

A NOVEL ENVIRONMENTALLY FRIENDLY 2,4,6-TRINITROTOLUENE (TNT) BASED EXPLOSIVE

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A novel bioremediation technology has been developed. This technology involves the incorporation of a newly isolated *Pseudomonas putida* GG04 and *Bacillus* sp. SF into an explosive formulation to enhance biodegradation of TNT residues and explosives which fail to detonate due to technical problems. The incorporation of these microorganisms into the explosive did not affect the quality of the explosive in terms of detonation velocity while complete degradation of TNT moieties upon transfer in liquid media was observed after 4 days. The incorporated microorganisms sequentially reduced TNT leading to the formation of hydroxylamidnitrotoluenes (HADNT), 4-amino-2,6-dinitrotoluenes; 2-amino-4,6-dinitrotoluenes, different azoxy compounds; 2,6-diaminonitrotoluenes and 2,4-diaminonitrotoluenes. Aminodinitrotoluenes (AMDNT) and diaminonitrotoluenes (DAMNT) constituted the predominant metabolites which steadily increased achieving 41 μ M and 63 μ M in *P. putida* GG04 cultures and, 73 μ M and 109 μ M in *Bacillus* SF cultures, respectively. Although both microorganisms use NAD(P)H dependent enzymes to transform TNT, *P. putida* GG04 has a preference for NADPH. The accumulation of AMDNT and DAMNT was effectively prevented in the presence of guaiacol and catechol. A 89 % reduction of AMDNT and a 80 % of DAMNT was achieved in *P. putida* GG04 cultures, while in *Bacillus* sp. SF, 91% and 70 % reduction was achieved. This demonstrates that biodegradation of TNT in the presence of humic material is effective in immobilizing TNT metabolites. Addition of acetonitrile (1:4) to TNT and to its biodegradation products with sequential freezing of the samples at -20 °C was effective in concentrating and enhancing detection signals to identify TNT contaminates sites.

Key words: 2,4,6-trinitrotoluene (TNT); explosive; biotransformation; immobilization; humic monomers

НОВ И ПРИЈАТЕЛСКИ ЗА ОКОЛИНАТА ЕКСПЛОЗИВ НА БАЗА НА 2,4,6-ТРИНИТРОТОЛУЕН (ТНТ)

Развиена е нова технологија на биопречистување. Оваа технологија вклучува внесување на нови изолати на *Pseudomonas putida* GG04 и *Bacillus* sp. SF во експлозивни за да се зголеми биодеградијата на остатоците од тринитротолуен (TNT) и експлозивите кои не експлодирале поради технички проблеми. Внесувањето на овие микроорганизми во експлозивот не влијае врз квалитетот на експлозивот во поглед на брзината на детонација додека целосна деградација на TNT по негово пренесување во течна средина е забележано по 4 дена. Внесените микроорганизми го редуцираат последователно TNT, што води до создавање на хидроксиламинодинитротолуени (HADNT), 4-амино-2,6-динитротолуени, 2-амино-4,6-динитротолуени, различни азокси соединенија, 2,6-диаминонитротолуени и 2,4-диаминонитротолуени. Аминонитротолуените (AMDNT) и диаминонитротолуените (DAMNT) ги сочинуваа доминантните метаболити кои постојано растеа достигнувајќи 41 μ M и 63 μ M во културите на *P. putida* GG04 и 73 μ M и 109 μ M во културите на *Bacillus* SF. Иако и двата микроорганизма за трансформација на TNT користат ензими чиј кофактор е NAD(P)H, *P. putida* GG04 го преферира коензимот NADPH. Присуството на гвајакол и катехол води до запирање на создавањето на AMDNT и DAMNT. Со културата на *P. putida* GG04 се постигнува редуција од 89 % на AMDNT и 80 % на DAMNT, додека со *Bacillus* sp. SF се постигнува редуција од 91 % и 70 %. Ова покажува дека биодеградијата на TNT во присуство на хумидни материи е корисна за имобилизацијата на метаболитите на TNT. Додавањето на ацетонитрил (1 : 4) кон TNT и кон него-

вите продукти на биодеградација со последователно замрзнување на примероците на $-20\text{ }^{\circ}\text{C}$ се покажа како ефикасна постапка за концентрирање и зголемување на сигналите за детекција со кои се идентификуваат контаминирани делови со TNT.

