the arrow). Curve designations as in Fig. 1.

nitrite was oxidized incompletely into nitrate (Fig. 2). According to the literature data, catalase and peroxidases may be involved in the oxidation of nitrite into nitrate in the presence of hydrogen peroxide [18].

The pathway of the TNT nitro group reduction was less pronounced by strain *G. candidum* AN-Z4. The concentration of 4-HADNT exceeded that of its isomer, 2-HADNT, which is in agreement with earlier observations indicating that biological reduction of the TNT nitro groups was preferentially directed to the NO_2 group at the *para* position [19, 20].

We earlier studied TNT transformation by yeasts under static conditions at pH from 5.0 to 8.0 [14]. In the course of TNT transformation by *G. candidum* AN-Z4, no 2,4-DNT production was observed at initial pH of 7.0 or 8.0. This result may be explained by slight acidification of the medium because of low production of organic acids; an increase in 2,4-DNT formation occurred only at pH below 4.2 (Fig. 1). Low acidification of the medium was also responsible for increased nitrite accumulation during TNT transformation at initial pH varying from 7.0 to 8.0. Intense

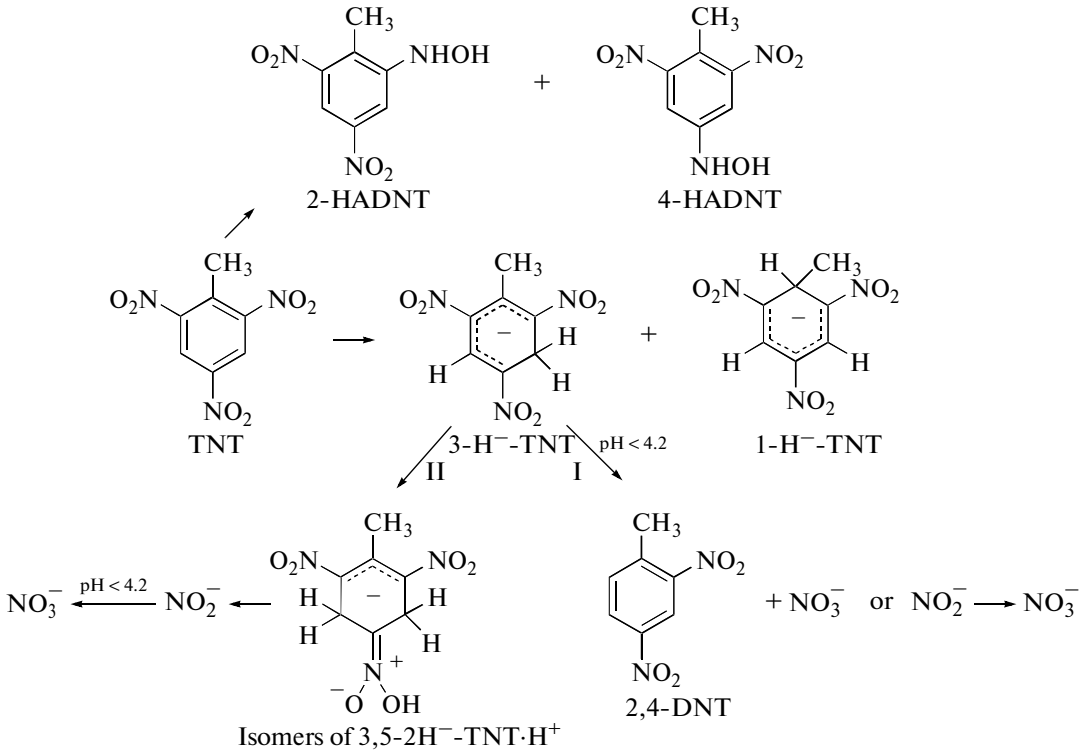

Fig. 3. Proposed pathways of TNT transformation by *G. candidum* AN-Z4. Roman numerals designate two possible variants of the xenobiotic degradation.

acidification of the medium was observed at initial pH values of 5.0 and 6.0 that initiated rapid oxidation of nitrite to nitrate. Moreover, intense aeration promoted the formation of HADNTs as the sole intermediates of the TNT nitro group conversion, whereas amino-dinitrotoluenes were not found under these conditions (Fig. 1).

The supposed pathways of TNT transformation by *G. candidum* AN-Z4 during aeration are illustrated in Fig. 3. Earlier, we described the scheme of TNT transformation by the yeasts under static conditions [14].

We suggest two possible pathways of TNT degradation via the formation of the intermediate hydride forms; according to the first one, at low pH values, direct elimination of a nitro group from 3-H⁻-TNT occurs with simultaneous accumulation of 2,4-DNT. The formation of 2,4-DNT from TNT means the conversion of this xenobiotic into a dinitroarene; such compounds are known to be more easily biodegradable than TNT [21, 22].

It was earlier suggested that the accumulation of nitrite ion in the course of TNT transformation by *Y. lipolytica* NCIM 3589 was associated with the elimination of a nitro group from 3-H⁻-TNT [13]. Since in this work separation and identification of TNT-hydride complexes were not performed, the mechanisms involved in elimination of the nitrite ion remained unclear. The ability of the strain to oxidize nitrite ion was not also studied.

The second pathway of transformation of the TNT aromatic ring by the strain *G. candidum* AN-Z4 involves degradation of one of the isomers of 3,5-2H⁻-TNT · H⁺. The release of nitrite ion into the medium began at the initial stages of TNT transformation, when 2,4-DNT was not yet revealed, but dihydride derivatives of 3-H⁻-TNT were already formed.

According to preliminary data, which require stricter confirmation, nitrite accumulation catalyzed by pentaerythritol-tetranitrate reductase from *Enterobacter cloacae* PB2 was accompanied by simultaneous disappearance of "orange products," during a decrease in the content of the TNT-dihydride complexes [10, 23]. The authors suggested that nitrite originated from one of the isomers of 3,5-2H⁻-TNT · H⁺ and that this pathway led to the destruction of the non-aromatic structure and formation of an alcohol or a ketone.

Our work provides new insight into the mechanism of TNT transformation by lower eukaryotes; the revealed nitrite and nitrate ions are indicative of partial mineralization of the toxicant. The understanding of this mechanism may improve the technologies for treatment of the TNT-contaminated zones.

The yeast strain *Geotrichum candidum* AN-Z4, due to its unique ability to degrade 2,4,6-trinitrotoluene, is promising for the development of a biotechnology for remediation of explosive- contaminated territories.

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