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EXPERIMENTAL ARTICLES

Alternative Pathways of the Initial Transformation of 2,4,6-Trinitrotoluene by Yeasts

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Abstract—A new model for the initial transformation of 2,4,6-trinitrotoluene (TNT) by facultatively anaerobic and aerobic yeasts is presented. The model is based on the data that *Saccharomyces* sp. ZS-A1 was able to reduce the nitrogroups of TNT with the formation of 2- and 4-hydroxyaminodinitrotoluenes (2-HADNT and 4-HADNT) as the major early TNT metabolites (the molar HADNT/TNT ratio reached 0.81), whereas aminodinitrotoluenes (ADNTs) and the hydride-Meisenheimer complex of TNT (H-TNT) were the minor products. *Candida* sp. AN-L13 almost completely transformed TNT into H-TNT through the reduction of the aromatic ring. *Candida* sp. AN-L14 transformed TNT through a combination of the two mechanisms described. Aeration stimulated the production of HADNT from TNT, whereas yeast incubation under stationary conditions promoted the formation of HADNT. The transformation of TNT into HADNT led to a tenfold increase in the acute toxicity of the TNT preparation with respect to *Paramecium caudatum*, whereas the increase in the toxicity was about twofold in the case of the alternative attack at the aromatic ring.

Key words: 2,4,6-trinitrotoluene, initial transformation, yeasts, toxicity.

The ever-increasing pollution of the environment with nitroaromatic compounds is due to their wide use as pesticides, chemical semiproducts, and explosives. In particular, the manufacture, storage, and use of 2,4,6-trinitrotoluene-based industrial and munition explosives have resulted in TNT being one of the major pollutants of the environment. For instance, soils and groundwaters in about 60 regions in Germany, where munition plants producing explosives were situated during World War II, are severely polluted with TNT [1]. The persistence of TNT in the polluted areas implies that it is poorly metabolized by microorganisms and hence is insignificantly involved in the biogeochemical cycles of carbon and nitrogen.

The toxicity of TNT and related compounds, which are also specified by the US Environmental Protection Agency as possible carcinogens [2], makes the ecological risks associated with the TNT persistence still greater. According to the results obtained using the *Salmonella typhimurium* fluctuation test, TNT and its mono- and diamino derivatives possess comparable mutagenicity [3].

The diversity and high metabolic potential of microorganisms can successfully be used to develop advanced technologies for the bioremediation of TNT-contaminated areas. This work is an attempt to study the metabolic potential of yeasts for initial attack on TNT as the ecologically hazardous pollutant of the environment.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Experiments were carried out with the yeasts *Saccharomyces* sp. ZS-A1, *Candida* sp. AN-L13, and *Candida* sp. AN-L14 obtained from the collection at the Kazan State University. The yeasts were cultivated at 28°C in shaken (120 rpm) flasks in a medium containing (g/l distilled water) glucose, 10.0; peptone, 7.0; yeast extract, 5.0; and NaCl, 1.0 (pH 6.5).

The ability of the yeasts to utilize TNT as the sole source of nitrogen was tested by growing them in a synthetic medium containing (g/l distilled water) glucose, 10.0; $MgSO_4$, 0.25; NaCl, 0.125; Na_2HPO_4, 8.7; KH_2PO_4, 5.3; and TNT, 0.1 (pH 6.0).

Cell biomass was evaluated by measuring the optical density of cell suspensions at 600 nm (OD_{600}) with reference to the culture or incubation liquid separated from yeast cells.

The transformation of TNT by cell suspensions. Yeast cells were cultivated until the late exponential phase, harvested by centrifugation at 5000 *g* for 15 min, washed with 16 mM phosphate buffer (pH 6.0), and resuspended in the buffer to an optical density OD_{600} = 1.0. The cell suspension was supplemented with 0.44 mM TNT (TNT was added in the form of ethanol solution). The incubation mixture contained 5 mM glucose as the main source of reducing equivalents.

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In aerobic experiments, 250-ml flasks containing 50 ml of the incubation mixture were shaken at 120 rpm. In stationary experimental variants, incubation was performed in test tubes with a thick (16 cm) layer of the incubation mixture. In both experimental variants, cells were removed by centrifugation after the incubation of the mixture at 30°C, and the supernatant was analyzed for TNT and its transformation products.

