

## Degradation of 2,4,6-trinitrotoluene by *P. aeruginosa* and characterization of some metabolites

Hatice Aysun Mercimek<sup>1</sup>, Sadik Dincer<sup>2</sup>, Gulcihan Guzeldag<sup>1</sup>, Aysenur Ozsavli<sup>1</sup>,  
Fatih Matyar<sup>3</sup>, Afet Arkut<sup>4</sup>, Fikret Kayis<sup>2</sup>, Melis Sumengen Ozdenefe<sup>2</sup>

<sup>1</sup>Department of Molecular Biology and Genetics, Kilis 7 Aralik University, Kilis, Turkey.

<sup>2</sup>Department of Biology, Cukurova University, Adana, Turkey.

<sup>3</sup>Department of Science and Technology Education, Cukurova University, Adana, Turkey.

<sup>4</sup>Department of Nutrition and Dietetics, Faculty of Health Sciences, Cyprus International University, Nicosia, Turkey.

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### Abstract

Degradation of 2,4,6-trinitrotoluene (TNT), a nitroaromatic explosive found in the soil and ground water, was investigated using *Pseudomonas aeruginosa* in *in vitro* experiments. Biodegradable ability of this bacteria was performed with 50 and 75 mg L<sup>-1</sup> TNT concentrations in a defined liquid medium for 96 h time period. Treatment of TNT in supernatant samples taken at 0, 6, 12, 24, 48, 72 and 96 h from agitated vessels was followed by reverse-phase high-performance liquid chromatography (HPLC). In cultures supplemented with 50 and 75 mg L<sup>-1</sup> TNT, after 96 h of incubation 46% and 59% reduction were detected respectively. Two metabolites as degradation intermediates with nitrite release into the medium, 2,4-dinitrotoluene (2,4-DNT) and 4-aminodinitrotoluene (4-ADNT), were elucidated by thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS). These findings clearly indicate that *Pseudomonas aeruginosa* can be used in bioremediation of TNT contaminated sites.

**Key words:** *Pseudomonas* sp, biotransformation, nitroaromatic explosives, TNT.

### Introduction

Oligonitroaromatic compounds are well-known environmental pollutants which are used in many industrial processes, including the production of pesticides, dyes, polymers, plastics, herbicides, explosives, textile, paper and pharmaceuticals (Ju and Parales, 2010). These pollutants over several decades have leaked to soils, groundwaters, surfacewaters and wastewaters around manufacturing sites as a result of their release in industrial effluents (Robertson ve Jjemba, 2005; Kalderis *et al.*, 2008; Lotufo *et al.*, 2010).

2,4,6-trinitrotoluene is a nitroaromatic explosive which has been extensively used for military purposes due to its low melting point, chemical and thermal stability, low sensitivity to impact and friction (Park *et al.*, 2002; Robertson ve Jjemba, 2005; Kalderis *et al.*, 2008; Lin *et al.*, 2013). It was reported that its production reached a maximum dur-

ing World War II (Zheng *et al.*, 2009; Ayoub *et al.*, 2010; Zhang *et al.*, 2011). Toxic and mutagenic effects of TNT to various organisms ranging from microorganisms and humans have been described even at low concentration (its lethal dose 0.8-13 g/kg) (Seth-Smith 2002; Dodard *et al.*, 2004; Ayoub *et al.*, 2010). TNT as possible human carcinogenic (class C) has been classified by United States Environmental Protection Agency (Gandia-Herrero *et al.*, 2008) and also referred to cause diseases such as rashes, toxic hepatitis, dermatitis, cyanosis, sneezing, cough, peripheral neuritis, cataract, muscular pain, aplastic anemia and kidney damage when exposure to it (Kalderis *et al.*, 2008; Yang *et al.*, 2008).

Owing to these impacts, improving the effective technology for remediation of TNT-polluted sites is immediately needed (Fleischmann *et al.*, 2004). Until today, several methods for treating of TNT contaminated site have

been developed, including incineration, compositing, chemical oxidation and adsorption (Baretto-Rodrigues *et al.*, 2009; Wang *et al.*, 2010). Limitations containing the retention of untreated and undefined compounds, costliness, the requirement of additional nutrients and incomplete degradation of TNT, recent researches have caused to focus on biotreatment processes which are an efficient, cost effective and environmental friendly (Fu *et al.*, 2012).

This hazardous chemical which is persistent to degradation in environmental, is sensitivity to microbial attack. Evidences of TNT transformation by aerobic, anaerobic and combined processes are available in the literature (Robertson and Jjemba, 2005; Moshe *et al.*, 2009; Montgomery *et al.*, 2013). The present paper aims to investigate the transformation capacity of TNT to non-toxic byproduct by *Pseudomonas aeruginosa* which was isolated from soil.

## Materials and Methods

### Isolation of *Pseudomonas*

For isolation of microorganism, soil sample was collected from the NATO military industry region, Izmir, Turkey. 1 g of soil sample in 99 mL of Glutamate Starch Phenol Red (GSP) medium (Coral and Karagoz 2005) containing the following (g L<sup>-1</sup> distilled water) sodium L(+) glutamate (10), soluble starch (3.5), KH<sub>2</sub>PO<sub>4</sub> (2), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5), phenol red (0.36), was incubated on an orbital shaker (180 rpm as aeration speed) at 30 °C for 72 h. And then 100 µL of growth culture was transferred to an GSP agar plate for preliminary isolation. The plate was incubated at 30 °C for 72 h under aerobically conditions. After incubation the selected colonies which were 2-3 mm in diameter, red and red-zone around theirs on plate, were maintained on an Nutrient agar slant. Isolates tested by Gram staining and biochemical tests were defined using VITEK bacterial identification system.

