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Mineralization and Uptake of TNT by Microorganisms: Effect of Pretreatment with Alkali

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Abstract: Four bacterial strains capable of growing on nitrotoluenes were incubated in basal salt medium containing either 2,4,6-trinitrotoluene (TNT) or its alkali hydrolysate (starting pH 12; duration 24 h) as carbon sources. The bacterial strains were identified as *Burkholderia cepacia* SH-1, *Pseudomonas aeruginosa* SH-2, *Pseudomonas putida* MC-I, and *Pseudomonas* sp. X. *Burkholderia cepacia* SH-1 was able to grow in medium containing TNT as the sole carbon source and it transformed approximately 80% TNT into metabolic products within six days. Addition of glucose or succinate improved both the growth of cells and TNT uptake. When supplemented with glucose, the Pseudomonas strains achieved significantly higher medium optical density as well as TNT transformation. The *Pseudomonas aeruginosa* SH-2 cells were most active in transforming TNT. Optical densities of all the cultures were higher after incubation with TNT-hydrolysate when compared to TNT only. In experiments with uniformly labeled ^{14}C-TNT and *Burkholderia cepacia* SH-1, the distribution of radioactivity was 0.15% in CO_2 produced, 4.88% in cell mass, 71.9% in solution, and 23% unaccounted. The same distribution when strain SH-1 was incubated in alkali hydrolysate of TNT, was 0.44% $^{14}CO_2$, 6.9% in cell mass, 22.5% in aqueous solution, and 70% unaccounted for.

Keywords: biodegradation, additional carbon source, bacteria, mixed culture

Introduction

2,4,6-trinitrotoluene (TNT) has contaminated large surface and subsurface areas at the production and testing ranges in several countries [1]. TNT is a priority pollutant and is potentially carcinogenic [2]. Technologies for clean up of TNT-contaminated areas have been investigated for over 20 years. Microbial transformations are considered desirable technology for remediation of TNT-contaminated sites and these have been subject of a large number of publications. Several recent reviews have documented the progress made in this field [3-6]. Although TNT is rapidly transformed by several microorganisms [7], the three symmetrically placed nitro-groups in TNT present a strong steric hindrance to electrophilic attack on ring-carbons by the microbial enzymes [8, 9]. In most cases, microbial transformations of TNT take place through successive reduction of the nitro-groups [7, 10-12]. Under anaerobic conditions, toluene (but not carbon dioxide) is the ultimate product in absence of any other nitrogen source [13] but not in presence of N-rich conditions. Under aerobic conditions, the aminonitrotoluenes polymerize easily into dead-end products [12, 14] and little mineralization of the parent contaminant into carbon dioxide takes place [5, 12]. Few microorganisms utilize TNT as a sole source of carbon and energy and most transform TNT by a cometabolic process in presence of other carbon sources as terminal electron acceptors [7, 15, 16]. On the other hand, reactions with alkali, ozone, or Fenton's reagent result in removal of nitro-groups from TNT [17-22]. Treatment of TNT solutions with alkali starting from solution pH of 12.0, results in rapid disappearance of > 95% TNT from solution [23]. Treatment with alkali results also in appearance of nitrate and nitrite in solution [24], suggesting that alkali action takes place via nucleophilic attack on ring carbon containing nitro groups. Although no reaction products have been identified, it is suspected that alkali action may proceed via formation of hydroxylated di- and mono-nitrotoluenes and eventually ends up in destruction of the ring structure [22]. This is especially interesting since several microorganisms utilize mono- and di-nitrotoluenes as sole carbon and energy source [25]; microorganisms extensively mineralize these nitrotoluenes also. It has been suggested that treatment of TNT-contaminations with alkali should be followed by bioremediation [14]. It would be especially desirable if such a scheme resulted in increased mineralization of TNT without needing an additional carbon source. The objective of the present work

was to investigate the effect of alkali hydrolysis on microbial growth on TNT and on mineralization of TNT. In this paper, we report several microorganisms that are capable of degrading TNT. One of the strains was able to grow on TNT as sole source of carbon and energy. Effect of alkali hydrolysis of TNT on microbial growth and mineralization of TNT is also reported.