Acute toxicity test. Acute toxicity was determined using a modified express test with *Paramecium caudatum* [4]. 16 mM phosphate buffer (pH 6.0) was used as the control. All measurements were conducted in quintuplicate. The results were expressed as the death rate of 10 individual paramecium organisms exposed to 0.3 ml of the tested liquid for 1 h. The highest detected acute toxicity level was taken to be 1.0, whereas 0 corresponded to the absence of lethal effect.

Analytical methods. TNT and its transformation products were analyzed by HPLC using an LKB 2150 liquid chromatograph equipped with a reversed-phase column (4.0 × 240 mm) packed with Spherisorb OD52 (bead size 4 μm), a UV detector at 254 nm, and a controller (Pharmacia–LKB Biotechnology, Sweden). The column was eluted at 30°C with a methanol–water (40 : 60) mixture at a flow rate of 1.0 ml/min. To analyze H-TNT, the column was eluted, at a flow rate of 1.0 ml/min, with an acetonitrile–water (45 : 55) mixture containing 20 mM tetrabutylammonium iodide as the ion-pair compound. H-TNT was detected at 546 nm. The absorption spectrum of H-TNT in the UV–visible spectral region was recorded in a Lambda 35 Perkin-Elmer spectrophotometer after the complete extraction of nonpolar compounds from the incubation mixture with diethyl ether and chloroform.

2-HADNT and 4-HADNT were separated and identified by the Wang *et al.* method [5]. HADNT and ADNT were synthesized as described earlier [6]. H-TNT was kindly donated by H.-J. Knackmuss from Stuttgart University (Germany).

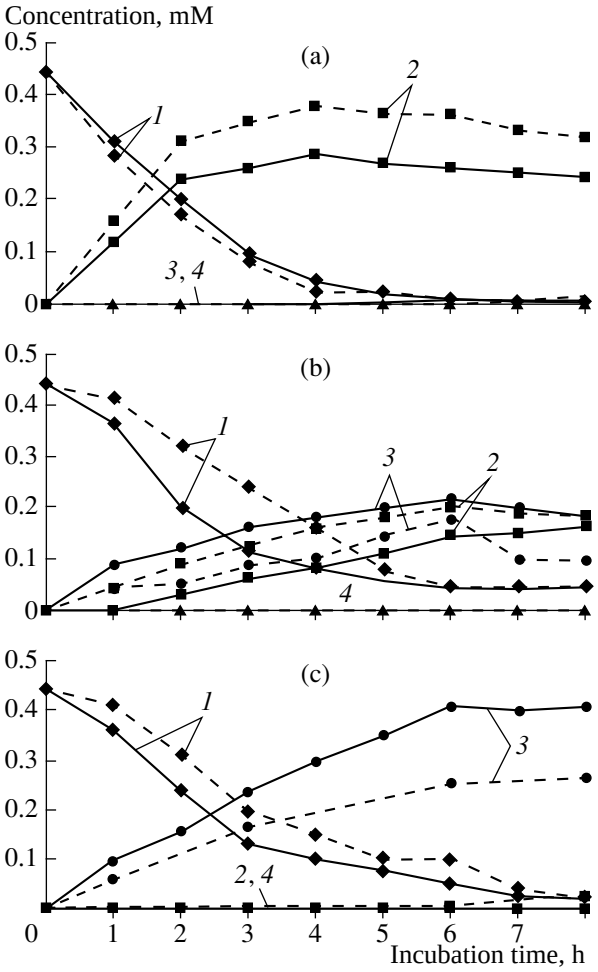

Fig. 1. The transformation of TNT by cell suspensions of (a) *Saccharomyces* sp. ZS-A1, (b) *Candida* sp. AN-L14, and (c) *Candida* sp. AN-L13 under (—) aerobic and (–––) stationary conditions: (*1*) TNT, (*2*) HADNT, (*3*) H-TNT, and (*4*) ADNT.

