ELSEVIER

FEMS Microbiology Letters 217 (2002) 213–217

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Models of 2,4,6-trinitrotoluene (TNT) initial conversion by yeasts

S.A. Zaripov [a], A.V. Naumov [b], J.F. Abdrakhmanova [a], A.V. Garusov [a], R.P. Naumova [a,*]

[a] *Department of Microbiology, Kazan State University, 18 Kremlevskaya str., Kazan 420008, Russia*
[b] *Montana State University, Bozeman, MT, USA*

Received 31 July 2002; received in revised form 4 October 2002; accepted 16 October 2002

First published online 6 November 2002

Abstract

The original models of the initial steps of 2,4,6-trinitrotoluene (TNT) conversion by yeasts are presented. *Saccharomyces* sp. ZS-A1 reduced nitro groups of TNT producing isomeric monohydroxylaminodinitrotoluenes (HADNT) as the key initial metabolites (molar ratio HADNT/TNT was up to 0.81), whereas aminodinitrotoluenes (ADNT) and the hydride–Meisenheimer complex of TNT (H-TNT) were the minor products. Conversely, *Candida* sp. AN-L13 transformed TNT almost quantitatively into H-TNT, thus realizing the alternative attack, consisting of the TNT aromatic ring reduction. The third type of conversion, revealed in *Candida* sp. AN-L14, is the combination of both above mechanisms and produces an equimolar mix of HADNT and H-TNT. In the toxicity tests with *Paramecium caudatum*, the supernatant of *Saccharomyces* sp. ZS-A1, which converts TNT into HADNT, was most toxic while the supernatant of *Candida* sp. AN-L13 (TNT → H-TNT) was least toxic. The microorganisms converting TNT quantitatively to the reactive metabolites can be useful for their immobilization through the detoxifying interaction with the soil components such as humic compounds.
© 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: 2,4,6-Trinitrotoluene; Initial transformation; Reactive metabolite; Yeast; Toxicity; Explosives and oil contamination

1. Introduction

The widespread dissemination of the nitroaromatic compounds in the environment is due to their use as pesticides, in the production of chemical precursors, explosives, and a number of other applications. 2,4,6-Trinitrotoluene (TNT) is a major component of military and industrial explosives. It is well known that technological operations related to its production, storage, and use result in a widespread dispersion of TNT in the environment as a characteristic pollutant. For example, in Germany, where TNT was the main explosive produced during World War II, in about 60 former production sites TNT may persist in the solid state and act as a long-term source of soil and groundwater contamination [1]. The presence of TNT in the polluted areas, decades after the halting of its production, suggests that there are metabolic barriers preventing the biodegradation and entrance of this pollutant into the global biogeochemical carbon and nitrogen cycles. The existence of such barriers is also substantiated by the poor mineralization rate of TNT and its metabolites in different composting systems [2].

In TNT biotransformation, the most commonly detected metabolites are aminodinitrotoluenes (ADNT) and, in some cases, diaminonitrotoluenes (DANT) [3–7]. On the other hand, more recent investigations have discovered the early reduced metabolites of TNT, namely hydroxylaminodinitrotoluenes (HADNT), which are the products of four-electronic reduction of the 2- or 4-nitro groups by fungi [8] and bacteria [9–11]. Some lactobacilli are able to transform up to 600 μmol of TNT stoichiometrically to HADNT [9,12]. It has been shown that a partially reduced alternative intermediate, the hydride–Meisenheimer complex of TNT (H-TNT), can be produced by some bacteria through the transfer of hydride ion to the aromatic ring of TNT [14].

While this restricted reductive conversion of TNT has been demonstrated for some bacteria, actinomyces, and fungi [14,15], the possibility of analogous conversion by yeasts has not been elucidated. Thus the aim of this

* Corresponding author. Tel.: +7 (8432) 726129.
E-mail address: nrp@ksu.ru (R.P. Naumova).