### Evaluation of tolerance

Tolerance of the isolated strains to TNT was analysed by growing in an aqueous medium contained different concentrations of TNT (5, 10, 15, 30, 50, 75 and 100 mg L<sup>-1</sup>). This degradation medium which consisted of (g L<sup>-1</sup>) K<sub>2</sub>HPO<sub>4</sub>, 6.18; KH<sub>2</sub>PO<sub>4</sub>, 1.93; yeast extract, 1; pH adjusted to 7.2 using 1 N NaOH, was prepared by adding from the stock solution of TNT prepared in acetone (1000 mg L<sup>-1</sup>) was filtered through (0.22 µm) sterilized (Tope *et al.*, 1999).

### Bioremediation

The capability of the selected isolate to remove TNT was investigated by two processes: (i) Preproduction: The isolate was grown in liquid medium including (g L<sup>-1</sup>) meat extract, 10; meat peptone, 10; NaCl, 5; K<sub>2</sub>HPO<sub>4</sub>, 2; KH<sub>2</sub>PO<sub>4</sub>, 1; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; pH 7.2, (Tope *et al.*, 1999) at 30 °C with shaking (180 rpm) for 24 h. After incubation,

separated cells by centrifuged (9000 x g for 10 min +4 °C) were washed twice with 50 mM potassium phosphate buffer (pH 7.3). A final optical density (OD<sub>600</sub>) of acquired bacterial suspension was adjusted about 4.8 and 50 mL of this biomass was evaluated as inoculum culture for second process. (ii) Incubation of inoculums in different TNT concentration (50 and 75 mg L<sup>-1</sup>) containing degradation medium and monitoring of bacterial growth: In shaking flask, the biomass was grown at 30 °C (180 rpm) in the dark for 96 h. Throughout the experiment, the growth was followed by recording the absorbance at 600 nm and counting viable colonies on plate count agar (PCA). Uninoculated flasks were utilized as controls, as well as flasks containing the bacteria without TNT.

### Following TNT transformation

#### Extraction

To identify the major intermediates of TNT by *Pseudomonas aeruginosa*, 50 mL of bacterial cultures and control groups were sacrificed at each time point (0, 6, 12, 24, 48, 72 and 96 h), centrifuged at 9000 x g for 1 h at 4 °C. The acidified culture supernatants (20 mL) with 1 N HCl 160 µL for per 1 mL sample, were extracted twice with equal volume of ethyl acetate. After acidification, H<sub>2</sub>O was removed from the extracts over anhydrous Na<sub>2</sub>SO<sub>4</sub>. And then the extracts were concentrated by removing excess solvent with rotary evaporation at 35 °C. The reconstituted residues in 1 mL of acetonitrile, were preserved at +4 °C in 1.5 mL teflon coated brown glass vial for HPLC, GC MS and TLC analysis (Schackmann and Müller 1991).

### Analysis of TNT

Analysis of TNT transformation was carried out using a Shimadzu, Japan HPLC equipped with a UV detector at 254 nm. Chromatographic separation was performed by injected a sample volume of 20 µL into a C18 reverse phase column at 30 °C. The mobile phase comprised of 21% (v/v) acetonitrile; 35% methanol and 44% deionized water. This phase mixture was isocratically delivered to column at a flow rate of 0.7 mL min<sup>-1</sup>. TNT concentration in samples was determined by comparison a peak height that yielded with reference standarts (Mercimek *et al.*, 2013).

### Identification of degradation products by TLC and GC MS analyses

Final confirmation of target products made whereby GC/MS and TLC. In GC/MS analyses, TNT intermediates were identified by comparison of retentions times and mass spectra between samples and standart chemicals. The samples were chromatographed with a gradient temprature programme using TR5-MS column (60 mx0.25 mm, 0.25 µm film thickness) and ionization detector. Initial oven temperature 80 °C for 1 min was ramped to 300 °C with a rise 10 °C min<sup>-1</sup> and held for 12 min final hold. The injector

temperature was 240 °C. Helium flow rate was adjusted at 1 mL min<sup>-1</sup> and the injection volume was 1 µL (Oh and Kim, 1998; Mercimek *et al.*, 2013).

In TLC analyses, the metabolic products were separated by the ascending method with a solvent system consisting of ethyl acetate/hexan (40:60 v/v) on pre-coated plates of silica gel F254 purchased from Merck (Nam 1997). For identification of spots indicating TNT metabolites, TLC sheets were visualized under UV illumination. Rf values of the spots were determined by comparing those of the authentic standards.

### Other analyses

During the degradation, demineralization of TNT that is responsible for nitrite ion release was analyzed by spectrophotometrically (Nyanhongo *et al.*, 2006). From 20 mL of the cultures withdrawn, was centrifuged at 9000 xg for 1 h. The acquired supernatant (200 µL) was transferred to test tubes including 50 µL sulphanilamide solution which was prepared by dissolving 5 g sulphanilamide in 26 mL HCl and adjusting total volume of solution to 500 mL in distilled water. After incubation at room temperature for 5 min, 50 µL *N*-(1-naphthyl) ethylenediamine dihydrochloride solution (0.5 g *N*-(1-naphthyl) ethylenediamine dihydrochloride in 500 mL deionized water) as the coloring reagent was added to reaction mixture and incubated for 20 min at room temperature. Total volume of reaction mixture was adjusted at 1 mL with deionized water and nitrite concentration was determined the OD at 540 nm. The oxidation of released nitrite to nitrate was followed by mixing 5 mL of supernatant with 1 mL 1 N HCl and then examining at 220 nm (Al-Absi 2008).

## Results

### Isolation and identification of the *Pseudomonas* sp

In NATO soil sample, bacterial population that is capability of growth in the presence of TNT, was screened on GSP agar. This medium contains glutamate and starch as its sole nutrients. While many microorganisms can not metabolize these compounds, *Aeromonas* was able to hydrolyze with acid production the starch, causing phenol red to change yellow but not by *Pseudomonas*. These bacteria formed a red precipitation zone surrounding colonies, 2-3 mm in diameter, on GSP agar plates. All of the selected strains were revealed belonging to the *Pseudomonas* genus, with possible similarities such as Gram negative, bacilli-shaped, oxidase and citrate positive. It was finally defined that isolated ten strains were *Pseudomonas* sp. by VITEK bacterial identification system.