RESULTS AND DISCUSSION

Data on the transformation of TNT by the cell suspensions of fermenting (*Saccharomyces* sp. ZS-A1) and respiring (*Candida* sp. AN-L13 and *Candida* sp. AN-L14) yeasts are presented in Fig. 1. It can be seen that TNT was reductively transformed, irrespective of the incubation conditions used. *Saccharomyces* sp. ZS-A1 transformed TNT mainly into HADNT, stationary incubation conditions being more favorable for the reduction of the nitrogroup than aerobic incubation conditions (as can be seen from Fig. 1a, the HADNT/TNT ratio in these two cases was equal to 0.81 and 0.65, respectively). At the same time, *Candida* sp. AN-L13 transformed TNT with the formation of H-TNT in almost stoichiometric amounts (Fig. 1c). In this case, after 6 h of incubation, the H-TNT/TNT ratio was equal to 0.93 and 0.77 under aerobic and stationary conditions, respectively. As for *Candida* sp. AN-L14, this yeast exhibited a combination of the described reductive attacks on the TNT molecule (Fig. 1b). Aeration exerted the same stimulating effect on the formation of H-TNT from TNT as in the case of *Candida* sp. AN-L13, whereas the production of HADNT was higher under stationary conditions, as in the case of *Saccharomyces* sp. ZS-A1. ADNT was detected in small amounts (no more than 4% of the initial content of TNT in the incubation mixture) only at the end of the incubation period (Fig. 1).

The strain *Candida* sp. AN-L13, which transformed TNT with the formation of H-TNT, was able, to a certain degree, to utilize TNT as the sole source of nitrogen (Fig. 2). However, in the course of further repeated subculturing in the synthetic medium with 0.44 mM TNT, *Candida* sp. AN-L13 exhibited a gradual decrease in its growth rate. A similar tendency was

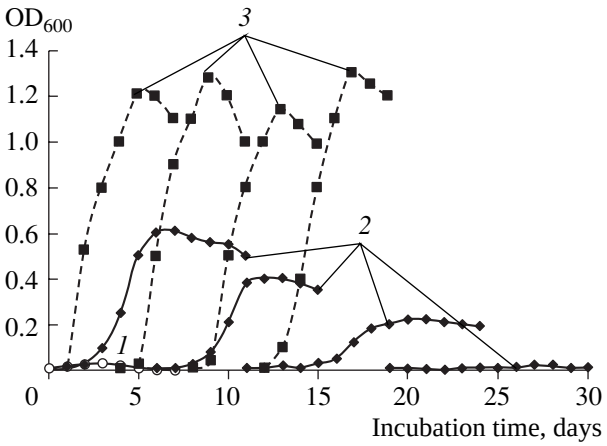

Fig. 2. Growth of *Candida* sp. AN-L13 on different nitrogen sources: (*1*) medium without any nitrogen source; (*2*) medium with TNT; and (*3*) medium with an equivalent concentration of $(NH_4) \cdot 2SO_4$ with respect to the nitrogen content.

observed by Vorbeck *et al.* for three TNT-degrading bacterial strains isolated from contaminated soil [7].

As can be seen from the table, the amount of the TNT transformed by the yeast species increased with its initial concentration (three initial TNT concentrations were tested, 0.11, 0.22, and 0.44 mM).

Experiments with some other yeasts from the collection of the Kazan State University showed that three saccharomycetes transformed, to certain degrees, TNT into 2-HADNT and 4-HADNT, whereas *Candida* sp. AN-L7 and *Candida* sp. AN-L20 transformed TNT into a mixture of HADNT and H-TNT (data not presented). It should be noted that no yeast strains other than *Candida* sp. AN-L13 that would be able to transform TNT into H-TNT in stoichiometric amounts have so far been found.

The ability of *Saccharomyces* sp. ZS-A1 and other saccharomycetes to attack the TNT molecule through the reduction of the nitrogroups is likely related to their fermentative metabolism and the ability to ferment glucose in considerable amounts even under aerobic conditions [8]. Conversely, the respiratory metabolism of glucose, typical of *Candida* yeasts, may be responsible for the hydrogenation of TNT and the formation of H-TNT in some way or other.

