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Cover Page Footnote

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TRANSFORMATION OF 2,4,6-TRINITROTOLUENE (TNT) BY IMMOBILIZED AND RESTING CELLS OF *ARTHROBACTER* SP.

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ABSTRACT

Arthrobacter sp. transformed 2,4,6-trinitrotoluene (TNT) into two isomeric monoaminodinitrotoluenes viz, 4-amino-2,6-dinitrotoluene (4-ADNT), 2-amino- 4,6 –dinitrotoluene (2-ADNT) and also 2,4- diamino-6-nitrotoluene (2,4-DANT) under aerobic resting cell conditions. Experiments were carried out at 30° C (±1°C) in phosphate buffer with 60 mg per liter TNT at a pH of 7.2 (±0.2). Complete transformation of TNT occurred within 36 hours, yielding a mixture of monoaminodinitrotoluenes and diaminonitrotoluene. The major component was found to be 4-ADNT. Under the same experimental conditions, transformations were studied using barium alginate immobilized cells of *Arthrobacter* sp. Appropriate controls were run simultaneously. In both the cases, reactions were monitored every six hours by thin-layer chromatography (TLC) and gas chromatography (GC). The products were identified by gas chromatography coupled with mass spectrometry (GC-MS). Immobilization of *Arthrobacter* sp. in barium alginate resulted in more efficient TNT transformations, which were complete in 24 hours. The immobilized cells could be utilized at least for eight cycles and a decrease in rate of transformation was observed with each cycle.

Key words: *monoaminodinitrotoluenes, dinitrotoluene, Arthrobacter sp., gas chromatography, mass spectrometry*

INTRODUCTION

Due to relative inertness of the nitro group, many of the nitroaromatic compounds can be recalcitrant in the environment. 2,4,6-Trinitrotoluene (TNT) is one such compound that is widely used as an explosive. TNT exhibits toxicity to natural microbial flora of soil and water. The physicochemical methods reported so far for treatment of TNT require extreme conditions (Tsai, 1991), adding to the cost of production, maintenance, and disposal. Moreover, none of these methods is environmentally benign. Hence the search for a satisfactory method of microbial/ biological treatment for such hazardous substances is of interest.

Several bacteria have been reported to transform TNT but not mineralize it (Boopathy and Kalpa, 1994; Duque et al., 1993; Fiorella and Spain, 1997; McCormick et al., 1976; Regan and Crawford, 1994; Schackmann and Muller, 1991; Vanderberg et al., 1995). For the past two decades, a search for a suitable microbe that could mineralize TNT has gone on. TNT is known to be retained for long periods of time in soil and water, and hence it poses an environmental hazard. It is known to cause toxicity to fish when found in concentrations of 2 mg/ml (Mathews et al., 1954). It is a strong mutagenic agent (Won et al., 1976) but its mutagenecity decreases with the reduction of nitro groups to amines. The usually detected nitroso and hydroxylamino intermediates of TNT are known to disrupt DNA structure (Hanne et al., 1993). One fungal member viz,

Phanerochaete chrysosporium, has been reported to mineralize TNT (Pasczynski and Crawford et al., 1995).

In the utilization of TNT, a variety of microbes opt for either of the two principal pathways. In the first pathway, nitro groups are eliminated as nitrite/ nitrate (Boopathy et al., 1994; Vorbeck et al., 1994; Duque et al., 1993; Vanderberg et al., 1995). In the second pathway the nitro group is reduced to amines. This is usually observed in organisms that can draw energy by using the nitro group as the final electron acceptor, transforming it to amine (Cartright and Cain et al., 1959; Gorontzy et al., 1994; Pruess et al., 1993).

A number of reduction products of TNT other than 4-ADNT, 2- ADNT 2,4- DANT, and 2,4,6-triaminotolouene (TAT) are known. Lewis et al. (1996) identified 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT) in the fermentation broth of *Clostridium perfringens*. In addition to these compounds, the azo-derivatives viz, 2-2', 6-6'-tetranitro-4-azoxytoluene, and 2-2', 4-4'-tetranitro-6-azoxytoluene are also known to be formed (Fiorella and Spain, 1997; Regan and Crawford 1994; McCormick et al., 1976).

