Isolation of TNT Tolerant *Pseudomonas* Species (Strain KA) from TNT Contaminated Soil–Biotransformation of TNT

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ABSTRACT

A 2,4,6-trinitrotoluene (TNT) utilising bacterium, *Pseudomonas* species (strain KA) was isolated from soils of a munitions processing unit and studied for its ability to grow and metabolise TNT. The result indicated that the isolate could grow aerobically in a minimal salt medium containing 0.25 mM/l TNT at 30 °C. It could completely transform 0.25 mM/l TNT in 5 days giving 2-isomeric monoaminodinitrotoluenes, namely 4-aminodinitrotoluene and 2-aminodinitrotoluene. Products of TNT transformation were analysed and confirmed by thin layer chromatography and gas chromatography coupled with mass spectrometry. Both, growth of the isolate and biotransformation rates were better supported on sugar and ammonium salt when added to the medium separately. *Pseudomonas* species (strain KA) showed maximum TNT transformation efficiency in the presence of mannose. For degradation of TNT, this strain can be employed to initiate the process in association with the other microbial members that can collectively degrade TNT.

Keywords: Biotransformation, TNT metabolism, microbial degradation, *Pseudomonas* species

1. INTRODUCTION

Nitroaromatic compounds are largely produced for their use in the syntheses of dyes, insecticides, pharmaceuticals and explosives. 2,4,6-trinitrotoluene (TNT) in its manufacturing, loading, assembling, and packing as well as in field-testing is reported to contaminate soil and water[1,2]. It is known to be toxic to many microorganisms[3-6] and to fish when found in concentration[7] of 2µg/ml. TNT and its reduction products like hydroxylamino derivatives are found to interact with DNA, and hence are mutagenic in nature[8]. However, its mutagenicity has been reported to decrease with compounds having increasing number of nitro groups being converted to amino[9]. The present-day methods of destroying unused TNT or contaminated soils by detonation or incineration are the worst options as they are unsafe, environmentally highly hazardous and expensive. Based on its toxic properties, United States Environment Protection Agency (USEPA) has identified TNT as a priority environmental pollutant.

TNT gets metabolised both by aerobes and anaerobes through reductive pathways. Amongst the biological agents employed for the remediation of TNT, anaerobic consortia have been reported to be more efficient[10] than pure cultures of either the aerobes[11-13], which predominantly comprise *Pseudomonas* species or the anerobes[14-16]. The fungus *Phanerochaete chrysosporium* degrades a small percentage[17] of TNT when found in concentrations[18] < 24 mg/l.

The aim of the present study is to screen and isolate microbes of significance, which could grow and degrade TNT. Hence, soil from a defence establishment's munitions processing unit situated in Hyderabad was collected, and isolated colonies of the microbes were evaluated for the above-mentioned properties.

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Govt. sources, while analytical quantities of 4-aminodinitrotoluene (4-ADNT) and 2-amino dinitrotoluene (2-ADNT) were gifted by Dr Ron Spanggord, SRI–Internationals, Palo–Alto, Ca, USA. All other chemicals used were of reagent grade.

2.2 Screening

The soil sample collected aseptically from a spot approx. 7.62 cm downstream of the waste-water collection tank of a munitions processing plant was processed for microbial screening. The soil sample (50 mg) was suspended in 5 ml sterile saline and serially diluted. A dilution of 10^{-5} was selected for further work. A fixed volume (0.5 ml) was used as inocculum while transferring this onto a solid minimal salt medium before incubation.

2.3 Medium Composition

The minimal salt medium of the following composition was used unless specified otherwise, KH_2PO_4 (400 mg), K_2HPO_4 (200 mg), $NaCl$ (600 mg), $MgSO_4.\ 7H_2O$ (50 mg), and $CaCl_2$ (5 mg) were dissolved in 1l distilled water and the p*H* was adjusted to 6.8 (± 0.2). To this 0.25 mM (57 mg) TNT, pre-dissolved in 5 ml acetone was added, and autoclaved at 120 °C for 15 min. For solid minimal salt medium, 1.5 per cent (w/v) agar-agar (Hi-Media) was added. Separate media were prepared using different sugar (glucose, arabinose and mannose) in which a 3 per cent solution of each sugar was separately added to the minimal salt medium after sterilising it at 110 °C for 10 min.