### Selection of *Pseudomonas* sp that used in TNT transformation

It was observed that only one of the ten isolates was capable to grow in an aqueous medium contained 50 and 75 mg L<sup>-1</sup> TNT concentrations. So, this isolate, *Pseudomonas aeruginosa*, was selected to be used for further degradation studies.

### Growth of *P. aeruginosa* and biodegradation of TNT as sole carbon source

*P. aeruginosa* was inoculated in liquid mediums containing 50 and 75 mg L<sup>-1</sup> TNT, to evaluate its capability to utilization of TNT as carbon source by aerobic pathway. It was observed that color of culture mediums that were treated by *P. aeruginosa*, turned to red color from yellow during degradation. Within 4 days of culturing, degradation efficiency for different concentrations (50 and 75 mg L<sup>-1</sup>)

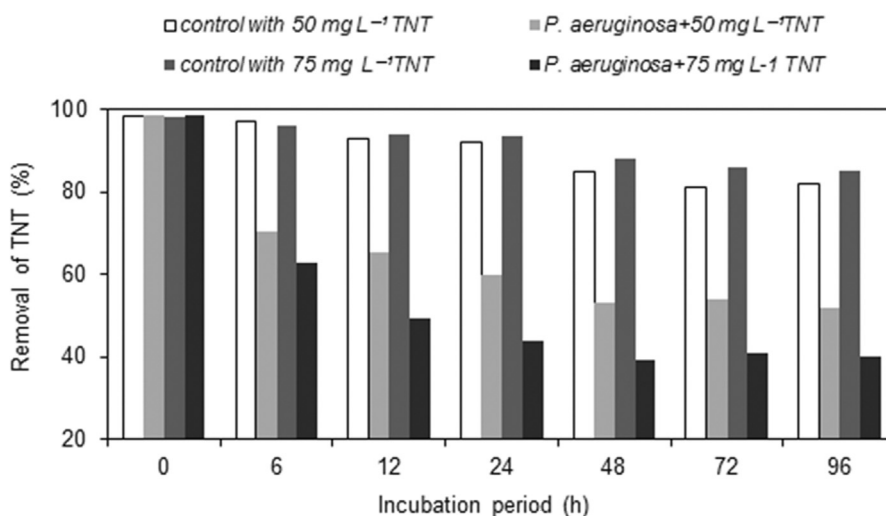


Figure 1 - Degradation of TNT by cell suspensions of *P. aeruginosa*.

of TNT, was recorded as 46% and 59% respectively (Figure 1).

The role of abiotic processes on biotransformation of TNT, was also examined in the uninoculated controls. In these control cultures, at initial concentrations of TNT were determined a decline with time by HPLC (Figure 1). The certain microbial degradation of TNT by *P. aeruginosa* was calculated by expulsion values of chemical hydrolysis.

During incubation, correlation between decreasing TNT concentrations and increasing density of cells in cultures was followed to prove that *P. aeruginosa* used TNT as nutritional source. Time-dependent changes in microbial growth (turbidity and colony-forming units (CFU)) were reported in Figure 2. Bacterial growth based on the initial inoculum that was about  $102.5 \times 10^5$  cfu mL<sup>-1</sup>, arose following degradation and reached maximum levels at 12 h ( $178 \times 10^5$  cfu mL<sup>-1</sup>) and 24 h ( $208.5 \times 10^5$  cfu mL<sup>-1</sup>) of incubation in liquid cultures including respectively 50 and 75 mg L<sup>-1</sup> TNT (Figure 2a). From that period to by the end of incubation, it was determined that bacterial growth in both concentrations decreased gradually (Figure 2a-b). But biotransformation was shown to proceed with the residual viable cells. In control cultures without TNT, maximum growth was recorded as  $131 \times 10^5$  cfu mL<sup>-1</sup> at 48 h (Figure 2a).

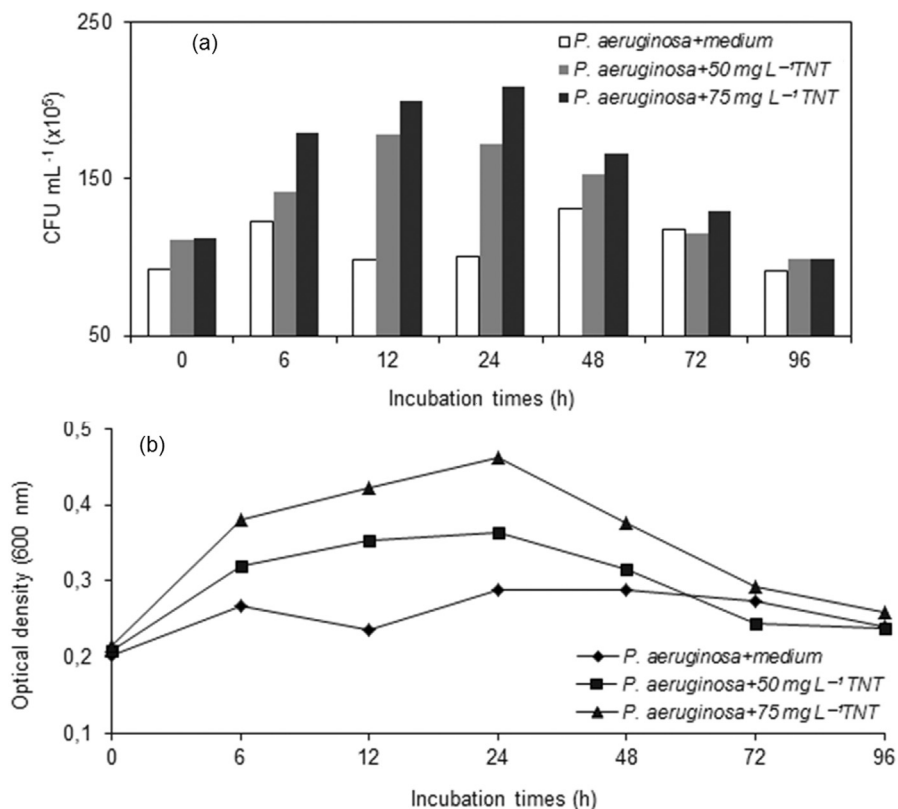
### Estimation of nitrite and nitrate from TNT