Members of the genus of *Arthrobacter* are universally distributed in all soils, having considerable nutritional versatility. Strains have been isolated that decompose herbicides, caffeine, nicotine, polychlorinated biphenyls, and other compounds (Gilbert and Crowly, 1997). Some strains have been reported to grow using nitrophenol, finally yielding hydroquinone and nitrite (Hanne et al., 1993). No reports of studies employing *Arthrobacter* sp. for degradation of TNT are known.

Though the idea of using immobilized microorganisms for TNT biotransformation is not new, work in this area is limited to immobilization of only one fungus viz, *P. chrysosporium* (Sublette et al., 1992). In the present work, an attempt has been made to increase the efficiency of TNT transformation by an *Arthrobacter* sp. through immobilization. The metabolites were identified and a possible route of transformation is proposed.

MATERIALS AND METHODS

Pure TNT (99%) was procured from government sources, while 4-ADNT and 2-ADNT were generous gifts from Dr. Thomas Lewis, University of Idaho, Moscow, Idaho. 2,4- DANT was kindly made available by Dr. Ron Spanggord, SRI International, Palo Alto, California. Ethyl acetate, glacial acetic acid, toluene, ethanol, acetone, methanol, HCl, and sodium alginate were A.R. grade from Merck. Beef extract, peptone, sodium chloride, KH2PO4, K2HPO4, and MgSO4. 7 H2O were from Hi-Media, Mumbai, India.

Culture: *Arthrobacter* sp. *8929* was procured from the National Collection of Industrial Microorganism (NCIM), Pune, India**.**

Complex Medium: *Arthrobacter* sp. was grown on the complex medium with the following contents:

Beef extract-1%, peptone –1%, NaCl- 0.5%,K2HPO4- 0.2%, KH2PO4- 0.1%,

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MgSO4.7H2O- 0.02%, and pH of 7.2 (±0.2).

Culture conditions

Cells of *Arthrobacter* sp**.** were grown in two liters of broth containing complex medium at 25°C (±1°C) for 24 hours in an incubated shaker at 175 rpm with 50 ml freshly grown cells as inocculum.

Resting cell suspension

Cells from complex medium were separated by centrifugation at 15,000g for 20 minutes at 4°C, using Hitachi Cold Centrifuge 52. Separated cells were suspended in one liter of 50 mM potassium phosphate buffer, pH 7.2(±0.2). The optical density (OD) of the suspension at 600 nm was adjusted to 1.75 with plain buffer as blank. To this buffer, 60 mg of TNT, predissolved in 3 ml of acetone, was added.

CONTROL

Uninocculated phosphate buffer (one liter), containing 60 mg TNT (pre-dissolved in 3 ml of acetone), was maintained in an incubated orbital shaker at 30°C (±1°C) and a speed of 175 rpm. It was used as a control throughout the experiment.

Whole-cell immobilization on barium alginate

Sodium alginate (3g) was dissolved in 50 ml of sterilized distilled water and shaken on a magnetic stirrer for 30 minutes, until a homogenous solution was obtained. To this, 50 ml of phosphate buffer containing *Arthrobacter* sp. with an OD of 2.0 at 600 nm was mixed and shaken for 30 minutes at 175 rpm. This solution was added dropwise in 1% BaCl2 to obtain barium alginate beads of uniform diameter (2 mm). Sterilized conditions were maintained throughout the procedure.

Measurement of viability

Throughout the experiment, growth was monitored every 12 hours. As inocculum, 0.1 ml of liquid culture was withdrawn from the suspended cells and inoculated in 100 ml of complex medium. It was incubated at 30° C in an orbital shaker maintained at 200 rpm for 24 hours. Growth was monitored by recording the absorbance at 600 nm.