Materials and Methods

TNT

TNT was obtained as an aqueous solution (concentration ~50 mg L^{-1}, pH 7.0) from the US Army Engineer Research and Development Center (USAERDC), Vicksburg, MS. This stock solution of TNT was stored in refrigerator in dark bottles to prevent any photo-induced transformation of TNT. Working solutions were obtained by dilution of the stock solution. New England Nuclear Life Science Products, Boston, MA, produced the uniformly labeled 14C-TNT, which was procured from Perkin Elmer Life Science, Inc. The radiolabeled TNT was in a powder form (40 μCi $mmol^{-1}$) and it was dissolved in acetonitrile to produce a stock solution containing 100 μCi mL^{-1} radiolabeled TNT.

Isolation and growth of bacteria

A bacterial strain, *Pseudomonas* sp. strain X was obtained from Dr. Herbert Fredrickson of US Army Engineer Research and Development Center, Vicksburg, MS, USA. This was enriched from a TNT-contaminated soil in USA.

A mixed culture capable of degrading mono- and di-nitrotoluenes was enriched from TNT-contaminated soil from a TNT-production facility at Pardubice, Czech Republic, using a mixture of mono- and di-nitrotoluenes in basal salt medium (BSM). The bacterial consortium was stored and propagated in Basal Salt Medium (BSM) supplemented with 1 g L^{-1} succinate as supplemental carbon source. From this mixture of microorganisms, a pure culture (strain MC-1) was isolated by successive plating on BSM agar containing a set of nitrotoluenes including TNT (BSM-NT agar). From the aqueous solution of TNT obtained from US Army Engineer Research and Development Center, Vicksburg, MS, USA, two pure cultures (SH-1 and SH-2) were isolated by successive plating on BSM-NT agar. These strains were stored and propagated on tryptic-soy agar slants. All the media compositions are listed in Table 1.

Strains were characterized by standard procedures of microbiology including Gram´s staining procedure and microscopy. Gram negative rods were further characterized by detection of oxidase-activity (Beckton Dickonson & Co.).

This was followed by growth of the cells on selective and differentiating media as MacConkey (DIFCO) and Pseudomonas P and F agar media (DIFCO). MacConkey medium selects for Gram-negative bacteria and differentiates between lactose-fermenting and lactose non-fermenting microbes. Pseudomonas P and F agar media differentiate between cells based on formation of pyocyanin and/or pyorubin, and fluorescein. Simultaneous use of both of these culture media permits identification of most *Pseudomonas* species. Subcultures from single colonies were used for biochemical identification. Bacteria were identified with the API™ system (BioMérieux).

Table 1. Media compositions

Medium	Ingredients	Concentration
BSM solution/ media	K_2HPO_4	4.3 g L^{-1}
	KH_2PO_4	3.4 g L^{-1}
	$(NH_4)_2SO_4$	3.0 g L^{-1}
	$MgCl_2.6H_2O$	0.34 g L^{-1}
Trace elements solution (TES)	$FeSO_4.7H_2O$	5.0 g L^{-1}
	$ZnSO_4.7H_2O$	5.0 gL^{-1}
	$MnSO_4.H_2O$	5.0 g L^{-1}
	$CuSO_4.H_2O$	5.0 g L^{-1}
	$CoCl_2.6H_2O$	1.1 gL^{-1}
	Na_2B_4O	1.1 gL^{-1}
	Na_2MoO_4	0.1 g L^{-1}
BSM-NT Agar	BSM solution (25x)	40.0 mL L^{-1}
	TNT solution (stock)	100.0 mL L^{-1}; final conc. ~5 ppm
	Agar granulated	20.0 g L^{-1}
	2-NT	5.0 ml L^{-1}
	4-NT	5.0 mg L^{-1}
	2,4-DNT	5.0 mg L^{-1}
	2,6-DNT	5.0 mg L^{-1}

1 ml of TES was added to 1 L of BSM and BSM-NT Agar, respectively.

For inoculum preparation, the selected strains were first streaked on a new agar plate and incubated at 37 °C overnight. For first preculture, a single colony from the fresh agar plate was transferred into 15 mL tryptic soy broth (DIFCO) and cultivated overnight. Cells from this preculture were used to inoculate 50 mL BSM containing 4 g L^{-1} glucose at 5% (v/v) level for a second preculture and incubated. Mid log-phase cells from the second preculture were used to inoculate flasks at 5% (v/v) level for growth, transformation, and mineralization experiments. All the liquid media were incubated at 30 °C and 150 rpm.