Клучни зборови: 2,4,6-тринитротолуен (TNT); експлозив; биотрансформација; имобилизација; хумидни мономерни

INTRODUCTION

Naturally occurring nitroaromatic compounds are rare in nature; however, these compounds are produced extensively for application in the explosive, dye, polymer, pesticide and pharmaceutical industries [1]. Among these compounds, TNT is perhaps one of the most widely produced and used nitroaromatic compound. TNT is an explosive which has dominated the military production industry since 1902 [2]. Persistence of TNT is not the only major concern – the electrophilic nature of its nitro groups yields to prompt oxidation of biological molecules [3]. Occupational exposure to TNT results in pathological effects ranging from liver damage, cyanosis, sneezing, cough, sore throat, peripheral neuritis, muscular pain, kidney damage, cataracts, sensitization dermatitis, leukocytosis or leukopenia and aplastic anemia [4]. These adverse effects make the development of new methods for avoiding human exposure a necessity.

Although incineration is the most widely used remediation technology, there is great interest in developing biologically-based technologies which are environmentally friendly and cost effective. However, TNT biodegradation studies up to now have resulted predominantly in biotransformation leading to the accumulation of AMDNT, DAMNT and azoxy compounds [5, 7]. Interestingly, some studies have shown the possibility of immobilizing TNT and its metabolites onto complex soil organic matter during anaerobic TNT degradation [8, 9]. Coupling of TNT metabolites during TNT biodegradation under aerobic conditions has been shown to be effective, resulting in reduced toxicity [10, 11].

Current biological treatment technologies have focused mainly on heavily TNT contaminated sites (production and storage sites). Occupational health risks arising from residues after incomplete detonation of the explosive in the mining and construction sites, or explosives which fail to detonate due to technical failure, have been overlooked. For the first time, the current work attempts to address the destruction of these residual explosives. This is achieved by incorporating microorganisms or their

enzyme systems into the explosives formulation. Since biodegradation studies have resulted predominantly in biotransformation, screening for more efficient biodegraders and immobilization of TNT metabolites onto humic monomers are subjects of investigation in this work.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade; 2,4,6-trinitrotoluene (TNT); 2,6-dinitrotoluene and p-nitrotoluene were supplied by a local explosives producer, while 2,4-dinitrotoluene; 2,4-diamino-6-nitrotoluene; 2,6-diamino-4-nitrotoluene; 2,2,6,6-tetraazoxynitrotoluene; 4-amino-2,6-dinitrotoluene; 2-amino-4,6-dinitrotoluene; 2,6-dinitrotoluene and 2,4-dinitrotoluene were purchased from Accustandards Inc. (New Haven, USA). Contaminated soils and wastewater samples for isolation of microorganisms were also provided by the local explosives producer and stored at $4\text{ }^{\circ}\text{C}$. All other chemicals were obtained from Sigma-Aldrich and Merck.

Isolation of microorganisms

TNT contaminated soil and wastewater samples were used as source for the isolation of microorganisms. The enrichment procedure and the detection of TNT removal activity was as described by Gunnison *et al.* [13]. Pure isolated microorganisms were sent for identification to the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. In addition, TNT degradation capability of the thermoalkalophilic *Bacillus sp.* SF strain isolated previously from wastewater [12] was investigated.

Liquid cultures

A medium containing (g l^{-1}) 10 g glucose, 6 g yeast extract, 5 g $(\text{NH}_4)_2\text{SO}_4$ was autoclaved at $121\text{ }^{\circ}\text{C}$ for 15 min, cooled and then supplemented with

100 ml sterile basal salt medium. The salt medium contained (g l^{-1}) K_2HPO_4 0.1, $(\text{NH}_4)_2\text{SO}_4$ 0.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, KCl 0.2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.005, $\text{FeCl}_2 \cdot \text{H}_2\text{O}$ 0.005. TNT solution was prepared by suspending 50 g TNT in 500 ml deionized water and heating it at 85 °C for 20 minutes with intermittent stirring. The solution was allowed to cool at room temperature and then filtered to remove undissolved TNT. The culture medium (25 ml), dispensed into a 250 ml buffered flask, was then supplemented with 65 ml TNT solution (190 mg TNT l^{-1}). Each flask was then inoculated with 10 ml bacterial culture in its exponential phase (approximately 3.2 absorbance units at 600 nm). The inoculated flasks (100ml working volume) were incubated at 30 °C and at 150 rpm. Sampling was done periodically by withdrawing 4 ml which were then centrifuged at 10 000 rpm. Apart from using the clear supernatant directly for TLC and HPLC analysis, acetonitrile (99 %) in the ratio of 1 : 4 (acetonitrile to sample) was added to the clear supernatant, thoroughly mixed and the mixture frozen at -20 °C over night. The resulting organic top phase was then used for TLC and HPLC analysis.