3. GROWTH OF MICROORGANISM

From the selected dilution (10^{-5}), 0.5 ml was used as the inoculum. This was inoculated to the minimal salt medium agar by spread plate method and incubated at 30 °C. After 5 days, a lawn of separate, pin-headed, yellow, smooth, raised, circular colonies with even boundaries were observed. On persistent incubation for two weeks, the colonies were found to be rough, desiccated, highly reticulated against red (due to TNT).

Arginine	+
Indole	–
Esculine	–
Simmons Citrate	+
Phosphatase	–
Urease	–
Mannitole	–
Xylose	+
Maltose	–
Lactose	–
Nitrate	+
Oxidase	+

*** + = Growth, enzyme activity**
– = No growth, no enzyme activity

Colonies were collected in sterile saline (100 ml, absorbance at 600 nm–0.682 nm). Ten per cent by volume of this suspension was used as the inoculum while transferring this to the liquid minimal salt medium (1l). Further growth was recorded by monitoring absorbance at 600 nm, coupled with determination of protein concentration by Lowry method[19]. The strain was identified as *Pseudomonas* species on the basis of its microscopic examination and biochemical characteristics[20] and was assigned *Pseudomonas* species (strain KA) (Table 1).

3.1 Analytical Methods

Biotransformation of TNT was monitored every 24 hr. For analysis, a fixed volume (50 ml) was drawn from the culture and the cells were separated by centrifugation at 10000 rpm for 20 min at 4 °C using a Hitachi 52 preparative centrifuge. The supernatant was collected and extracted with ethyl acetate (3 × 50 ml). The organic phase was separated and dried over anhydrous sodium sulphate. The ethyl acetate was dried in vacuum and the contents were reconstituted in small quantities of acetone.

Metabolites were separated using thin layer chromatography (TLC). For TLC, pre-coated plates of silica gel (G/254) from Merck were used. Compounds were separated using the solvent system of toluene/ethyl acetate/acetic acid (60 : 30 :10 v/v/v),

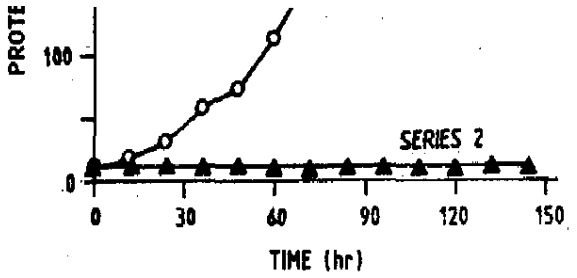

Figure 1. Growth (measured as protein) of *Pseudomonas* species (strain KA) in medium containing TNT as the sole source of carbon and nitrogen (series 1), series 2 indicates killed control.

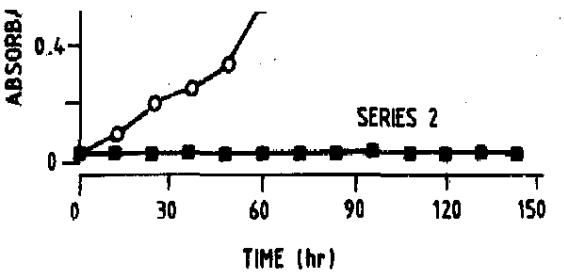

Figure 2. Growth (measured as absorbance) *Pseudomonas* species (strain KA) in presence of mannose as carbon source along with TNT (series 1), series 2 indicates killed control.

respectively. The metabolites were identified on the basis of their respective R_f values which matched with that of the standard compounds.

Final confirmation of these compounds were made on the basis of gas chromatography (GC) coupled with mass spectrometry (GC-MS) analyses. GC was performed using HP-5889 II model connected to mass spectrometer (MS) on electron ionisation mode using HP-1659 model. The samples were chromatographed with a gradient temperature programme using HP-5 glass capillary column (25 M x 0.25 mm id). The initial temperature was 55 °C and a final temperature of 240 °C was maintained with a ramp of 10 °C/min. Injection temperature was set at 240 °C and the transport line was kept at 200 °C. Helium flow was adjusted at 15 ml/min and the injection volume was 2µl.