In case of immobilized cells, a bead was added to the complex medium (100 ml), incubated at 30°C in an incubated shaker, and maintained at 200 rpm for 24 hours. Viability was recorded with increase in absorbance at 600 nm.

Isolation of reaction products

Every 6h, 10 ml of buffer were removed from the controls, resting cells, the immobilized system, and the complex medium. It was acidified with 1 M HCl and extracted with 3x10 ml ethyl acetate. After centrifugation at 20,000g for 10 minutes at 4°C, the organic phase was separated and dried with sodium sulfate and evaporated on a vacuum rotavapor. The respective residues were reconstituted in small quantities of acetone for analytical work.

For TLC, pre-coated plates of silica gel G/254 from E.Merck, Dermstadt, Germany, were used. Compounds were separated with a solvent system of toluene/ethyl acetate/acetic acid (60:30:10) v/v/v. Amino groups were detected by spraying the plate with sodium nitrite (0.1 % w/v) solution in 1 N HCl, followed by N-1- naphthyl ethylene diamine HCl (0.04% w/v) solution prepared in ethanol. The Rf values of the bands were TNT-0.7, 4-ADNT-0.5, 2-ADNT-0.46, and 2,4- DANT-0.3, respectively. These values matched those of the standards.

GC analysis was carried out every six hours using an HP-5 capillary column and flame-ionization detector. Helium was the carrier gas with a flow rate of 15 ml per minute. The oven temperature was maintained at 50°C initially and ramped at 5°C rise per minute to a final temperature of 220°C, using an HP-5890 II GC system. The retention times of TNT, 4-ADNT, 2-ADNT, and 2,4-DANT were 3.36, 8.76, 10.792, and 7.430, respectively.

Final confirmation of 4-ADNT, 2-ADNT, and 2,4- DANT was done by GC-MS at conditions mentioned above using a Finnigan-Mat 1020 B system. Characteristic molecular ion of 197 and the major fragment ion at 180 confirmed 4-ADNT, while distinctive reduction in the abundance of the fragment ion of 167 marked the presence of 2-ADNT from that of 4-ADNT. However, the two isomers had different Rf values as well as different retention times for GC. 2,4-DANT was confirmed with its characteristic molecular ion of 167 and major fragment ion of 150. In addition, the mass spectrum of each compound matched with those of the standards.

Estimation of nitrite ($NO2^-$)

Nitrite release was monitored once every 24 hours. From the 20 ml of the buffers withdrawn, 10 ml was used for analysis for nitrite. After centrifugation at 20000g, the supernatant was filtered through 0.25 mM microbial filter (Pall Gelman Laboratory, Pall Pharmalab Filtration, Mumbai) and the filtrate was used for analysis. Nitrite was estimated by Bratton and Marshall method, using N-(1-Napthyl) ethylenediamine dihydrochloride (NED-dihydrochloride) as the coloring reagent.

Estimation of nitrate ($NO3^-$)

Analysis for nitrate release was carried once every 24 hours. The remaining 10 ml was centrifuged at 20,000g; the supernatant was filtered through 0.25 m M microbial filter (Pall Gelman Laboratory, Pall Pharmalab Filtration, Mumbai). Nitrate was estimated by the phenol-disulfonic acid method.

RESULTS

In the present work, no nitrite/nitrate release was observed (data not shown). Despite being an obligate aerobe, *Arthrobacter* sp. metabolized TNT, like many anaerobes, to give the reduction products. Though TNT was completely transformed in 36 hours, reaction studies were continued for another 40 hours in order to detect any other metabolite. Further, when TNT was incorporated in the complex medium, cells of *Arthrobacter* sp. did not prefer it in the growth phase.

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The TLC and GC profiles indicated that TNT was completely transformed by the resting cells in 36 hours. Within 12 hours, 4-ADNT appeared as the first reduction product; 2-ADNT appeared after 24 hours; and 2,4-DANT appeared after 30 hours. In the alginate-immobilized state, cells of *Arthrobacter* sp. took 24 hours to completely transform 60 mg per liter TNT with the appearance of ADNTs at the end of 12 hours and 2,4-DANT at the end of 18 hours (Table 1).