Identification of enzymes involved in initial TNT degradation

Cells of *P. putida* and of *Bacillus sp.* SF harvested from a 2-L culture medium in their late exponential phase (approximately 3.2 absorbance units at 600 nm) were washed twice with 50 mM phosphate buffer (pH 7.0) and re-suspended in 100 ml of the same buffer. The suspended cells were cooled on ice and broken with ultrasonic pulses at 2 min intervals (Model Bandelin Sonorex Super RK102H, Berlin, Germany). Cell breakage was monitored microscopically. Cell debris was removed by centrifugation at 20,000 rpm for 20 min, at 4 °C. The extracellular fluid, the broken cells and the intracellular supernatant were then separately incubated with TNT in the absence or presence of cofactors (NADPH, NADH and ATP) for 1h. The reaction mixture contained 150 μl of the individually separated fraction, 800 μl 50 mM phosphate buffer at pH 7.0 containing 200 μM TNT and 1.5 mM cofactor concentration. A control was prepared with denatured fractions (boiled at 100°C for 10 minutes) and TNT transformation was also monitored as described below.

Immobilization of TNT metabolites on humic monomers during TNT degradation

P. putida GG04 and *Bacillus sp.* SF cultures in the mid exponential phase were inoculated with 220 μM TNT and then supplemented with 800 μM of the respective humic monomers (guaiacol, ferulic acid and catechol). Three controls were prepared. One control flask was supplemented with TNT only while another was also supplemented with the respective humic monomers in the absence of TNT and the third was boiled at 100 °C for 20 min to inactivate the bacteria and their enzymes before supplementing it with TNT only. The decrease or reduction in the formation of major TNT metabolites in cultures was recognized as the effect caused by the humic monomers. This was justified by comparing cultures with and without humic monomers.

Monitoring TNT degradation

During incubation periods residual TNT and TNT degradation products were monitored using different approaches as described below.

Thin layer chromatography (TLC) analysis of metabolites

Samples were spotted on silica gel 60 plates with fluorescent background (J T. Backer Inc, Phillipsburg, NJ, USA), and developed in benzene: hexane (3:1) as mobile phase. For identification of the metabolites, developed plates were observed under UV at 254 nm. The plates were further sprayed with 10 % tetramethylammonium hydroxide [14] and immediately dried. This allowed the differentiation of the metabolites based on color as well as on R_f-values. The metabolites were identified by comparing R_f values and color to authentic standards. Detection of TNT degradation products with amino groups was done by immersing developed TLC plates in a solution of NaNO_2 (0.1 % wt/vol) in a 1 N HCl for 1 to 2 minutes enough to make the plates wet. Afterwards the plates were dried. The plates were then further immersed in N-1-naphthylethylenediamine HCl (0.04 % wt/ vol) solution [15] for 2 minutes and allowed to stand for another 3 minutes before drying. Colors resulting from this reaction were compared with colors from the authentic standards.

HPLC analysis of TNT and its degradation products

For detection and confirmation of the products of TNT and its metabolites already identified by TLC, HPLC analysis was used. The analysis was performed by HPLC system from Dionex with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector (UV 220 nm). Identification and quantitative determination of TNT and its transformation products was done by reversed phase HPLC, on a Discovery HS C18 column (5 μm ; 25 cm \times 4.6 mm, Supelco, Bellefonte, USA) using 50/50 methanol: deionized water as solvent at a flow rate of 1 ml min^{-1} and at an oven temperature of 40 $^{\circ}\text{C}$.

Preparation of microorganisms for incorporation into explosives

Preparation of Bacillus sp. SF for incorporation into explosives

A sporulation medium for *Bacillus sp. SF* containing (per liter) 8 g bacterial nutrient broth, 1 g KCl, 0.25 g MgSO_4 and 0.5 ml NaOH (1 M) was prepared and autoclaved at 121 $^{\circ}\text{C}$ for 15 minutes. The medium was left to cool down to 50 $^{\circ}\text{C}$ before supplementing it with 1 ml of the medium containing $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (1 M); MnCl (0.01 M) and 1 ml FeSO_4 (1 mM) [16]. The medium was inoculated by aseptically transferring the microorganisms from an agar plate into a 1 liter Erlenmeyer flask containing 500 ml medium, and it was incubated at 40 $^{\circ}\text{C}$ at 150 rpm for 2 days to allow the formation of high biomass. The cultures were then supplemented with 50 g wood flour to enable the organism to adsorb on to the flour and further incubated while monitoring spore formation. Spore formation was monitored microscopically in the following way. A drop from the culture was mixed with malachite green on a glass slide and fixed by gently heating for 2 to 3 min. The fixed slide was then washed with distilled water and further counter stained with safranin for one minute. The spores stained green while the vegetative cells stained red. Finally the cultures were harvested by carefully decanting excess medium. The remaining filter cake was transferred into 1 liter plastic bottles and frozen at -20°C .