During degradation time, nitrite analysis was continued to determine the accumulation of nitrite from TNT, as a positive evidence of demineralization and degradation. The findings of nitrite analyses were graphically demonstrated in Figure 3. In bacterial cultures containing 50 mg L<sup>-1</sup> TNT, the increase in measurable nitrite concentrations after incubating for 6 h, continued up to 48 h, reached a maximum of 0.28 mg L<sup>-1</sup>, at 72 h. This value showed the negligible decrease (0.01 mg L<sup>-1</sup>) and thereafter it remained constant level at 0.27 mg L<sup>-1</sup>. During the biotransformation, the nitrite amounts in biotic cultures with 75 mg L<sup>-1</sup> TNT showed a gradual rise and the highest nitrite concentration released from TNT was defined 0.47 mg L<sup>-1</sup> at 72 h.

In uninoculated media including TNT, nitrite accumulation was observed depending upon the chemical degradation. At the end of 96 h when was arrived maximum biotransformation, amount of nitrite that measured for both of abiotic cultures was 0.15 mg L<sup>-1</sup>. A significant result with regard to forming nitrate was not achieved. The results of nitrate assays are presented in Figure 4.

### The conversion of TNT to reduction products

In all of the experiments conducted, removal of TNT was associated to an accumulation the possible degradation products. These products in biotic and abiotic cultures



**Figure 2** - Changes in bacterial growth following incubation period; (a) viable bacterial counts (cfu mL<sup>-1</sup>) and (b) turbidity of culture mediums with and without TNT inoculated *P. aeruginosa*.



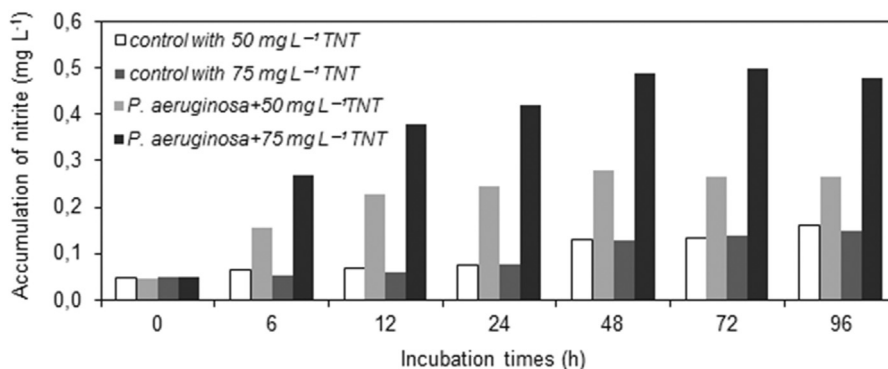


Figure 3 - Release of nitrite during TNT removal by *P. aeruginosa*.

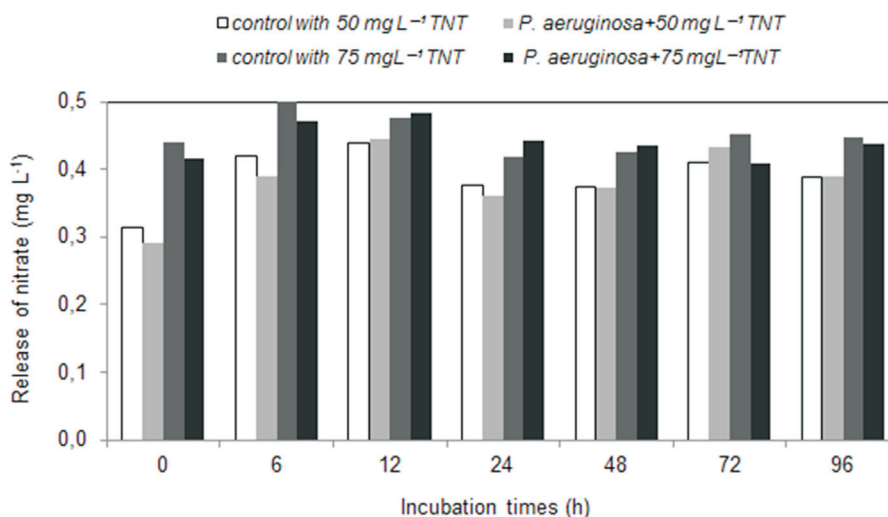


Figure 4 - Detection of nitrate by forming via nitrite reduction.

