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EXPERIMENTAL ARTICLES

Hydride-Mediated Reduction of 2,4,6-Trinitrotoluene by Yeasts as the Way to Its Deep Degradation

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Abstract—Broad screening of microorganisms from natural and anthropogenic ecological niches has revealed strains *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4 which transform 2,4,6-trinitrotoluene (TNT) via alternative pathways (with the domination of hydride ion-mediated reduction of the aromatic ring) and produce relatively high amounts of nitrites. According to the spectrophotometry data, the hydride attack of TNT by *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4 grown at pH 5.0–8.0 leads to the mono- and dihydride complexes of TNT (H^--TNT and $2H^-$-TNT, respectively) and to protonated forms of the latter. Analysis by HPLC, GC-mass spectrometry, and ion chromatography revealed the products of deep conversion of TNT. The growth of the yeast strains in a weakly acidic medium with TNT (440 μM) is accompanied by formation of 2,4-dinitrotoluene (2,4-DNT, up to 18.2 μM). Together with accumulation of nitrites (up to 76.0 μM, depending on pH of the medium), these findings demonstrate the capacity of both strains for TNT denitration. Formation of 2,4-DNT reflects the realization of one of the possible mechanisms of TNT *ortho*-nitro group elimination and switching over to the pathways of metabolism of dinitrotoluenes, which are much more easily biodegradable than TNT. Simultaneously with the dominating TNT hydride attack, the mechanism of 4- and 6-electron reduction of the nitro group also functions in *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4. Realization of the studied mechanisms of TNT transformation under growth of *Candida* sp. AN-L15 on *n*-alkane is important for bioremediation in the cases of combined pollution by oil products and explosives.

Key words: 2,4,6-trinitrotoluene, yeast, hydride complexes, 2,4-dinitrotoluene, nitrites.

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Production and application of 2,4,6-trinitrotoluene, the most commonly used explosive in the world, results in pollution of soils, surfaces, and ground water. TNT and its nitroreduction intermediates are toxic and stable pollutants, which can circulate in the environment for a long time [1]. International agencies for environmental protection have assigned TNT to toxic and potentially mutagenic xenobiotics; prevention of pollution by these compounds is therefore an important environmental problem [2].

Beginning from the mid-1990s, concepts of TNT microbial transformation pathways have undergone significant changes; in particular, the concept of the dominant position of aminodinitrotoluenes (ADNT) as major TNT metabolites was reconsidered. It has been shown that the barrier on the pathway of reductive transformation of its nitro groups occurs mainly at the stage prior to ADNT formation. Accumulation of hydroxylamino-dinitrotoluenes (HADNT) as major metabolites was first demonstrated in single strains, including lactic acid bacteria [3], obligate anaerobic clostridia [4], and yeasts [5, 6], and was subsequently confirmed for a wide range of prokaryotes [7].

The school of H.-J. Knackmuss [8, 9] initiated the study of an alternative pathway of TNT biotransformation based on its hydride-mediated reduction. The works in this field revealed the ability for hydride transformation of TNT only in a narrow range of bacteria: *Mycobacterium* sp. HL 4-NT-1; *Rhodococcus opacus* HL PM-1; *Enterobacter cloacae* PB2; *Pseudomonas fluorescens* I-C; and *Pseudomonas* sp. clone A [8–12].

As regards eukaryotes, we were the first to characterize three models of TNT transformation by facultatively anaerobic (*Saccharomyces* sp. ZS-A1) and aerobic (*Candida* sp. AN-L13 and *Candida* sp. AN-L14) yeasts [5, 6]. The first of the abovementioned strains demonstrated traditional TNT reduction with the dominating formation of HADNT, whereas in *Candida* sp. AN-L13 the most pronounced mechanism was hydride ion-mediated aromatic ring attack resulting in formation of a TNT monohydride complex as the major metabolite. The intermediate model (*Candida* sp. AN-L14) demonstrated a combination of both mechanisms.