Preparation of P. putida GG04 for incorporation into explosives

For incorporation of *P. putida* GG04 into the explosive, different cryo-protective media were compared. The screened cryoprotectants were milk, honey, raffinose, Na-glutamate, and trehalose [17]. Preliminary screening in cryovials showed 5 % raffinose is the best protective media as evidenced by the viability test of 49 % and was therefore chosen for incorporation. *Pseudomonas putida* GG04 culture (500 ml) was grown in the medium described before, up to mid-exponential phase (approximately 1.2 absorbance units monitored at 600 nm). Two aliquots of 500 ml culture were then transferred into a 2 l Erlenmeyer flask containing 50 g sterile wood flour to enable the organism to adsorb on the wood flour. This mixture was further incubated and later harvested in the late exponential phase by filtering excess water through a 0.45 μm filter membrane under sterile conditions. The filter cake (wood flour and microorganisms) was then packed into 1 l plastic bottles which were supplemented with 200 ml 5 % raffinose solution as a protective medium and frozen at -20°C .

For the lyophilization and incorporation of the filter cake of both cultures into the explosive, frozen samples were freeze dried using the Labconco Freeze Dry System / FreeZone $^{\circ}$ 4.5 Liter Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of -48°C and at a vacuum pressure of 3×10^{-4} mbar. The freeze dried filter cakes of *P. putida* GG04 and of *Bacillus sp. SF* were mixed and then incorporated into a TNT based explosive by the local explosives producer. The quality of the explosive was assessed by measuring the detonation velocity of the explosive.

Recovery of microorganisms from detonation residues and explosive formulation

To investigate if *P. putida* and *B. subtilis* were not inhibited after incorporation into the explosive formulation, 3 g of the TNT based explosive was inoculated in 200 ml culture medium containing 200 μM TNT solution (final concentration) in 1-l Erlenmeyer flasks and incubated under similar conditions described before. The degradation process was monitored by TLC and HPLC. Agar plates overlaid with TNT crystal as in the isolation procedure were also inoculated with the explosive

formulation containing microorganism. The agar plates were scored for growth and no growth. Long term survival of the microorganism was monitored over a period of 3 years by inoculating 1 g of the ammonium nitrate explosive containing microorganism onto agar plates and then scoring the colony forming units.

RESULTS AND DISCUSSION

TNT degradation

Microbial TNT degradation was detected on plates inoculated with enriched TNT contaminated soil samples. The activity was observed as the disappearance of TNT crystals, the formation of dark red colonies and also some watery transparent spots on agar plates. A bacterial strain (GG05) isolated from the watery transparent spots was identified as *Bacillus cereus* while another strain (GG04) isolated from the deep red colored colonies was identified as *P. putida*. The ability of the microorganisms to degrade TNT crystals overlaid on agar plates shows overwhelming tolerance of high TNT concentrations, remarkable ability and suitability for *in situ* degradation of TNT.

TNT degradation profiles in liquid cultures varied from one strain to the other as shown in

Fig.1. TNT degradation by *P. putida* GG04 started immediately after addition of the inoculum in liquid cultures (Scheme 1) and the culture turned to a deep red color. The highest degradation rate of 1 μM TNT per minute was achieved during the period between 2 – 8 h of incubation and thereafter the rate drastically declined to 0.1 μM per minute. The decrease in TNT degradation may be attributed to either inhibition of the organism by TNT metabolites, or formation of slimlike material excreted by the organism (an attribute of the genus) and accumulating on the walls. Therefore the slim material complexes the organism and some TNT metabolites.

P. putida GG04 required 24h to completely degrade 220 μM TNT (Fig. 1), while *B. cereus* GG05 achieved only 68 % TNT degradation in the same time. In contrast, the alkalothermophilic *Bacillus* sp. SF completely degraded this amount of TNT within 22 h and thus was chosen for further studies as representative of the *Bacillus* genus. In previous studies, Kalafut *et al.* [18], observed different ability to transform TNT by *Pseudomonas aeruginosa*, *Bacillus* sp and a *Staphylococcus* sp. . Generally, the TNT degradation efficiency observed in this study is consistent with other studies where similar organisms have been used [19, 20].