0378-1097/02/$22.00 © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0378-1097(02)01085-6

work was to elucidate the initial steps of TNT transformation by yeasts, from the viewpoint of the principal possibility as well as the depth of biodegradation of this pollutant.

2. Materials and methods

2.1. Microorganisms and cultivation conditions

This work was performed using the yeast strains from our collection at the Kazan State University. *Saccharomyces* sp. ZS-A1, *Saccharomyces* sp. ZS-A3, *Saccharomyces* sp. ZS-A8, and *Saccharomyces*. sp. ZS-A9 were the baking yeast strains. *Candida* sp. AN-L7, *Candida* sp. AN-L13, *Candida* sp. AN-L14, and *Candida* sp. AN-L20 were isolated from oil-polluted peat bogs (Langepas, Western Siberia, Russia).

Yeasts were grown on the medium containing glucose (10.0 g/l), peptone (7.0 g/l), yeast extract (5.0 g/l), NaCl (1.0 g/l), with pH adjusted to 6.5. The cultivation was performed in shaking flasks (120 rpm) at 28°C. The growth of yeasts was monitored at A_{600}, with the cell-free culture media serving as blanks.

2.2. Transformation of TNT by yeast cell suspensions

The strains were grown to the late exponential phase, harvested (5000×*g*, 15 min), washed with 16 mM phosphate buffer (pH 6.0), and resuspended in the same buffer to the density $A_{600} = 1.0$. TNT was added as ethanol solution to the final concentration of 0.44 mM. The incubation mixture contained 5 mM of glucose as the main potential source of reducing equivalents. Aerobic incubation was performed in shaking flasks (250 ml) containing 50 ml of the incubation mixture. In the static variant, the same amount of mixture was incubated without shaking in a 21 mm diameter tube completely filled with the incubation mix. After various periods of incubation at 30°C, the cells were harvested with the following determination of TNT and its metabolites in the supernatants.

2.3. Toxicity test

The toxicity of cell-free incubation fluids and chemical standards was determined using the modified *Paramecium caudatum* express-test [16]. The test was based on the mortality rate of the test organisms exposed to 0.5 ml of the analyzed liquid for 1 h, using 10 individual cells of *P. caudatum*. Phosphate buffer (16 mM, pH 6.0) was used as a control. The analysis was repeated five times for each sample. The toxicity was expressed as the relative lethal effect. For the scale of this test, the highest value of the toxicity level was accepted as one, and nil was accepted as the survival rate in control.

2.4. Chemical analyses

High-performance liquid chromatography analyses of TNT and its nitroreduction products were performed using a liquid chromatograph LKB2150 equipped with reverse phase column (4.0×240 mm, 4 μm Spherysorb OD52, LKB), UV-detector (254 nm), and controller (Pharmacia LKB Biotechnology, Sweden). Elution was accomplished isocratically, with a methanol:water solvent system (40:60), flow rate of 1.0 ml/min, and at 30°C. For H-TNT analysis, elution was achieved with an acetonitrile:water solution system (45:55) supplemented by tetrabutyl ammonium iodide as an ion pair component, with an elution rate of 1.0 ml/min, and detection at 546 nm. UV-VIS absorption spectrum of these metabolites was recorded on a Lambda 35 spectrophotometer (Perkin Elmer, USA), after exhaustive extraction of apolar components of the incubation fluid with diethyl ether and chloroform.

Separation and identification of 2- and 4-isomers of HADNT were performed as described by Wang et al. [17].

Chemical synthesis of HADNTs and ADNTs was performed as described earlier [13]. Chemical standard of hydride complex of TNT was kindly provided by Prof. H.-J. Knackmuss (University of Stuttgart, Germany).