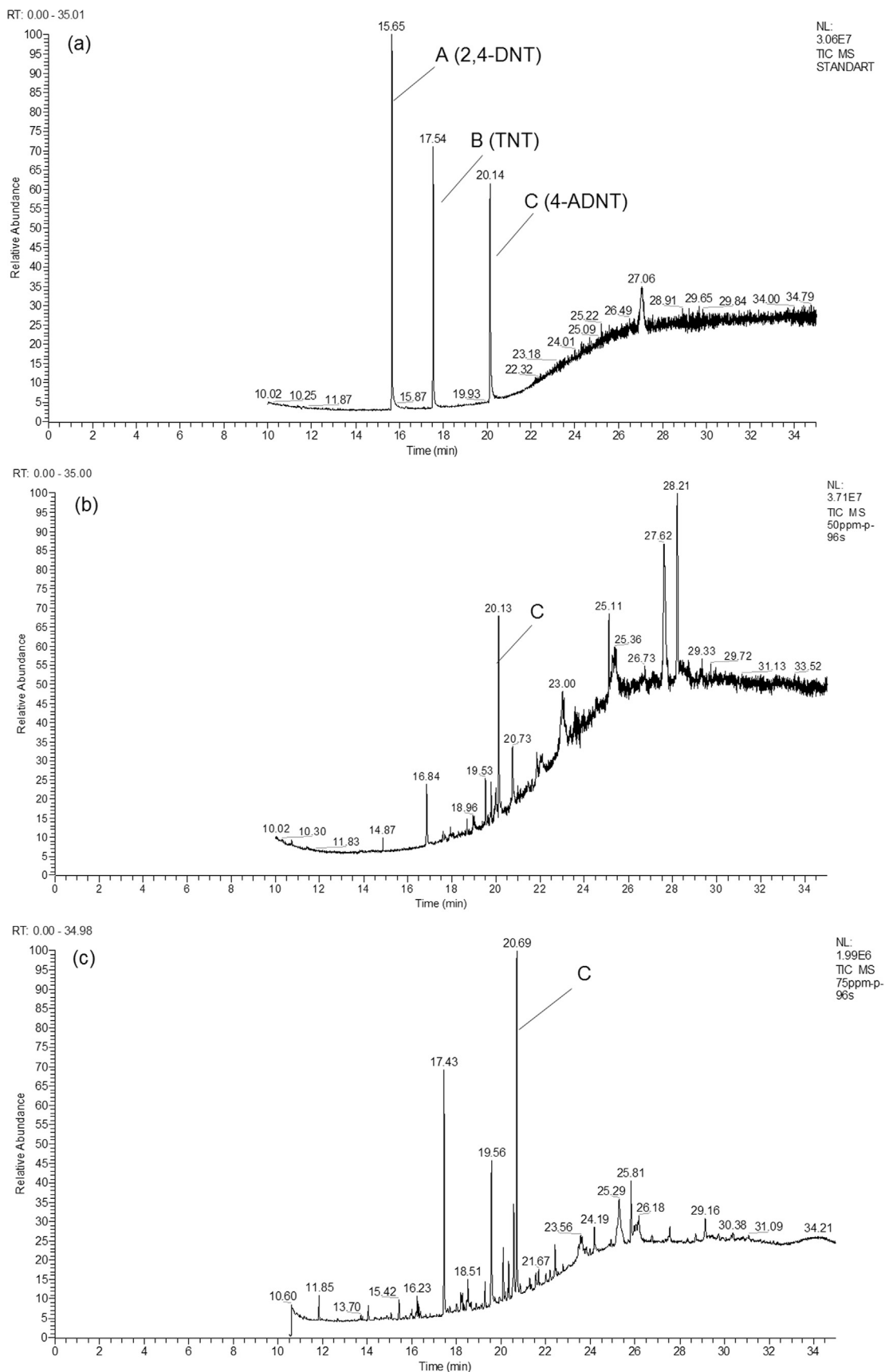
supernatants were decomposed by GC-MS and preparative TLC plates. The assignment of TNT and its metabolites were based on the match with standard mass spectrum in the GC-MS library database. The retention times of TNT, 2,4-DNT and 4-ADNT standards based on mass/charge were detected as 17.54, 15.65 and 20.14 min, respectively (Figure 5a). From degraded samples of TNT, retention times of peaks at 20.13 and 20.69 min were identified as 4-ADNT by comparison of standards (Figure 5b-c). The metabolite containing amino group (aminodinitrotoluenes) could only be determined in the biotic culture supernatants by GC-MS.

TLC analyses were performed to monitor the removal of TNT and formation of its intermediates. TNT, 2,4-DNT and 4-ADNT standards migrated with R<sub>f</sub> values 0.83, 0.67 and 0.5 (Figure 6a). At the end of degradation, it was revealed the presence two spots on TLC plates, corresponding to the spots belonging to the standards (Figure 6b-c). These spots at R<sub>f</sub> values 0.8 and 0.63 were accepted as TNT and 2,4-DNT. 2,4-DNT couldn't be encountered in the un-inoculated media.