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The mechanism of hydride ion-mediated aromatic ring attack may contribute to deep TNT conversion with emission of nitrogen as NO_2^- [10, 11]. The possibility of such denitration was assessed at the level of enzymatic reactions, including those with the involvement of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB 2 [10] and xenobiotic reductase B from *Pseudomonas fluorescence* I-C [11], though in the former of these works nitrite was identified only by the Griss color reaction, which reqires confirmation.

According to one of the versions [11], denitration can occur not only on the basis of the hydride pathway intermediates but also at the interaction (including nonbiological one) of the products of TNT conversion via both alternative pathways. Thus, it seemed advisable to supplement the criteria for strain selection for the study of deep TNT degradation. If previously [5, 6] we have ranged the strains mainly by their ability for maximal TNT conversion into its monohydride complex, then, in the view of a more objective assessment of the depth of xenobiotic degradation, it was necessary to combine this criterion with an estimation of the degree of TNT denitration.

The goal of this work was therefore to continue the search of microorganisms which attack the TNT molecule by means of hydride ion-mediated reduction of the ring in combination with denitration and to characterize the dynamics of formation of hydride complexes as well as the scale of nitrite formation at varied pH of the medium.

MATERIALS AND METHODS

Yeasts and cultivation conditions. Experiments on the dynamics of TNT transformation were performed with the yeast strains *Candida* sp. AN-L15 isolated from oil-polluted peat bogs (Langepas, West Siberia) and *Geotrichum* sp. AN-Z4 isolated from oily sludge, the waste of Nizhnekamskneftekhim petrochemical plant (Nizhnekamsk). Both strains are stored in collections of the Kazan State University and Montana State University.

Yeast cultures were maintained under aerobic conditions on agarized Sabouraud medium containing (g/l distilled water): glucose, 10.0; peptone, 10.0; yeast extract, 5.0; NaCl, 0.25; and agar, 20.0. TNT transformation was studied in a synthetic medium with $(NH_4)_2SO_4$, 7.6 mM and $MgSO_4$, 2 mM. Phosphate buffer (pH 5.0 to 8.0) was sterilized separately and introduced prior to inoculation in the final concentration of 16 mM. As a source of carbon and reducing equivalents, the medium contained either glucose (28 mM) or hexadecane (10.5 mM). TNT was introduced as crystals, 440 μM, before autoclaving.

When studying the dynamics of TNT transformation, the inocula were precultivated in a liquid Sabouraud medium at required pH (*Candida* sp. AN-L15 for 24 h and *Geotrichum* sp. AN-Z4 for 48 h); the media with the same pH (50 ml in 250-ml flasks) were then inoculated. The cultivation was carried out at 30°C without shaking. The initial optical density of the culture (A_{600} 0.1).

Spectrophotometric measurements were carried out using a Lambda 35 spectrophotometer (Perkin Elmer, United States). Cell biomass was assessed by measuring the optical density at 600 nm. The cell-free culture fluid was used as a control. H^--THT was determined at its peak of absorption at 476 nm (A_{476}), and the dihydride complex and the total of its protonated forms were revealed by the spectral shifts according to literature data [9–11].

High performance liquid chromatography. TNT, its nitroreduction metabolites, and 2,4-dinitrotoluene (2,4-DNT) were analyzed by HPLC in a Series 200 chromatograph (Perkin Elmer, United States) in the reversed-phase variant using a column (150 × 4.60 mm 5u micron–Luna 5u C18 (2), Phenomenex), with a wavelength detector at 254 nm. Elution was performed in the isocratic mode in an acetonitrile–water (40 : 60) system of solvents. The flow rate was 1 ml/min, at 25°C.

GC–mass spectrometry. Additional separation and identification of 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), and 2,4-DNT was performed in a Turbo Mass Clarus 500 device (Perkin Elmer, United States) with a column of 30 m × 0.25 mm ID, at a film thickness of 0.25 μm (Elite-5MS). The mobile phase was helium; the flow rate was 37 cm/sec. The injector and detector temperatures were 240°C and 250°C, respectively. The temperature was increased from 120°C to 240°C at a rate of 6°C/min.