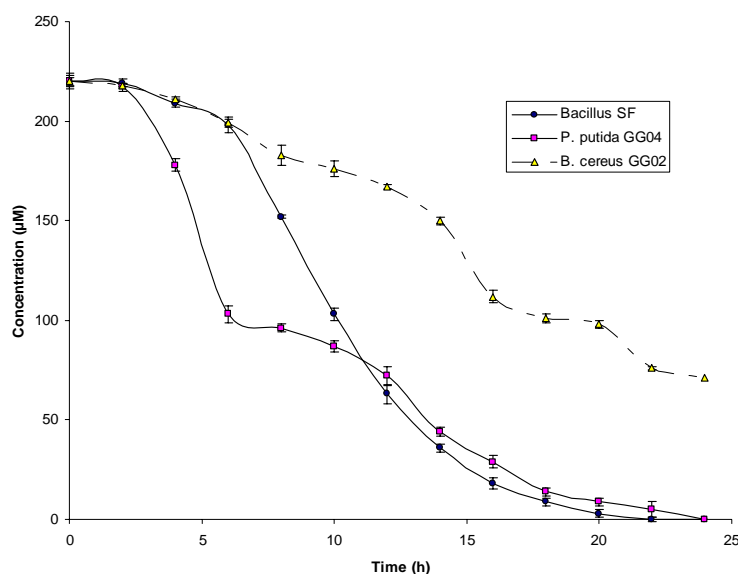


Fig. 1. Biodegradation of TNT by *P putida* GG04, *B. cereus* GG02 and *Bacillus* SF.

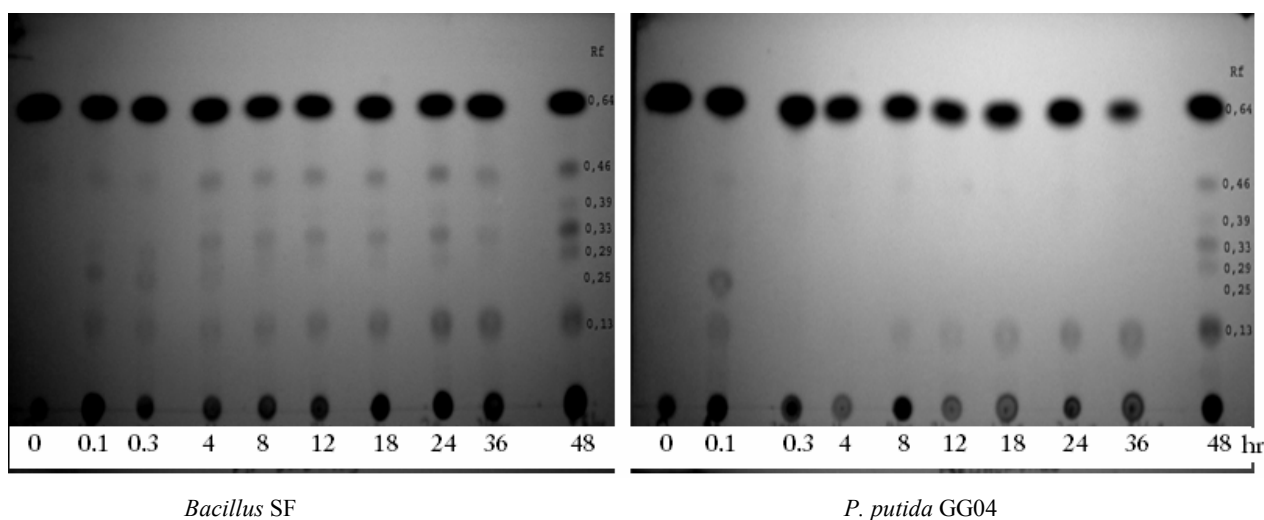
TNT biodegradation metabolites

A total of 8 different TNT metabolites were identified during TNT degradation by TLC and confirmed by HPLC (Table 1 and Scheme 1). Hydroxylaminodinitrotoluenes (2-HADNT and 4-HADNT) were the first detectable metabolites observed after 10 min of incubation in *P. putida* GG04 cultures (Scheme 1). In *Bacillus* sp. SF the products detected during the first 10 minutes were AMDNT (2-AMDNT and 4-AMDNT) and azoxytetranitrotoluene compounds (Scheme 1). Later on DAMNT (2,6-diamino-4-nitrotoluene and 2,4-diamino-6-nitrotoluene) and other azoxytetranitrotoluenes were detected after 4h of incubation on TLC for *Bacillus* SF and by HPLC for *P. putida* GG04. Although AMDNT were the first metabolites to accumulate, after 8h of incubation the concentration of DAMNT was higher than that of ADMNT in both cultures (Fig. 2). Further, an unidentified, highly hydrophilic, dark red metabolite which could not be extracted using most of the organic solvents like methylchloride, chloroform, ethylacetate or hexane appeared during the exponential phase and persisted during the whole incubation period in *P. putida* GG04 cultures. The metabolite could only be recovered by lowering pH below 2.5 and formed aggregates which precipitated at the bottom of the flask. The properties of the red metabolite in *P. putida* GG04 cultures are consistent with data reported by previous researchers for TNT-Meiseinheimer complexes [21, 22]. In addition, there were several other products detected in both cultures which were not positively identified and many more products were detected