3. Results and discussion

Results of evaluation of the TNT transforming ability by cell suspensions of fermenting (*Saccharomyces* sp. ZS-A1) and respiring (*Candida* sp. AN-L13 and *Candida* sp. AN-L14) yeasts are shown in Fig. 1. Independently of incubation conditions, TNT undergoes the reductive transformation. The most interesting aspect of these results is the ability of different yeast strains to transform a major part or all of TNT by alternative reductive pathways. Thus, *Saccharomyces* sp. ZS-A1 converted the parent

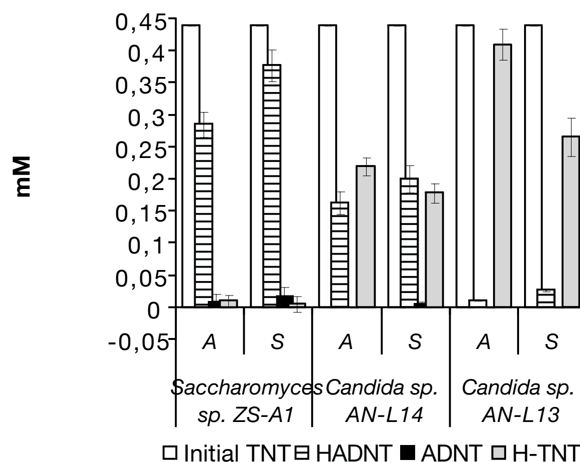

Fig. 1. Ratio of TNT transformation products formed by yeast under different aeration conditions during the maximum accumulation of intermediates (after 6 h). Conditions: A, aerobic; S, static.