Samples were analyzed after exhaustive extraction of nonpolar components by ethyl ether from 10 ml of culture liquid. The obtained extract was evaporated in a rotary evaporator, the solid residue was re-dissolved in 0.1 ml of acetone, supplemented with hexane to 1 ml, and introduced into the device in the amount of 1 μml.

Ion chromatography. Nitrites were detected by a Tzvet 3006M ion chromatograph (Russia) equipped with a separating column with anionite KhIKS-1 and precolumn KU-2-8. Elution was performed with 2 M Na_2CO_3 solution at a rate of 1.8 ml/min. $NaNO_2$ was used as a standard for plotting the calibration curve.

Chemical reagents. Chromatographically pure preparations of TNT and 2,4-DNT (99% purity after double recrystallization from ethanol) were used in the work. The H^--THT chemical standard was provided by Prof. H.-J. Knackmuss. Analytical standards of 2-ADNT and 4-ADNT have been synthesized previously by the method of Zbarsky [13] from *ortho*- and *para*-toluylic acids, respectively. All of the HADNT isomers were synthesized by reduction of TNT nitro groups with hydrogen sulfide according to Nielsen [14].

Table 1. The dynamics of TNT concentration (μmM) under growth of *Candida* sp. AN-L15 with pH variation

Time, h	*Candida* sp. AN-L15			
	pH			
	5.0	6.0	7.0	8.0
0	440	440	440	440
15	404	412	414	420
25	286	306	313	318
35	84	90	102	123
45	26	42	55	60
55	0	0	7	15
65	0	0	0	0

RESULTS

Screening for the ability to transform TNT via the hydride ion-mediated reduction pathway made it possible to plot a broad spectrum of microorganisms in respect to occurrence of this biochemical activity.

The overwhelming majority of the isolates of aerobic heterotrophic bacteria and yeast, both from anthropogenic or natural ecological niches (the water of natural basins, soil, solid petrochemical waste, and black oil-polluted peat bogs), attacked TNT only by the pathway of nitroreduction (data not presented). Only two yeast strains (identified as *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4) showed an alternative biochemical activity as the dominant mechanism, although it was less pronounced as compared with that of *Candida* sp. AN-L13 in respect to the scale of TNT hydride ion-mediated reduction assessed by the maximum of the monohydride complex [5, 6].

The comparative analysis of nitrite production in a synthetic medium with TNT was carried out with all of the yeast reducing TNT by the aromatic ring, including four isolates obtained previously from peat bogs [5]: *Candida* spp. AN-L7, -L13, -L14, and -L20. Nitrite accumulation was favored by static cultivation conditions (data not presented). The maximal nitrite content under growth on synthetic medium with TNT (440 μM) at pH 7.0 varied within ~20–70 μM. The maximal accumulation of nitrite ion was shown in *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4, which were therefore chosen as a model for further experiments.

TNT conversion by the growing yeast cells of *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4 under static conditions was accompanied by accumulation of dark-red monohydride TNT complex. Further change of the culture fluid color to orange and then to yellow agrees with the spectral characteristics of the intermediates of the reduction pathway under study [9–11].

In view of our preliminary data on pH dependence of TNT hydride ion-mediated reduction, it was studied under pH values ranging 5.0 to 8.0 (Fig. 1).

Strain *Candida* sp. AN-L15 grown under static conditions showed the most active production of the TNT hydride complex at pH 5.0. The dynamics of accumulation of H^--THT, $2H^-$-THT, and protonated forms of the latter in the course of growth in glucose-containing medium, pH 5.0, determined spectrophotometrically, is presented in Figure 1A. Sequential increase of absorption in the region of 470–476 nm continued until 34 h from the beginning of cultivation, which corresponded to the maximum absorption A_{476} 3.25. The data on the dynamics of TNT decrease are presented in Table 1.