Taking into account the high reactivity of the early TNT metabolites, we tested the toxicity of all incubation mixtures at the time of the maximum accumulation of the metabolites, i.e., after 6 h of incubation (Fig. 1). The results of the preliminary estimation of the toxicity of chemically synthesized TNT, HADNT, and H-TNT (Fig. 4a) suggested that the incubation mixture containing the yeast *Saccharomyces* sp. ZS-A1, which mainly produces HADNT from TNT, must be most toxic. Toxicity tests confirmed this supposition (Fig. 4b). The lowest toxicity was observed for the incubation mixture containing the yeast *Candida* sp. AN-L13, which transforms TNT with the formation of H-TNT (the least toxic product of TNT transformation). In accordance with the above data that *Candida* sp. AN-L14 transforms TNT into a mixture of HADNT and H-TNT (Fig. 1b), the incubation liquid of this strain exhibited a medium toxicity (Fig. 4).

Since conditions in explosive-polluted soils and groundwaters are aerobic or microaerobic, the model presented may reflect actual processes in these anthropogenically impacted econiches, where microbial cells growing on readily metabolizable suitable substrates can easily transform TNT into relatively stable early transformation products.

Experimental data on the acute toxicity of the HADNT standard and HADNT-containing incubation mixtures agree well with the inhibitory action of HADNT on the lignin peroxidase of *Phanerochaete chryzosporium* [9] and the key enzymes (glyceraldehyde 3-phosphate and glucose-6-phosphate dehydrogenases) of glycolysis and the pentose phosphate cycle [6]. It is HADNTs that may be responsible for a drastic decrease in the microbial diversity of petrochemical waste sludge, which was observed under the action of TNT (data not presented).

The formation of the early TNT transformation products by suspensions of yeast cells depending on the initial concentration of TNT and aeration conditions

Strains	Transformation products, mM											
	aerobic conditions						stationary conditions					
	H-TNT			HADNT			H-TNT			HADNT		
	a	b	c	a	b	c	a	b	c	a	b	c
Saccharomyces sp. ZS-A1	0.01	0.02	0.09	0.07	0.12	0.29	0	0	0.02	0.09	0.17	0.36
Candida sp. AN-L14	0.03	0.12	0.2	0.02	0.04	0.11	0.02	0.07	0.11	0.04	0.11	0.18
Candida sp. AN-L13	0.06	0.17	0.41	0	0.01	0.02	0.05	0.16	0.34	0.01	0.03	0.1

Note: The initial concentration of TNT was (a) 0.11, (b) 0.22, and (c) 0.44 mM.