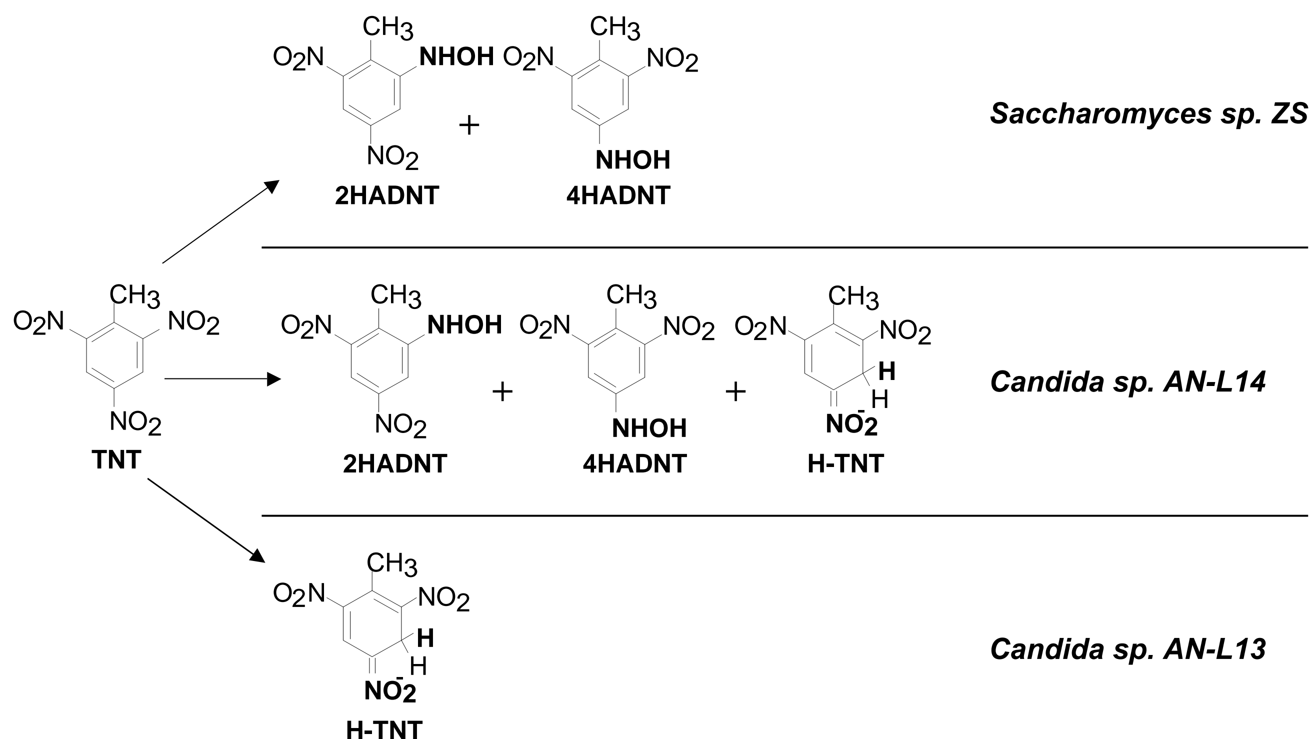

Fig. 2. Schematic representation of the initial TNT transformation steps by yeasts.

compound practically into HADNT only (Fig. 1). In this case, the nitro group reduction was more profound under the static incubation conditions in comparison with aerobic incubation: the ratio of HADNT/TNT reached 0.81 and 0.65, respectively (Fig. 1). On the contrary, the TNT transformation by the *Candida* sp. AN-L13 strain resulted in the stoichiometric accumulation of H-TNT (Fig. 1). The proportion of the end products under two aeration conditions was also reversed, with aerobic incubation favoring the formation of H-TNT: the H-TNT/TNT ratio after 6 h incubation was 0.93 under aerobic conditions and 0.77 under the static conditions. The *Candida* sp. AN-L14 strain displayed the combination of the two pathways, with accumulation of almost equal quantities of both intermediates of the TNT reductive attack, irrespective of aeration conditions (Fig. 1). Obviously, this strain may possess enzymes for both pathways, albeit the presence of reductases with very broad substrate specificity cannot be excluded. The general scheme of TNT transformation by yeasts is given in Fig. 2.

Some other yeast strains from our collection were tested for the formation of the TNT conversion products according to the presented scheme (Fig. 2). Indeed, three *Saccharomyces* strains transformed TNT to a greater or lesser extent into HADNT, while the additionally tested *Candida* strains (AN-L7 and AN-L20) produced a mixture of HADNT and H-TNT (Table 1). No strains analogous to *Candida* sp. AN-L13, which performs a practically unidirected reduction of the aromatic ring, were found, however, among other strains in our collection. Such microorganisms are also unknown in the literature. Interestingly, the taxonomic affiliation and the type of metabolism (fermentative vs. respiratory) of yeasts correlated with the pathway of TNT degradation. Thus, *Saccharomyces* sp.

Table 1
Concentration of TNT intermediates formed by yeast strains under aerobic (A) and static (S) conditions with 0.44 mM initial concentration of TNT

Strain	Intermediates (mM)					
	HADNT		ADNT		H-TNT	
	A	S	A	S	A	S
Saccharomyces sp. ZS-A3	0.24	0.36	0.01	0.02	0.01	0
Saccharomyces sp. ZS-A8	0.25	0.36	0.03	0.05	0.05	0
Saccharomyces sp. ZS-A9	0.30	0.34	0	0.02	0.015	0.005
Candida sp. AN-L7	0.11	0.18	0	0	0.24	0.18
Candida sp. AN-L20	0.19	0.23	0	0.01	0.16	0.11

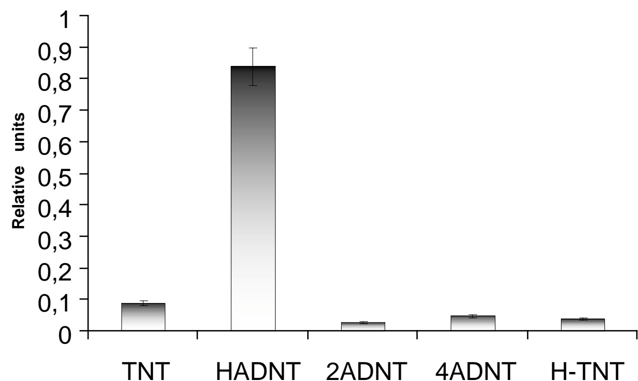

Fig. 3. Toxic effect of TNT and its metabolites in the toxicity test with *P. caudatum*. Concentrations of all compounds were equal to the initial TNT concentration in transformation experiments with cell suspensions, e.g. 0.44 mM.