Further growth of this culture was accompanied by the shift of the maximum to the short-wave region of the spectrum (440–445 nm), which indicated large-scale formation of dihydride TNT complex and its protonated forms (Fig. 1A). According to the data of HPLC and GC–mass spectrometry, 2,4-DNT appeared in the medium 34 h after the beginning of cultivation in the final concentration of 18.2 μM (Table 2). Simultaneously, a non-stoichiometric amount (27.4 μM) of nitrite ion was revealed by the method of ion chromatography.

As the intermediates of nitroreduction of the original nitroaryl, the products of its mononitroreduction were revealed: HADNT (109.1 μM) and a minor amount of 4-ADNT (5.2 μM). The delayed appearance of these products as compared with H^--THT is characteristic.

The dynamics of accumulation of hydride complexes and the ratio of TNT *ortho*-nitro group elimination product and nitrites (2,4-DNT/NO_2^-), as well as the level of intermediates of the nitroreductase pathway, were similar to those typical of TNT transformation by *Candida* sp. AN-L15 grown at pH 6.0 (Fig. 1B, Table 2).

At initial pH 7.0, in spite of the similar dynamics of accumulation of H^--THT and $2H^-$-$THTH^+$ isomers, the ratios of other metabolites were changed. It is interesting that a high concentration of nitrites (76 μM) was revealed in this case, while 2,4-DNT was absent. Along with HADNT and 4-ADNT, 2-ADNT was detected as well (Fig. 1B, Table 2). This tendency was maintained at further pH shift to 8.0. Under these conditions, 2,4-DNT was not revealed either, but nitrites and HADNT were present and the quantity of 2-ADNT and 4-ADNT increased to 16.2 and 29.5 μM, respectively (Fig. 1D, Table 2). Obviously, the neutral and weakly alkaline conditions of the medium intensify the six-electron reduction of nitro groups of the initial xenobiotic in positions 2 and 4 and probably lead to another mechanism of nitrite cleavage.