These results indicate faster and better performance of *Arthrobacter* sp. in an immobilized state than in resting conditions in transforming TNT. A significant observation was the twofold increase in the concentration of ADNTs in the immobilized state rather than in the resting cells at the end of 12 hours. Delayed appearance of DANT in the resting cells (24 hours) and its appearance at the end of 12 hours in the immobilized system was also a notable feature.

Viability of the resting and immobilized cells was monitored every 12 hours. It was observed that the resting cells lost most of their viability after approximately 96 hours. In contrast, the immobilized cells could retain their viability for 192 hours. Figure 1 shows the change in the viability of the resting cells with time.

Microscopic examination of cells of *Arthrobacter* sp., maintained in phosphate buffer containing TNT for more than 24 hours, indicated a change in cell morphology from coccoid to pleomorphic rods (data not shown).

DISCUSSION

Although biological reduction of TNT to 4-ADNT, 2-ADNT, and 2,4-DANT occurs more readily under anaerobic conditions, in the present study it was observed that the same reductions take place under aerobic conditions by *Arthrobacter* sp. This process involves a gradual reduction of nitro group/s to amino group/s without mineralization of TNT. Under the conditions of the experiment, no triaminotoluene (TAT) was detected, indicating that complete reduction of TNT to TAT is a forte of obligate anaerobes (Lewis et al., 1997; Hawari et al., 1998).

Arthrobacter sp. has been reported to degrade 4, 6- dinitro-o-cresol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol with the release of nitrite (Jenson et al., 1967). The present work indicates that *Arthrobacter sp*. does not release nitrite in metabolizing 2,4,6-TNT. This resistance exhibited by TNT towards ring cleavage could be due to the absence of a hydroxyl group on the aromatic ring. Hence, we confirm that even a versatile bacterium like *Arthrobacter* sp. fails to release nitrite from TNT and behaves like many *Pseudomonas* species, which anaerobically transform TNT to monoaminodinitrotoluenes and diaminomononitrotoluene (Schackmann and Muller, 1991).

Immobilization of pure cultures has a significant role to play in industrial processes but their significance in degradations has not been fully exploited. An immobilized consortium of bacteria in association with *Arthrobacter* sp. can be a viable strategy for degradation of TNT found in red and yellow water of TNT-producing units.

For mineralization of TNT directly from the red and yellow waters, *P.chrysosporium* has been suggested to be the organism of choice (Fernando et al., 1990), but it is reported to be sensitive to concentrations of TNT greater than 24 mg/liter (Spiker et al., 1992; Michels and Gottshalk, 1994). There are no reports about the toxicity of ADNTs. It is suggested to separately immobilize an efficient, non-pathogenic bacterial strain like *Arthrobacter sp*., which can initially convert TNT to ADNTs that in turn can be further mineralized by another microorganism like *P. chrysosporium*.

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Table 1(a). Quantification of Products of Transformation of TNT by Immobilized Cells of *Arthrobacter* sp. Values represented are mean ± S.D. of three runs in mg/L.

Immobilized Cells						
Time (hrs)	**6**	**12**	**18**	**24**	**30**	**36**
TNT	59.0 (± 1.0)	27.2 (± 0.2)	11.8 (± 0.02)	--	--	--
CONTROL	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)
ADNT	--	30.3 (± 1.5)	46.3 (± 1.5)	51.66(±0.35)	52.0 (± 0.35)	52.0 (± 0.35)
CONTROL	--	--	--	--	--	--
DANT	--	--	1.9 (± 0.1)	2.53 (± 0.1)	6.9 (± 0.15)	7.2 (± 0.15)
CONTROL	--	--	--	--	--	--

Table 1(b). Quantification of Products of Transformation of TNT by Resting Cells of *Arthrobacter* sp. Values represented are mean± S.D. of three runs in mg/L.