ZS-A1 as well as other yeast strains belonging to this genus attacked the TNT molecule predominantly through the nitro group reduction [18]. Remarkably, other representatives of fermenting organisms, lactic acid bacteria, transformed TNT under similar conditions in stoichiometric amounts of HADNT [9,12]. Conversely, the respiratory type of glucose catabolism by the representatives of the genus *Candida* could mediate the ring hydrogenation, giving rise to H-TNT.

The combination of alternative modes of TNT initial reduction was observed earlier in *Rhodococcus erythropolis* HL PM-1 [13] and *Enterobacter cloacae* PB2 [19]. This transformation mechanism is the most similar to that performed by *Candida* sp. AN-L14 (Fig. 2).

Since the environmental conditions in explosive-polluted soils and waters are typically aerobic or microaerobic, the presented models of TNT transformation can reflect many real situations in such anthropogenic ecological niches where the microbial cells, growing on suitable accompanying substrates, can convert TNT into the early metabolites.

The most important aspect of the environmental pollution with TNT and its metabolites is obviously the exposure of wildlife, food animals and humans to the toxic effects of these compounds. Because of the relatively unstable nature of both HADNT [20] and H-TNT [13], we have chosen the *P. caudatum* toxicity test, which allows one to perform the test within a reasonably short period of time. Our preliminary experiments revealed that the concentrations of both HADNT and H-TNT were basically unchanged during 1 h incubation with *P. caudatum* cells, the time frame usually recommended for this test. Because the test is rapid, simple, and allows one to collect statistically sound data inexpensively, it was chosen to test the relative toxicity of TNT and the products of its transformation.

Comparative toxicological assessment of TNT, HADNT, and H-TNT chemical standards revealed that HADNTs are the most toxic compounds (Fig. 3) and, supposedly, the supernatant of the *Saccharomyces* sp. ZS-A1 strain, which converts TNT almost exclusively to HADNT, must be the most toxic for *P. caudatum* cells. Indeed, incubation of *P. caudatum* cells with the supernatant from this incubation fluid resulted in the highest mortality rates in comparison with other variants (Fig. 4). The lowest mortality rates were observed with the supernatants of *Candida* sp. AN-L13, which converts TNT to H-TNT (Fig. 4). This mortality rate is higher in comparison with the pure chemical incubation (Fig. 3) and may be attributed to the presence of small amounts of highly toxic HADNT in the supernatant of *Candida* sp. AN-L13 (Fig. 1). The presence of both metabolites in equimolar quantities in the culture supernatant of *Candida* sp. AN-L14 (Fig. 1) stipulated the intermediate level of toxicity (Fig. 4).

The separation of HADNT isomers by two-dimensional thin-layer chromatography according to [17] revealed the predominance of 4-HADNT over 2-HADNT in incubation fluids of all three strains demonstrating the models of TNT attack (Fig. 1 and 2, Table 1). The sum of HADNT rather than the isomers' ratio seems responsible for the heightened toxicity levels.

Some aspects of the cellular targets for the HADNT attack and the corresponding toxicity mechanisms have been studied in a limited number of microorganisms. It has been shown that HADNT inhibits lignin peroxidase of *Phanerochaete chrysosporium* [8] and key dehydrogenases (3-phosphoglyceraldehyde and glucose-6-phosphate) of glycolysis and pentose phosphate pathway [12]. The latter inhibitory effect may be the case with our test organism, *P. caudatum*. Presumably, the hydroxylamino derivatives of TNT, as the most toxic metabolites, may be responsible for the sharp reduction in both microbial numbers and population diversity in the petrochemical waste sludges exposed to TNT (data not shown). Our toxicological study suggest that the preferential pathway for TNT transformation could be via the hydrogenation of the aromatic ring, thus avoiding the formation of highly toxic products which may kill or inactivate the members of TNT degradation consortia. Thus, our results support the suggestion that the pathway leading to H-TNT may be the most attractive in terms of creating catabolic potential for the effective TNT biodegradation [21].