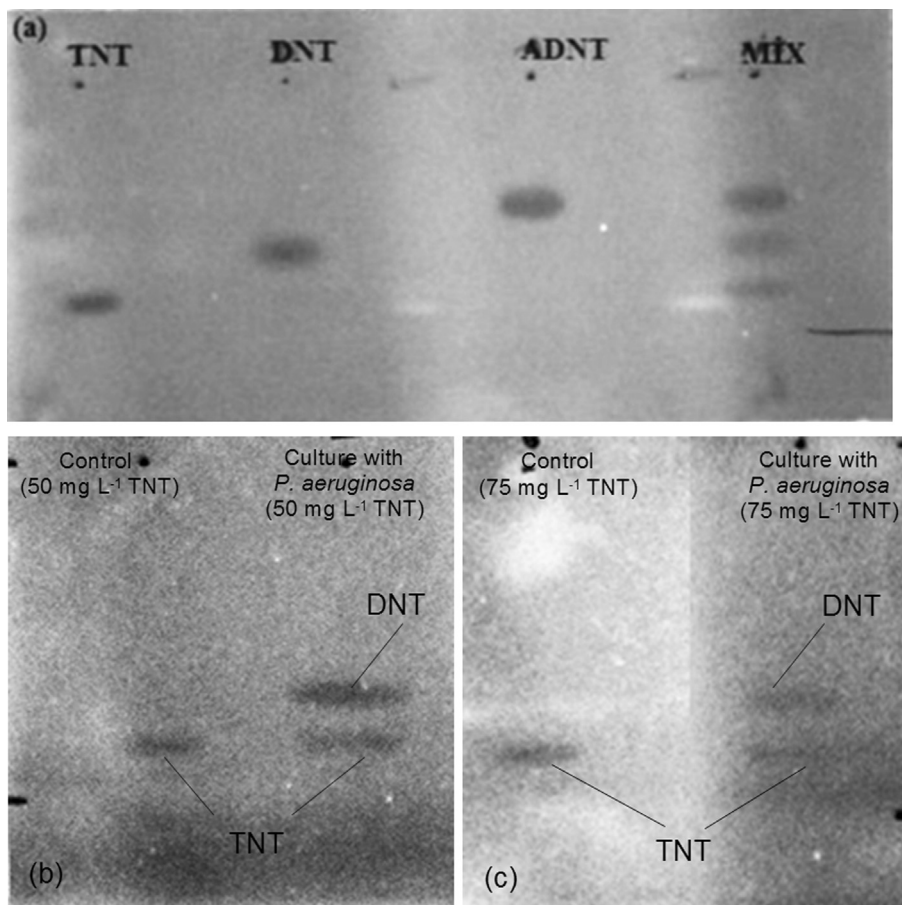
## Discussion

In our experience, it was researched the possibility that TNT degradation by *Pseudomonas aeruginosa* strain, carried out in liquid medium. In the presence of *P. aeruginosa*, maximum specific removal rates for cultures including 50 and 75 mg L<sup>-1</sup> TNT were calculated as 46% and 59% within 96 h, respectively. These differences among experiments in the degradation efficiency were mainly related to differences in initial TNT concentrations. Similarly, some workers were published that *P. aeruginosa* and other *Pseudomonas* sp were ability to grow in the presence of TNT and transform it (Alvarez *et al.*, 1995; Tope and Jamil, 2000; Park *et al.*, 2003; Zaripov *et al.*, 2004; Maeda *et al.*, 2006; Kubota *et al.*, 2008; Nyanhongo *et al.*, 2009). Accordingly our results, chemical hydrolysis of TNT based on agitating in the present studies, was also reported (Oh *et al.*, 2003; Claus *et al.*, 2007).

Generally, we observed that TNT metabolism was correlated with the increasing in microbial growth of *P. aeruginosa* accompanied by decreasing of TNT amounts in the cultures.



**Figure 5** - GC-MS spectra of peaks belonging to analytical standarts (a) and samples obtained from cultures containing respectively 50 (b) and 75 (c) mg L<sup>-1</sup> TNT.



**Figure 6** - TLC monitoring of intermediates formed during transformation by *P. aeruginosa*. (a) represents the reference standards on TLC plates. (b) and (c) present residual TNT and its metabolites that accumulated in biotic and abiotic cultures.

In time-dependent TNT biotransformation, bacteria-mediated reduction was detected to elevate with a high cell density of *P. aeruginosa* in the log phase. In control cultures without TNT, low levels of bacterial population indicated that *P. aeruginosa* can be used TNT as nutrient in building their cells. Also, in this present work was documented that the rate of TNT removal by *P. aeruginosa* declined as it entered stationary phase, which may be due to nutrient depletion and a reduction in the microbial population based on toxicity of the accumulating transformation products. These data are in agreement with those acquired by prior researches on TNT-biodegrading ability of *P. aeruginosa* (Tope and Jamil, 2000; Oh *et al.*, 2003; Maeda *et al.*, 2006). Which is contrast to our investigations, in a number of earlier experiments was noted that efficient of TNT removal rised with decreasing in the bacterial growth (Tope *et al.*, 1999).

The findings with regard to nitrite accumulation corresponded to distribution of TNT from culture medium throughout incubation. A rise in the level of recorded nitrite ions was substantially revealed by the decrease in TNT concentrations. In the light of the above findings, it was clearly a evidence that liberation nitrite by utilization TNT

as a growth substrate under enrichment cultures with yeast extract. The occurrence of nitrite ions manifests a release of nitrogen from the aromatic ring during degradation reaction. Besides, nitrite ions as single transformation product in abiotic cultures were identified. So, estimation of nitrite in cultures without bacteria, was verified chemical degradation correlated with decrease of TNT concentrations. Previous researchers denoted supportively our results on nitrite release by reduction during microbial processes (Oh *et al.*, 2003; Litake *et al.*, 2005; Maeda *et al.*, 2006; Mercimek *et al.*, 2013).

The further reduction of the released nitrite to nitrate was associated with decreasing of nitrite in all cultures during incubation time. But a distinct formation of nitrate as a result of direct oxidation of nitrite to nitrate by bacteria and chemical reactions, was not encountered. In contrast to our data, some searchers indicated the findings of nitrate in the cultures of *Clavibacterium*, *Sphingomonas* and their mixture during destruction of TNT (Rahal and Moussa, 2011).

During removal process of TNT by *P. aeruginosa*, all of the cultures except abiotic were appeared color changes. It was observed that the color of the culture mediums returned to pinkish red to brown around 10-12 h of incubation.

tion, gradually darkened and changed a deep red color for a prolonged period. In previous reports of TNT biotransformation, the hydride-Meisenheimer complex of TNT formed by aerobic microbial degradation that occurred via the elimination of nitro groups, were detected to be responsible for these color changes (Kim and Song, 2000; Pak *et al.*, 2000; Litake *et al.*, 2005; Nyanhongo *et al.*, 2009).

Intermediates of TNT from red-coloured supernatant were analyzed to clarify the mechanisms by which metabolic pathway of TNT removal. The incorporated bacteria converted TNT to 2,4-DNT and 4-ADNT as well as nitrite, was determined by TLC and GC-MS analyses. The presence of two degradation products in *P. aeruginosa* cultures with nitrite, and in abiotic controls demonstrated that absence of 2,4-DNT and 4-ADNT, indicated that transformation TNT was biologically mediated by *P. aeruginosa*. The presence of impurities in originating from TNT was also shown by this analytical methods. This results supported to HPLC datas that indicated TNT was not completely used by *P. aeruginosa*. Formation of 2,4-DNT and 4-ADNT from TNT proves that reduction by two different biochemical routes: 1. the elimination nitro groups to nitrite/nitrate; 2. the reduction of nitro groups to amines.