4. RESULTS & DISCUSSION

Biotransformation of TNT was monitored every 24 hr by preparative TLC. It was observed that TNT was completely eliminated and transformed to the reduction products in 5 days in the culture from the minimal salt medium. The same transformations could not be detected in the un-inoculated media and in the heat-killed culture (sterilised immediately after inoculation). This indicated that disappearance of TNT and its subsequent transformation was biologically mediated by *Pseudomonas* species (strain KA). Since growth was monitored both by total protein measurements (Fig. 1) and absorbance at 600 nm, it was observed that growth of the isolate was well-augmented with the addition of either a nitrogen source (NH_4^+) or sugar as carbon source (Fig. 2). In addition, when inoculated in medium containing no TNT, ammonium or sugar, no growth was observed. Of the different sugars added, growth of the isolate was found to be most rapid in mannose coupled with faster TNT transformation rates (3 days)

In a separate experiment, stationary phase pellet of cells were grown on TNT in the presence of 0.25 mM 2,4-DNT (45.5 mg/l) as the sole source of energy. The isolate transformed 2,4-DNT in 24 hr and the two monoaminonitrotoluenes, namely 4-amino-2-nitrotoluene (4-ANT) and

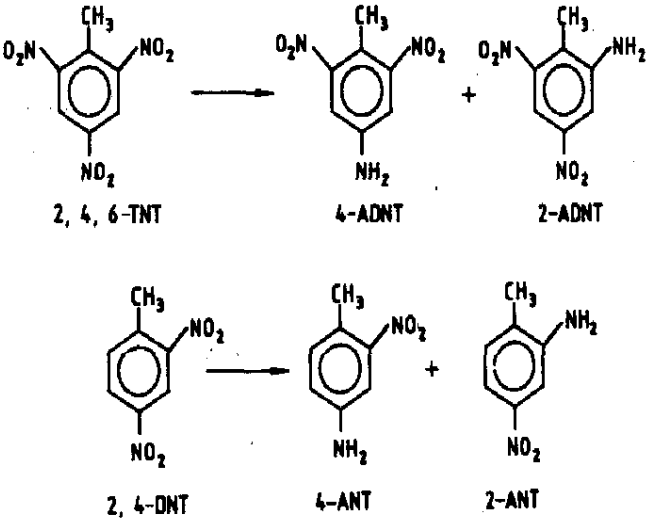

Figure 3. *Pseudomonas* species (strain KA) mediated transformation products of TNT and 2,4-DNT.

believed to be recalcitrant to microbial attack due to the ability of the nitro group to reduce the electron density in the aromatic ring, coupled with the stereochemical hindrance of the methyl group.

Earlier workers had unsuccessfully attempted to isolate a consortium of *Pseudomonas* strains that could metabolise TNT without the addition of a co-substrate[23]. In the present study, it was found that under aerobic conditions, the isolated strain of *Pseudomonas* species (strain KA) grew and metabolised TNT to its monoamino derivatives and preferred presence of sugar as the additional carbon source (Figs 1 and 2).

Schackmann and Muller[21] have reported the presence of nitro-reducing activity in five pure strains of *Pseudomonas*, none of the cultures or the purified enzyme have been evaluated for their relevance in TNT remediation process.

In the present study, the survival efficiency of the isolate not only tolerated high concentrations of TNT but also developed ability to use TNT as its nutrient (Figs 1 and 2). Pure cultures and consortia of anaerobes have been shown to metabolise TNT through the reduction pathway, forming the intermediates, viz., aminodinitrotoluenes (ADNT), diaminonit-rotoluene (DANT), triaminotoluene (TAT) and subsequently yielding unidentified metabolites. However, as has been found in many earlier studies[10,11,14-16], the initial transformation of TNT to ADNT is a rate- limiting step. Further, the highly oxidised nature of TNT also registers a toxic shock to many anaerobes. Keeping in view the well-induced activity of the isolate [*Pseudomonas* species (strain KA)] to grow in the presence of TNT as mentioned above, the schematic representation of *Pseudomonas* species induced transformation (Fig.3), also suggests that its potential can be fully harnessed by making it a member of an appropriate consortium in which it can initiate the process of TNT reduction. Subsequent degradation of 4-ADNT has been achieved through a cascade mechanism,

applications in remediation of TNT-contaminated soils and in treating waste water generated by TNT producing units.

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