Evidently, *Candida* sp. AN-L13, like the other H-TNT-

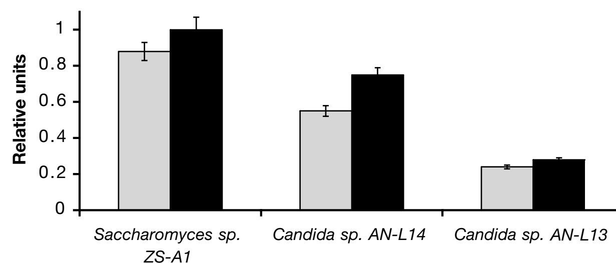

Fig. 4. *P. caudatum* toxicity test with incubation fluids at the time of maximal accumulation of specific metabolites (after 6 h incubation of yeast cell suspension with 0.44 mM TNT).

producing microorganism, *Mycobacterium* sp. [22], is not able to mineralize TNT, although the *Nocardia* strain was shown to possess the corresponding ability against another trinitroaromatic compound, picric acid [23]. Nevertheless, the reduced TNT metabolites, especially those with low toxicity in comparison with the parent xenobiotic and alternative intermediates, can be considered as the key intermediates from the viewpoint of the major problem to be resolved, namely 'mineralization versus immobilization' [14]. Their interaction with different compounds, such as humic acids of soil, can lead to the formation of environmentally stable, less toxic or harmless end products. These initial reduction steps are also creating the possibilities for other members of the biotransformation consortia (bacteria and plants) to perform further reduction and co-metabolic transformation steps.

Complex environmental contaminations such as explosives+petroleum hydrocarbons are not uncommon and in this regard the strain *Candida* sp. AN-L13, which was isolated earlier as one of the dominant microorganisms from oil-polluted peat bogs (Langepas, Western Siberia, Russia), deserves special attention. In addition to its ability to perform the initial TNT conversion step, it is able to utilize crude oil and several individual aliphatic and aromatic hydrocarbons (data not shown). This strain, as well as other microorganisms with comparable metabolic capabilities, is very interesting not only for academic research but it also possesses vast potential for bioremediation of areas with complex contaminations.

The work determining the mechanisms of the initial TNT transformation steps by yeasts is currently in progress.

Acknowledgements

The authors would like to thank Prof. H.-J. Knackmuss (University of Stuttgart, Germany) for providing the chemical standard of the hydride–Meisenheimer complex of TNT. This research was supported in part by the European Commission Grant ICA2-CT-2000-10006.

References

[1] Preuss, J. and Haas, R. (1987) Die Standorte der Pulver-, Sprengstoff-, Kampf- und Nebelstofferzeugung im ehemaligen Deutschen Reich. Geogr. Umschau. 39, 578–584.

[2] Semple, K.T., Reid, B.J. and Fermor, T.R. (2001) Impact of composting strategies on the treatment of soils contaminated with organic pollutants. Environ. Pollut. 112, 269–283.

[3] Esteve-Nunez, A., Lucchesi, G., Philipp, B., Schink, B. and Ramos, J.L. (2000) Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. J. Bacteriol. 182, 1352–1355.

[4] Johnson, M.S., Vodela, J.K., Reddy, G. and Holladay, S.D. (2000) Fate and the biochemical effects of 2,4,6-trinitrotoluene exposure to tiger salamanders (Ambystoma tigrinum). Ecotoxicol. Environ. Saf. 46, 186–191.

[5] Hannink, N., Rosser, S.J., French, C.E., Basran, A., Murray, J.A., Nicklin, S. and Bruce, N.C. (2001) Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. Nat. Biotechnol. 19, 1120–1121.