As earlier mentioned, TNT is a symmetrical nitroaromatic compound with nitro groups present on a carbon ring at the 2, 4 and 6 positions and a methyl group localate in the 1 position (Gallagher 2010). These arrangement that procured stability of aromatic ring and the electron-withdrawing properties of nitro groups surrounding its aromatic ring created steric hindrance (Claus *et al.*, 2007; Kubota *et al.*, 2008). Due to the presence of multiple nitro groups on the aromatic ring, TNT has electron deficient in  $\pi$ -orbitals. The potentiality of electrophilic attack that leading to ring fission declines as the number of nitro groups on aromatic ring increases (Han 2008). So, microbial activities on TNT be limited to elimination of nitro groups from aromatic ring and metabolism of the remaining carbon skeleton (Spain 1995).

Aerobic reduction of TNT was summarized in three categories: 1. ring oxygenation that leads to accumulation of nitrite ions, 2. nucleophilic attack by the addition of a hydride ion to form a hydride-Meisenheimer complex that reduce the aromatic ring to di-nitro derivate (Kulkarni and Chaudhari, 2007) 3. partial reduction of the nitro group to a hydroxylamine derivate (Spain 1995). In case of anaerobic processes, TNT was degraded either by respiration of the  $\text{NO}_2^-$  groups or by removal of the  $\text{NO}_2^-$  groups to be used as a terminal electron acceptor or as a nitrogen source (Gallagher 2010). TNT was catalyzed to 2,4,6-triaminotoluene (TAT) as final product in anaerobic conditions (Hawari *et al.*, 2000; Esteve- N  nuez *et al.*, 2001).

Upon review of the above information, in this study appearance of red color during degradation and the accumulation of 2,4-DNT, 4-ADNT and nitrite as biotransformation products, demonstrated that *P. aeruginosa* can

reduce the aromatic ring to dinitro-compounds by forming hydride-Meisenheimer complex, which subsequently caused the release of nitrite. And it was described that the further reduction of the nitro groups to amino groups occurred by using the nitro-group as electron acceptor in the second biochemical reaction.

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## References

- Al-Absi A (2008) Nitrate contamination of ground water and methemoglobinemia in Gaza strip. *J Al-Aqsa Univ* 12:1-14.
- Alvarez MA, Kitts CL, Botsford JL *et al.* (1995) *Pseudomonas aeruginosa* strain MA01 aerobically metabolizes the aminodinitrotoluenes produced by 2,4,6-trinitrotoluene nitro group reduction. *Can J Microbiol* 41:984-991.
- Ayoub K, Van Hullebusch ED, Cassir M *et al.* (2010) Application of advanced oxidation processes for TNT removal: A review. *J Hazard Mater* 178:10-28.
- Barreto-Rodrigues M, Silva FT, Paiva TCB (2009) Characterization of wastewater from the Brazilian TNT industry. *J Hazard Mater* 164:385-388.
- Claus H, Bausinger T, Lehmler I *et al.* (2007) Transformation of 2,4,6-trinitrotoluene (TNT) by *Raoultella terrigena*. *Biodegradation* 18:27-35.
- Coral G, Karagoz S (2005) Isolation and characterization of phenanthrene-degrading bacteria from a petroleum refinery soil. *Ann Microbiol* 55:255-259.
- Dodard SG, Powlowski J, Geoffrey I *et al.* (2004) Biotransformation of 2,4,6-trinitrotoluene (TNT) by enchytraeids (*Enchytraeus albidus*) in vivo and in vitro. *Environ Pollut* 131:263-273.
- Esteve-N  nuez A, Caballero A, Ramos JL (2001) Biological degradation of 2,4,6-trinitrotoluene. *Microbiol Mol Biol Rev* 65:335-352.
- Fleischmann TJ, Walker KC, Spain JC *et al.* (2004) Anaerobic transformation of 2,4,6-TNT by bovine ruminal microbes. *Biochem Biophys Res Commun* 314:957-963.
- Fu D, Zhang Y, Lv F *et al.* (2012) Removal of organic materials from TNT red water by Bamboo Charcoal adsorption. *Chem Eng J* 193-194:39-49.
- Gallagher EM (2010) Anaerobic Degradation of 2,4,6-trinitrotoluene (TNT): Molecular Analysis of Active Degraders and Metabolic Pathways. Ph.D. Dissertation, Rutgers The State University of New Jersey, New Jersey, 127 pp.
- Gandia-Herrero F, Lorenz A, Larson T *et al.* (2008) Detoxification of the explosive 2,4,6-trinitrotoluene in Arabidopsis: discovery of bifunctional O- and C-glucosyltransferases. *Plant J* 56:963-974.
- Han S (2008) In Situ Bioremediation and Natural Attenuation of Dinitrotoluenes and Trinitrotoluene. Ph.D. Dissertation, Georgia Institute of Technology, Atlanta, 262 pp.
- Hawari J, Beaudet S, Halasz A *et al.* (2000) Microbial degradation of explosives: Biotransformation vs. mineralization. *Appl Microbiol Biotechnol* 54:605-618.