after prolonged incubation times of up to 48h in both cultures (Scheme 1). Of all the products identified, AMDNT and DAMNT were the predominant metabolites, which steadily increased achieving 41 μM and 63 μM in *P. putida* GG04 cultures respectively and 73 μM and 109 μM in *Bacillus* sp. SF cultures respectively (Fig. 2). As evidenced by the TLC profiles, *P. putida* GG04 and *Bacillus* sp. SF use different pathways during TNT biodegradation. Surprisingly the products in both cultures are almost similar after prolonged incubation, that is after 48h of incubation (Scheme 1)

Identification of TNT degrading enzymes

To identify the enzymes involved in TNT degradation, the extracellular fractions, the cell debris and the intracellular fractions were incubated with TNT in the presence and absence of cofactors. TNT degradation activity was detected in intracellular fractions for both *P. putida* GG04 and *Bacillus* sp. SF in the presence of NADPH and NADH (Fig 3). In particular, *P. putida* GG04 transformed 171 μM TNT in the presence of NADPH and 123 μM in the presence of NADH. However, the amount of TNT transformed by *Bacillus* sp. SF intracellular fractions in the presence of NADPH and NADH was almost the same. In both cases nitroreductases have been shown to be the main enzymes involved in the initial TNT biodegradation, which is in agreement with previous results [5]. The preferred reduction of TNT is attributed to the strong electron withdrawing properties of the nitro group which confers a high electron deficiency on the TNT molecule [23].



Scheme 1. TLC monitoring of TNT degradation products during incubation with *Bacillus* sp. SF and *P. putida* GG04.

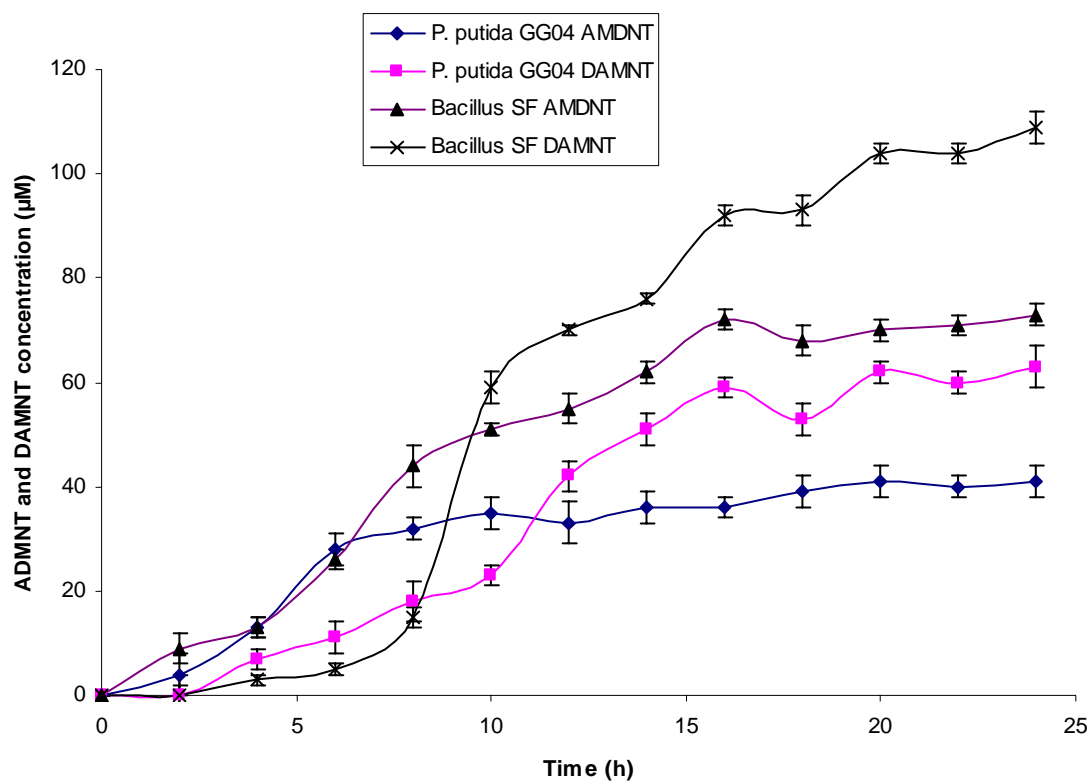
Table 1

TLC-Identification of TNT metabolites in *P. putida* GG04 and *Bacillus* sp. SF culture samples