[6] Pavlostathis, S.G. and Jackson, G.H. (2002) Biotransformation of 2,4,6-trinitrotoluene in a continuous-flow *Anabaena* sp. system. Water Res. 36, 1699–1706.

[7] Thiele, S., Fernandes, E. and Bollag, J.M. (2002) Enzymatic transformation and binding of labeled 2,4,6-trinitrotoluene to humic substances during an anaerobic/aerobic incubation. J. Environ. Qual. 31, 437–444.

[8] Michels, J. and Gottschalk, G. (1994) Inhibition of the lignin peroxidase of *Phanerochaete chrysosporium* by hydroxylaminodinitrotoluene, an early intermediate in the degradation of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 60, 187–194.

[9] Naumov, A.V., Zaripova, S.K., Suvorova, E.S., Khamidullina, E.T. and Wild, D.R. (1997) Transformation of 2,4,6-trinitrotoluene to hydroxylaminodinitrotoluenes by lactic acid bacteria. VINITI (Russia) #2614-B97.

[10] Fiorella, P.D. and Spain, J.C. (1997) Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl. Environ. Microbiol. 63, 2007–2015.

[11] Khan, T.A., Bhadra, R. and Hughes, J. (1997) Anaerobic transformation of 2,4,6-TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*. J. Ind. Microbiol. Biotechnol. 18, 198–203.

[12] Naumov, A.V., Suvorova, E.S., Boronin, A.M., Zaripova, S.K. and Naumova, R.P. (1999) Transformation of 2,4,6-trinitrotoluene by *Lactobacillus* with forming toxic hydroxylaminodinitrotoluenes. Microbiology (Moscow) 68, 65–71.

[13] Vorbeck, C., Lenke, H., Fischer, P., Spain, J. and Knackmuss, H.-J. (1998) Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 64, 246–252.

[14] Hawari, J., Beaudet, S., Halasz, A., Thiboutot, S. and Ampleman, G. (2000) Microbial degradation of explosives: biotransformation versus mineralization. Appl. Microbiol. Biotechnol. 61, 605–618.

[15] Spain, J., Hughes, J. and Knackmuss, H.-J. (2000) Biodegradation of Nitroaromatic Compounds and Explosives. CRC Press, London.

[16] Selivanovskaya, S.Y., Petrov, A.M., Egorova, K.V. and Naumova, R.P. (1997) Protozoan and metazoan communities treating a simulated petrochemical industry wastewater in a rotating disc biological reactor. World J. Microbiol. Biotechnol. 13, 511–517.

[17] Wang, C.Y., Bhadra, R. and Hughes, J.B. (1997) Rapid separation of reduction products of 2,4,6-trinitrotoluene using TLC. Biotechnol. Tech. 11, 519–521.

[18] Gancedo, C. and Serrano, R. (1989) Energy-yielding metabolism. In: The Yeast (Rose, A.H. and Harrison, J.S., Eds.), Vol. 3, pp. 206–259. Academic Press, London.

[19] French, P.D., Nicklin, S. and Bruce, N.C. (1998) Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetrenitrate reductase. Appl. Environ. Microbiol. 64, 2864–2868.

[20] Wang, C.-Y., Zheng, D. and Hughes, J.B. (2000) Stability of hydroxylamino- and aminointermediates from reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. Biotechnol. Lett. 22, 15–19.

[21] Lenke, H., Achtnich, C. and Knackmuss, H.-J. (2000) Perspectives of bioelimination of polynitroaromatic compounds. In: Biodegradation of Nitroaromatic Compounds and Explosives (Spain, J., Hughes, J. and Knackmuss H.-J., Eds.), pp. 91–126. CRC Press, London.

[22] Vorbeck, C., Lenke, H., Fisher, P. and Knackmuss, H.-J. (1994) Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. J. Bacteriol. 176, 932–934.

[23] Ebert, S., Fischer, P. and Knackmuss, H.J. (2001) Converging catabolism of 2,4,6-trinitrophenol (picric acid) and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. Biodegradation 12, 367–376.