- Ju KS, Parales RE (2010) Nitroaromatic Compounds, from Synthesis to Biodegradation. *Microbiol Mol Biol Rev* 74:250-272.
- Kalderis D, Hawthorne SB, Clifford AA *et al.* (2008) Interaction of soil, water and TNT during degradation of TNT on contaminated soil using subcritical water. *J Hazard Mater* 15:329-334.
- Kim HY, Song HG (2000) Transformation of 2,4,6-trinitrotoluene by white rot fungus *Irpex lacteus*. *Biotechnol Lett* 22:969-975.
- Kubota A, Maeda T, Nagafuchi N *et al.* (2008) TNT biodegradation and production of dhydroxylaminonitrotoluene by aerobic TNT degrader *Pseudomonas* sp. strain TM15 in an anoxic environment. *Biodegradation* 19:795-805.
- Kulkarni M, Chaudhari A (2007) Microbial remediation of nitro-aromatic compounds: An overview. *J Environ Manage* 85:496-512.
- Lin HY, Yu CP, Chen ZL (2013) Aerobic and anaerobic biodegradation of TNT by newly isolated *Bacillus mycoides*. *Ecological Engineering* 52:270-277.
- Litake GM, Joshi SG, Ghole VS (2005) TNT biotransformation potential of the clinical isolate of *Salmonella typhimurium*-potential ecological implications. *Indian J Occup Health* 9:29-34.
- Lotufo GR, Blackburn W, Marlborough SJ *et al.* (2010) Toxicity and bioaccumulation of TNT in marine fish in sediment exposures. *Ecotoxicol Environ Saf* 73:1720-1727.
- Maeda T, Kadokami K, Ogawa HI (2006) Characterization of 2,4,6-trinitrotoluene (TNT)-metabolizing bacteria isolated from TNT-polluted soils in the Yamada Green Zone, Kitakyushu, Japan. *J Environ Biotechnol* 6:33-39.
- Mercimek HA, Dincer S, Guzeldag G *et al.* (2013) Aerobic biodegradation of 2,4,6-trinitrotoluene (TNT) by *Bacillus cereus* isolated from contaminated soil. *Microb Ecol* 66:512-521.
- Montgomery MT, Coffin RB, Boyd TJ *et al.* (2013) Incorporation and mineralization of TNT and other anthropogenic organics by natural microbial assemblages from a small, tropical estuary. *Environ Pollut* 174:257-264.
- Moshe SSB, Ronen Z, Dahan O *et al.* (2009) Sequential biodegradation of TNT, RDX and HMX in a mixture. *Environ Pollut* 157:2231-2238.
- Nam SI (1997) On-site analysis of explosives in soil evaluation of thin-layer chromatography for confirmation analyte identity. *Cold Regions Research and Engineering Laboratory Special Report* 97-121.
- Nyanhongo GS, Aichernig N, Ortner M *et al.* (2009) Incorporation of 2,4,6-trinitrotoluene (TNT) transforming bacteria into explosive formulations. *J Hazard Mater* 165:285-290.
- Nyanhongo GS, Erlacher A, Schroeder M *et al.* (2006) Enzymatic immobilization of 2,4,6-trinitrotoluene (TNT) biodegradation products onto model humic substances. *Enzyme Microb Technol* 39:1197-1204.
- Oh B, Shea PJ, Drijber RA *et al.* (2003) TNT biotransformation and detoxification by a *Pseudomonas aeruginosa* strain. *Biodegradation* 14:309-319.
- Oh KH, Kim YJ (1998) Degradation of explosive 2,4,6-trinitrotoluene by s-triazine degrading bacterium isolated from contaminated soil. *Bull Environ Contam Toxicol* 61:702-708.
- Pak JW, Knoke KL, Noguera DR *et al.* (2000) Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. *Appl Environ Microbiol* 66:4742-4750.
- Park C, Kim TH, Kim S *et al.* (2002) Biokinetic parameter estimation for degradation of 2,4,6-Trinitrotoluene (TNT) with *Pseudomonas putida* KP-T201. *J Biosci Bioeng* 94:57-61.
- Park S, Kim TH, Kim S *et al.* (2003) Optimization of biodegradation of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas putida*. *J Biosci Bioeng* 95:567-571.
- Rahal AG, Moussa LA (2011) Degradation of 2,4,6-trinitrotoluene (TNT) by soil bacteria isolated from TNT contaminated soil. *Aust J Basic Appl Sci* 2:8-17.
- Robertson BK, Jjemba PK (2005) Enhanced bioavailability of sorbed 2,4,6-trinitrotoluene (TNT) by a bacterial consortium. *Chemosphere* 58:263-270.
- Schackmann A, Müller R (1991) Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. *Appl Microbiol Biotechnol* 34:809-813.
- Seth-Smith HMB (2002) Microbial Degredation of RDX. Ph.D. Dissertation, University of Cambridge, England, 139 pp.
- Spain JC (1995) Biodegradation of nitroaromatic compounds. *Annu Rev Microbiol* 49: 523-555.
- Tope AM, Jamil K (2000) Isolation of TNT tolerant *Pseudomonas* species (strain KA) from TNT contaminated soil biotransformation of TNT. *Def Sci J* 151:141-145.
- Tope AM, Jamil K, Baggi TR (1999) Transformation of 2,4,6-trinitrotoluene (TNT) by immobilized and resting cells of *Arthrobacter* sp. *J Hazard Subst Res* 2:3-9.
- Yang Q, Liang Y, Zhou T *et al.* (2008) TNT determination based on its degradation by immobilized HRP with electrochemical sensor. *Electrochem Commun* 10:1176-1179.
- Wang Z, Ye Z, Zhang M *et al.* (2010) Degradation of 2,4,6-trinitrotoluene (TNT) by immobilized microorganism-biological filter. *Process Biochem* 45:993-1001.
- Zaripov SA, Naumova AV, Suvorova ES *et al.* (2004) Initial stages of 2,4,6-Trinitrotoluene transformation by microorganisms. *Microbiology* 73:398-403.
- Zhang M, Zhao Q, Ye Z (2011) Organic pollutants removal from 2,4,6-trinitrotoluene (TNT) red water using low cost activated coke. *J Environ Sci* 23:1962-1969.
- Zheng W, Lichwa J, D'Alessio M *et al.* (2009) Fate and transport of TNT, RDX, and HMX in streambed sediments: Implications for riverbank filtration. *Chemosphere* 76:1167-1177.

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