<i>P. putida</i> GG04		<i>Bacillus</i> SF		Product identification
Rf values	Rf values	Treatment with 10 % TMAH*	Treatment with NaNO ₂ /NED*	
0	0	red	red-violet	2,4-DANT; 2,6-DANT
0.45	–		brown	4-HADNT
0.56	–			2-HADNT
0.13	0.13	yellow	red-violet	2A-DNT und 4A-DNT
0.23	–			?
0.25	0.25			?
0.29	0.29	red-violet		Azoxy-compound
0.33	0.32	intense red-violet		Azoxy-compound
0.39	0.39	violet		Azox- compound
0.46	0.46	brown -violet		2,2,6,6-tetraazoxytetranitrotoluene
0.64	0.64	brown		TNT

*TMAH – tetramethylammonium hydroxide

*NED – N-1-naphthylethylenediamine

Fig 2. Formation of ADMNT and DAMNT during TNT degradation by *P. putida* GG04 and *Bacillus* sp. SF

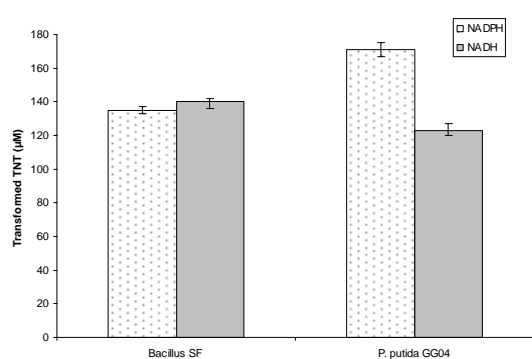


Fig. 3. TNT degradation by intracellular fractions of *P. putida* GG04 and *Bacillus* SF in the presence of NAD(P)H. There was no TNT biotransformation in the absence of these cofactors.

Immobilization of TNT metabolites onto lignin monomers

Since AMDNT and DAMNT constituted the major TNT metabolites which persisted during the incubation period, incorporation of humic monomers (catechol, ferulic and guaiacol) was investigated (Fig. 4) to prevent their formation. In the control experiments catechol and guaiacol were evidently transformed, forming a dense precipitate at the bottom and the walls of the flask. However in the control supplemented with ferulic acid no significant transformation was observed. Cultures supplemented with guaiacol were more efficient in preventing the formation of AMDNT and DAMNT (Figs. 4 and 5). An 89 % and 80 % reduction in the formation of AMDNT and DAMNT was achieved, respectively, in *P. putida* GG04 cultures (Fig. 4).

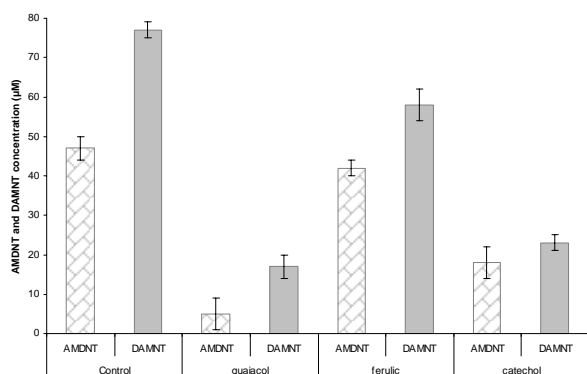


Fig. 4. Effect of incorporating humic monomers (guaiacol, ferulic acid and catechol) during TNT degradation process on the formation of AMDNT and DAMNT in *P. putida* GG04 cultures

Similarly, a 91 % and 70 % reduction in AMDNT and DAMNT was achieved, respectively, in *Bacillus* sp. SF cultures (Fig. 5). However both cultures supplemented with ferulic acid did not effectively inhibit the formation of AMDNT and DAMNT (Figs. 4 and 5). *P. putida* GG04 and *Bacillus* SF produced phenoloxidases as evidenced by the transformation of both catechol and guaiacol in the control experiments. Using different TNT metabolites, Nyanhongo *et al.* [10] demonstrated complete immobilization of HADNT in the presence of different humic monomers tested in the presence of fungal laccases. Therefore radicals generated by phenoloxidase from catechol and guaiacol may have reacted with HADNT thereby reducing the formation of AMDNT and DAMNT. The different humic monomers have a different coupling capability, as evidenced by ferulic acid. In similar previous studies a total of 71 % TNT was immobilized with catechol as co-substrate while only 25 was immobilized with humic acid as co-substrate [24]. Further, Achtnich *et al.* [25] and Thorn *et al.* [26] also provided evidence for different binding capacity of TNT metabolites by different humic fractions.