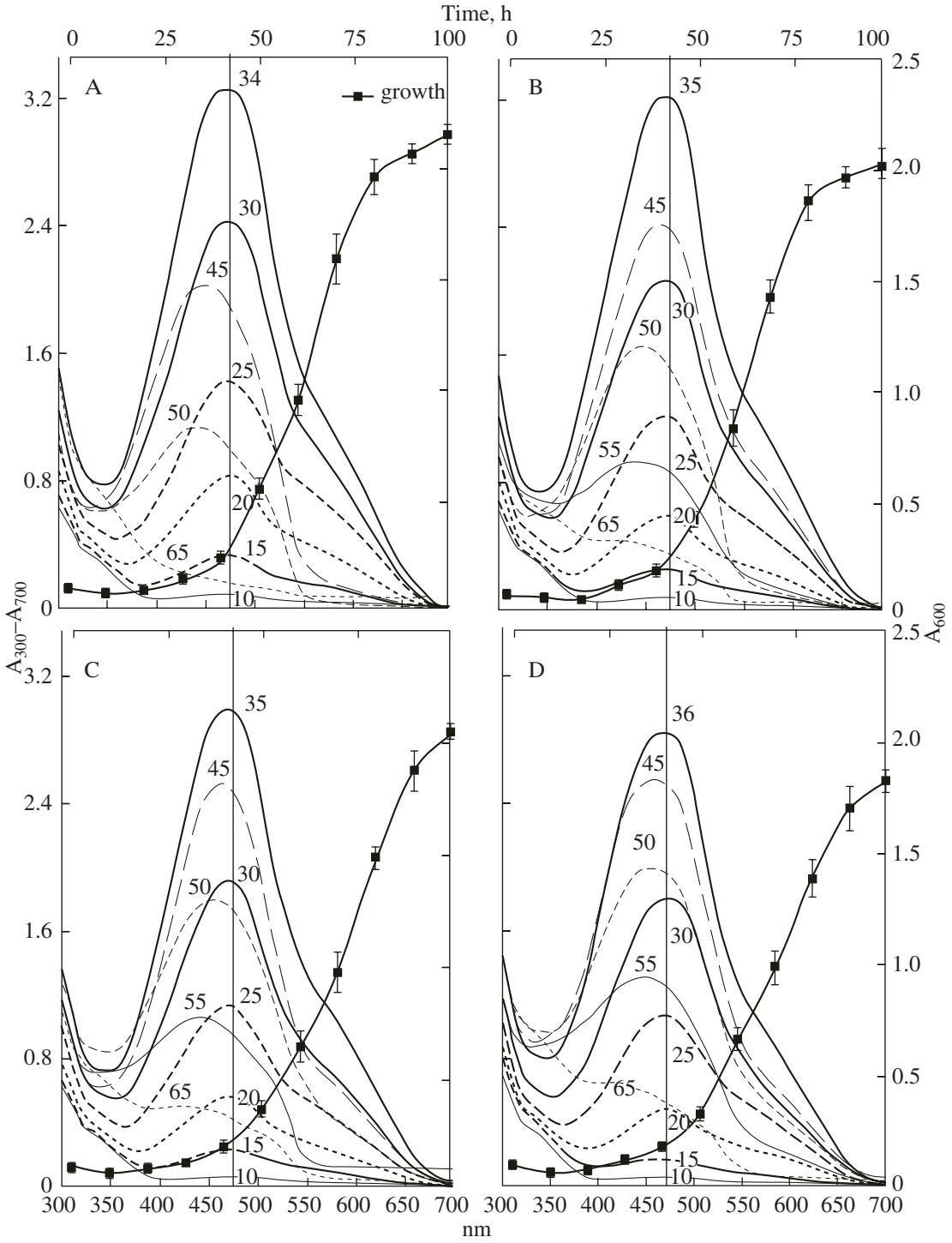

Fig. 1. Formation of the hydride complex and the total of dihydride complex isomers from TNT in the course of *Candida* sp. AN-L15 growth on glucose + TNT (440 μM). Growth medium pH: 5.0 (A); 6.0 (B); 7.0 (C); 8.0 (D). Time of conversion, h: 10, 15, 20, 25, 30, 34, 35, 36, 45, 50, 55, 65 (shown on spectral curves).

According to the above data on the effect of pH on TNT conversion, it would be interesting to compare its dynamics with yeast growth. Independent of pH, the growth of *Candida* sp. AN-L15 was activated at the stage of conversion of the hydride complex into its dihydride forms, whereas the final content of cell biomass was at a comparable level in all the variants (Fig. 1).

Data similar to those considered above were obtained for a member of another yeast genus, *Geotrichum* (strain AN-Z4) (Table 2). At the same time, the

Table 2. Maximal concentrations (μmM) of TNT nitro group elimination products (2,4-DNT (65 h*) and NO_2^- (48 h*)) and nitroreduction intermediates (HADNT (55 h*) and ADNT (65 h*)) under yeast growth at varied medium pH

Metabolites	*Candida* sp. AN-L15				*Geotrichum* sp. AN-Z4			
	pH				pH			
	5.0	6.0	7.0	8.0	5.0	6.0	7.0	8.0
2,4-DNT	18.2	16.0	ND**	ND	16.0	13.8	ND	ND
NO_2^-	27.4	32.3	76.0	70.2	26.2	30.4	61.2	65.4
HADNT	109.1	112.0	95.0	85.4	170.0	164.1	157.3	150.0
2-ADNT	ND	ND	5.6	16.2	2.1	5.1	7.2	14.8
4-ADNT	5.2	7.5	14.2	29.5	6.1	13.0	30.3	57.8

Notes: * Time (from the beginning of experiment) of detection of respective metabolite.
** Not detected.

relatively more pronounced orientation to reduction of the TNT nitro groups as compared with *Candida* sp. AN-L15 should be mentioned (Table 2).

The overall picture of *Geotrichum* sp. AN-Z4 growth and the final concentration of cell mass were similar to those considered above, although the growth rate was less than that of *Candida* sp. AN-L15.

When hexadecane was introduced into the nutrient medium instead of glucose (pH 5.0 and 6.0), the variant with *Candida* sp. AN-L15 showed growth impairment. However, as in the presence of glucose, TNT transformation was accompanied by appearance of 2,4-DNT and a nonstoichiometric quantity of nitrites; their concentrations were comparable with those under growth on glucose + TNT. Strain *Geotrichum* sp. AN-Z4 proved unable to utilize this alkane as a carbon and energy source.