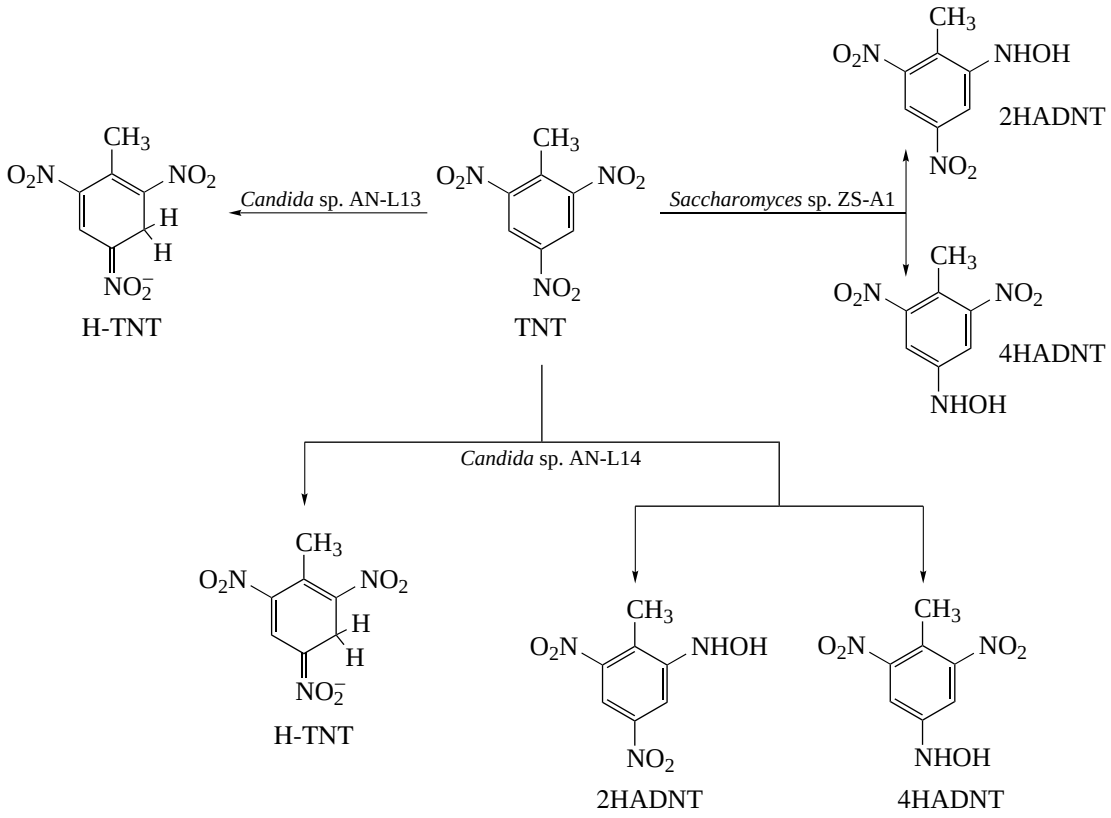

Fig. 3. Diagram showing the initial steps of TNT transformation by yeasts.

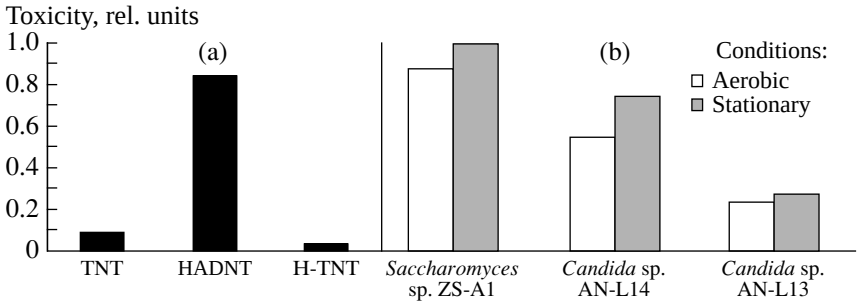

Fig. 4. The acute toxicity of (a) the chemical standards of TNT, HADNT, and H-TNT taken at a concentration of 0.44 mM and (b) the incubation liquids at the time of the maximum accumulation of TNT transformation products. The initial concentration of TNT in the incubation mixtures was 0.44 mM.

The combined reduction of TNT was observed earlier for *Rhodococcus erythropolis* HL PM-1 [7] and *Enterobacter cloacae* PB2 [10]. The flavoprotein oxidoreductase (XenB) of *Pseudomonas fluorescens* I-C was found to transform TNT both through the reduction of the nitrogroups and through the direct hydrogenation of the aromatic ring [11]. The latter mechanism is the closest to that implemented by *Candida* sp. AN-L14 (Fig. 3). To the best of our knowledge, the reduction of TNT to H-TNT in stoichiometric amounts by the yeast *Candida* sp. AN-L13 has not yet been described in the literature.

Toxicological testing performed in accordance with the proposed pathways of TNT transformation by yeasts (Fig. 3) showed the important role of the reductive attack at the aromatic ring of 2,4,6-trinitrotoluene, which makes it possible to a avoid drastic increase in the toxicity of the early TNT transformation products. The results of the present study confirm the prediction of Lenke *et al.* [12] that the transformation of TNT with

the formation of H-TNT is one of the most efficient ways of TNT biodegradation.

It should be noted that *Candida* sp. AN-L13, which was isolated from the oil-contaminated peats of Langepas (West Siberia) as one of the dominant microorganisms, is able to utilize crude oil and some individual aliphatic and aromatic hydrocarbons (data not presented). In view of this, *Candida* sp. AN-L13, along with other microorganisms with a similar metabolism, may be of practical importance for the bioremediation of areas contaminated with both explosives and petrochemicals.

Studies of the conditions promoting one or another mechanism of TNT transformation by yeasts are in progress in our laboratory.

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