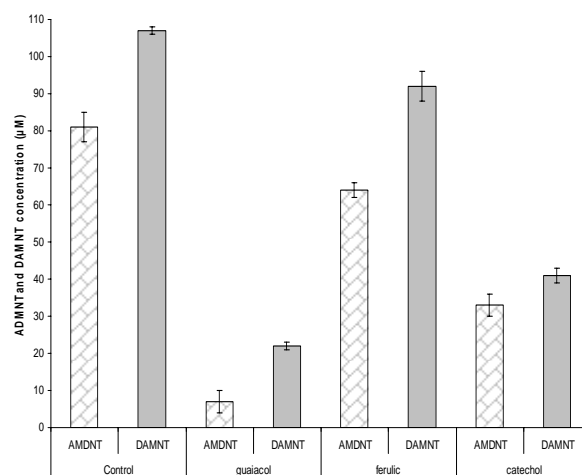


Fig. 5. Effect of incorporating humic monomers (guaiacol, ferulic acid and catechol) during TNT degradation process on the formation of AMDNT and DAMNT in *Bacillus* sp. SF cultures.

Incorporation of microorganisms into explosives

The ability of *Bacillus* sp. SF to produce spores was taken as an advantage for its incorporation into the explosive. Since *P. putida* GG04 does not produce spores, lyophilization was chosen as the best option. Several potential cryoprotectants

(milk, honey, Na-glutamate, trehalose, saccharose, lactose meso-inositol and sucrose) were tested and raffinose was observed to offer better protection achieving a survival rate of 49 % after lyophilization. Among the cryoprotectants used, 5 % raffinose gave the highest yield of 39.3×10^7 colony forming units (CFU) g^{-1} wood flour, while the worst was inositol which gave a yield of only 0.5×10^2 CFU g^{-1} . A large number of different cryoprotectants and bulking agents have been reported for preparing bacterial suspension including saccharose, lactose, trehalose, glycerol, sodium glutamate, meso-inositol, honey, raffinose, sucrose and skimmed milk [17; 27]. These substances generally improve resistance of organism to freezing but their performances depends on the organism. There is therefore an additional need for further studies to find the best protective medium. Again for this study 5 % raffinose was chosen as protective media for the incorporation of *P. putida* GG04 into the explosive. The wood flour samples coated with microorganisms (*Bacillus* sp. SF and *P. putida* GG04) were incorporated into the ammonium nitrate based explosive polonit and gelatine donarit explosives.

To confirm that the incorporation of the microorganisms into the explosive formulation did not affect the quality of the explosive, the detonation velocity (which is one of the most important parameter of an explosive) was tested using the ammonium nitrate explosive. This was compared to the reference, which in this case was a normal explosive without microorganisms. The "bioexplosive" (explosive with microorganism) showed better performance with a detonation velocity of 2006 m s^{-1} as compared to the reference with a velocity of 1701 m s^{-1} . After incubation of the bioexplosives on agar-plates overlaid with TNT crystals, the crystals disappeared forming a mixture of dark red colonies and watery transparent indicating degradation of TNT. TNT (200 μM) was completely degraded within 4 days when 3 g of explosive formulation containing *Bacillus* sp. SF and *P. putida* GG04 were added into the liquid cultures.

Long term storage at room temperature of wood flour coated with the *Bacillus* SF did not affect the organism over the 4 years (Table 2). However, *P. putida* GG04 cell count decreased with the increase of the storage time such that after 2 years all the cells were inactive (Table 2). Hence, there is still a need to optimize the protec-

tive media and lyophilization conditions, in particular those for *P. putida*.

Table 2

Long term survival of microorganisms in the explosive mixture coated onto wood flour at room temperature

Microorganism	Year (CFU/ mg wood flour)			
	0	1	3	4
<i>Bacillus</i> SF	29×10^5	28.9×10^5	29×10^5	28.5×10^5
<i>P. putida</i> GG04	39.9×10^7	17.2×10^7	–	–

In this work it was also observed that addition of acetonitrile to samples containing TNT and/or its metabolites (1:4) resulted in most of the products accumulating in the top solvent phase, except the highly polar metabolite, after subsequently freezing at $-20 \text{ }^\circ\text{C}$. This approach enhanced the detection signal of the products produced in low concentrations.

TNT degradation remains a complex process due to the unprecedented non enzymatic reactions which reduce the parent compound or the subsequent products. The non enzymatic reactions make it difficult to control directly the reaction process and/or even analyze the degradation process stoichiometrically. Incorporation of *P. putida* GG04 and *Bacillus* SF into the explosive is a very attractive option, especially for explosives that are used in the mining and construction industry since workers are exposed to great risks. Furthermore, the coupling of TNT metabolites is an interesting approach to avoid leaching and reduce environmental concerns.

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