DISCUSSION

Detection of microorganisms capable of deeper transformation and detoxification of TNT and its metabolites is of interest both from a theoretical point of view and as a scientific basis of relevant environment protection technologies.

In respect to the depth of TNT degradation, the attack directed to reduction of its aromatic ring and, probably, concomitant functioning of the alternative direction of TNT transformation are interesting as they may be coupled with the cleavage of at least one of the nitro groups from the carbon skeleton of a xenobiotic molecule. A decreased number of nitro groups means the conversion from trinitroaryls to the category of dinitro compounds, which are much more accessible for biodegradation [15].

The emerging concept of TNT transformation via the hydride ion-mediated reduction pathway until quite recently has been based on the study of only a few bacterial stains [9–12] and two representatives of microscopic eukaryotes [5, 6]. Rare occurrence of microorganisms with the appropriate phenotype in natural and anthropogenic biocenoses has been shown in the case of gram-positive and gram-negative bacteria [7] and in the results of the continued screening of TNT-transforming prokaryotes and lower eukaryotes performed in this work. So, whereas all randomly selected isolates (aerobic heterotrophs and yeasts) from different anthropogenic and natural ecological niches to some extent exhibited the ability to reduce TNT nitro groups, only two new strains, *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4, were capable of hydride ion-mediated reduction of the TNT aromatic ring as the dominating pathway of its transformation simultaneous with nitroreduction at a smaller scale.

The previously unknown ability of eukaryotes (exemplified by *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4) for intermediate formation of not only hydride complexes but also 2,4-DNT when grown in a synthetic medium with glucose in the presence of TNT is of special interest due to a peculiar position of the latter metabolite in the pathway of denitration of the TNT carbon skeleton (Fig. 2).

Owing to the fact that detection and quantification of nitrites is of exclusive value for the evaluation of efficiency of the mechanism and depth of TNT degradation, the criticism of Vorbeck et al. [9] is appropriate as concerns the works which used the colorimetric method on the basis of Griss reagent; this method gives a false positive reaction to nitrites in complex media, particularly in the presence of H^--THT The level of accumulated nitrites in *Candida* sp. AN-L15 and *Geot-*