Time (hrs)	**6**	**12**	**18**	**24**	**30**	**36**
TNT	58.9 (± 1.0)	51.73 (± 0.4)	14.83 (± 0.25)	8.0 (± 0.25)	2.0 (±0.05)	--
CONTROL	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)	59.0 (± 1.0)
ADNT	--	7.96 (± 0.15)	45.86 (± 0.32)	48.1 (± 0.2)	50.6 (± 0.36)	51.0 (± 0.36)
CONTROL	--	--	--	--	--	--
DANT	--	--	--	--	5.36 (± 0.25)	8.4 (± 0.17)
CONTROL	--	--	--	--	--	--

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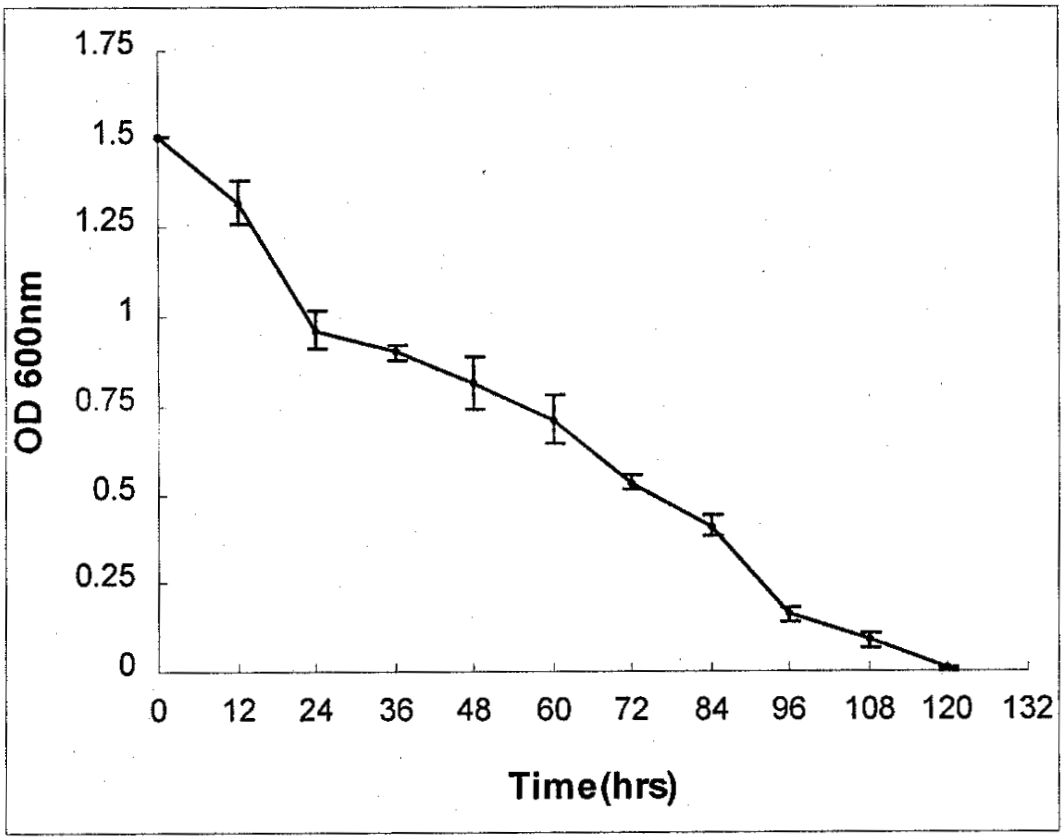

Figure 1. Viability Profile of Resting Cell of *Arthrobacter* sp. Viability was measured by recording the absorbance at 600 nm at an interval of 12 hours by inoculating 0.1ml of liquid culture in 100ml of complex medium, incubated at 30° C for 24 hours. Values represented above are mean of three runs and standard deviations are indicated with error bars.