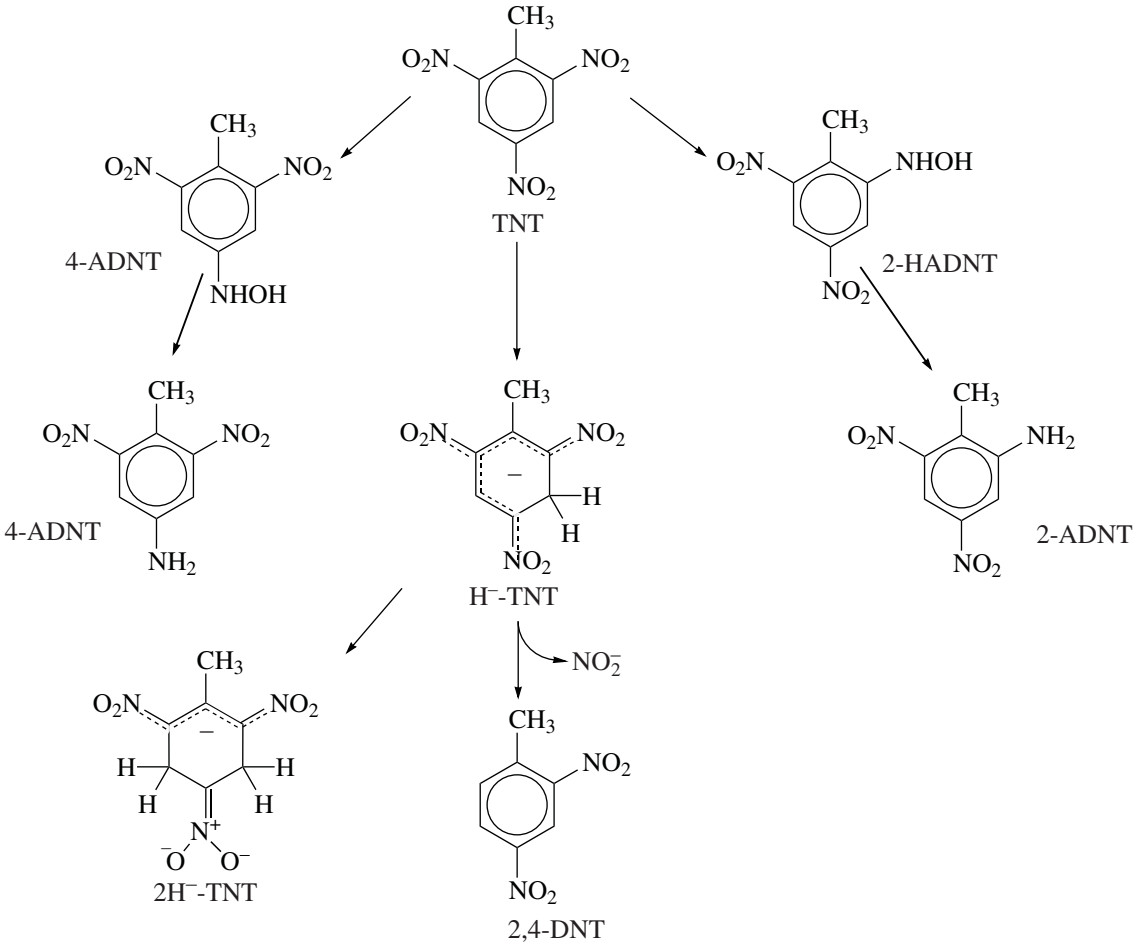

Fig. 2. Tentative scheme of concurrent functioning of alternative directions of TNT conversion in *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4.

richum sp. AN-Z4 significantly exceeds that in other yeasts from our collection. The maximal content of nitrites in culture fluids of *Candida* sp. AN-L15 and *Geotrichum* sp. AN-Z4, as evaluated by ion chromatography, was not commensurate with the amount of revealed 2,4-DNT but exceeded it 1.5–2-fold under weakly acidic conditions. When the strains are grown under weakly alkaline conditions, the ratio of 2,4-DNT and nitrites is still more contrasting: 0/70.2 μM for *Candida* sp. AN-L15 and 0/65.4 μM for *Geotrichum* sp. AN-Z4. These data imply a possibility of nitro group cleavage not only at the level of H^--THT (Fig. 2) but on a still larger scale during the conversions of TNT dihydride complexes or as a result of the interaction of the latter with the intermediates of TNT nitroreduction. At the same time, the assumption of Pak et al. [11] about the possible role of isomers of amino-dimethyl-tetranitrobiphenyl as intermediate products of such interaction was not confirmed in the case of TNT transformation by the yeasts under study, since these compounds were absent in all the variants of our experiments.

The dependence of TNT metabolism on pH in the yeasts under study shows itself in intensification of TNT conversion into monoamino derivatives under pH shift to weakly alkaline conditions. It probably reflects the response of the xenobiotic reductase type enzyme from *Pseudomonas fluorescens* I-C, which transforms TNT with formation of both nitroreduction products and intermediates of aromatic ring reduction [11].

The yeasts isolated from oil-polluted peat bogs and petrochemical wastes (oily sludge) are among the dominant microorganisms in these anthropogenic habitats. The ability to survive and dominate under such extreme conditions, in combination with the effective mechanism of TNT degradation, makes these microorganisms promising for bioremediation of industrial waste polluted with explosives. In this regard, the ability of *Candida* sp. AN-L15 to transform TNT by the combined scheme of hydride–nitroreductase reduction under growth on hexadecane instead of glucose is of particular interest. This property can be used in the case of complex pollution of soils with oil products and explosives, e.g. on the territories of military bases and other objects associated with manufacture and application of military products.

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