Viable cell counts were performed by spreading serially diluted liquid

samples on plate count agar (DIFCO) and counting colonies after an incubation period at 37 °C of either 24 or 48 hours.

TNT hydrolysate

TNT-hydrolysate was prepared by adding 1 N NaOH solution to TNT solution to bring its pH to 12 in a flask shielded from light and incubating it for 24 hours at room temperature with magnetic stirring. Uniformly labeled ^{14}C-TNT was added to the stock solution to prepare hydrolysate of radiotagged TNT.

Transformation and mineralization

For growth and TNT transformation experiments, 10 mL filter-sterilized TNT solution or TNT hydrolysate was added to autoclaved 37.5 mL BSM solution of appropriate concentration in baffled flasks covered with aluminum foil. In some flasks, additional carbon and energy sources (glucose, ethanol, and succinate) were also added at a level equivalent to 4 g L^{-1} glucose to investigate the effect of these supplemental carbon sources on cell growth and TNT degradation. The flasks were inoculated with actively growing cells prepared as described earlier and incubated in a shaking incubator at 25 ±2 °C at 150 rpm. Uninoculated flasks as well as those inoculated with heat-inactivated cells, formed controls.

Growth of cells was measured as optical density at 600 nm. Viable cell counts were performed as described earlier. Strain SH-1 was used for mineralization studies. Flasks were inoculated with about $4.5 \cdot 10^8$ living or heat inactivated (controls) cells. Mineralization was determined by measuring evolution of $^{14}CO_2$ in biometer flasks containing either ^{14}C-TNT or ^{14}C-TNT hydrolysate in BSM supplemented with 4 g L^{-1} glucose inoculated with cells. For mineralization studies, 10 mL filter-sterilized ^{14}C-TNT or ^{14}C-TNT hydrolysate was added to baffled respirometer flasks containing BSM and glucose (4 g L^{-1} final concentration). CO_2 produced was absorbed in KOH solution and a scintillation detector measured radioactivity in the KOH solution. At the end of the experiment, the broth was acidified to release CO_2 absorbed in it. The released CO_2 was also trapped in KOH solution and counted for radioactivity. Cells in broth were removed using centrifugation and radioactivity present in the cells and in the cell-free broth were also counted separately using a scintillation detector.

Analytical Methods

TNT concentration in the samples was measured by isocratic HPLC using a Bondasil C-18 4.6 x 250 mm column. The solvent system was 55% methanol and 45% water. Solvent flow rate was 0.6 mL min^{-1} and a UV/VIS detector detected peaks at 254 nm.

Results and Discussion

Characterization of microorganisms

All the microorganisms (strains SH-1, SH-2, MC-1, and X) were able to grow on BSM-NT agar. Thus all could be constructed as having the ability to grow on nitrotoluenes.

All strains tested were gram-negative and rod-shaped. Strains SH-2, MC-1, and X were strong and spontaneous oxidase-positive; strain SH-1 also needed only several seconds before it yielded a positive oxidase reaction. Thus it was concluded that all the four strains have cytochromes oxidase activity and belong most probably to the genera *Pseudomonas*. This was further confirmed by growth of the cells on selective and differentiating Pseudomonas media and by biochemically testing with the API 20E.

On the basis of these tests, the strains were classified as follows:

Strain SH-1	*Burkholderia cepacia*
Strain SH-2	*Pseudomonas aeruginosa*
Strain MC-1	*Pseudomonas putida*
Strain X	*Pseudomonas sp.*

Growth of microorganisms

Growth of *Burkholderia cepacia* strain SH-1 in BSM medium containing TNT or TNT-hydrolysate, with and without additional carbon-sources is shown in Figure 1. It appears that *Burkholderia cepacia* strain SH-1 is able to grow on TNT only, although the growth is not extensive. The extent of growth was some what more when additional carbon sources were also added, but it is still not very high even though considerable time (up to 30 days) was allowed for growth. Similar growth behavior was observed when plate counts were used to measure cell growth of strain SH-1. Maximum increase in cell count was noted in TNT-hydrolysate supplemented with 4 g L^{-1} glucose. Viable cell count passed through a peak on day 6.

The long-term growth data of all the cells in TNT supplemented with glucose are shown in Figure 2. It showed that *Pseudomonas* sp. strain X had the most prolific growth.

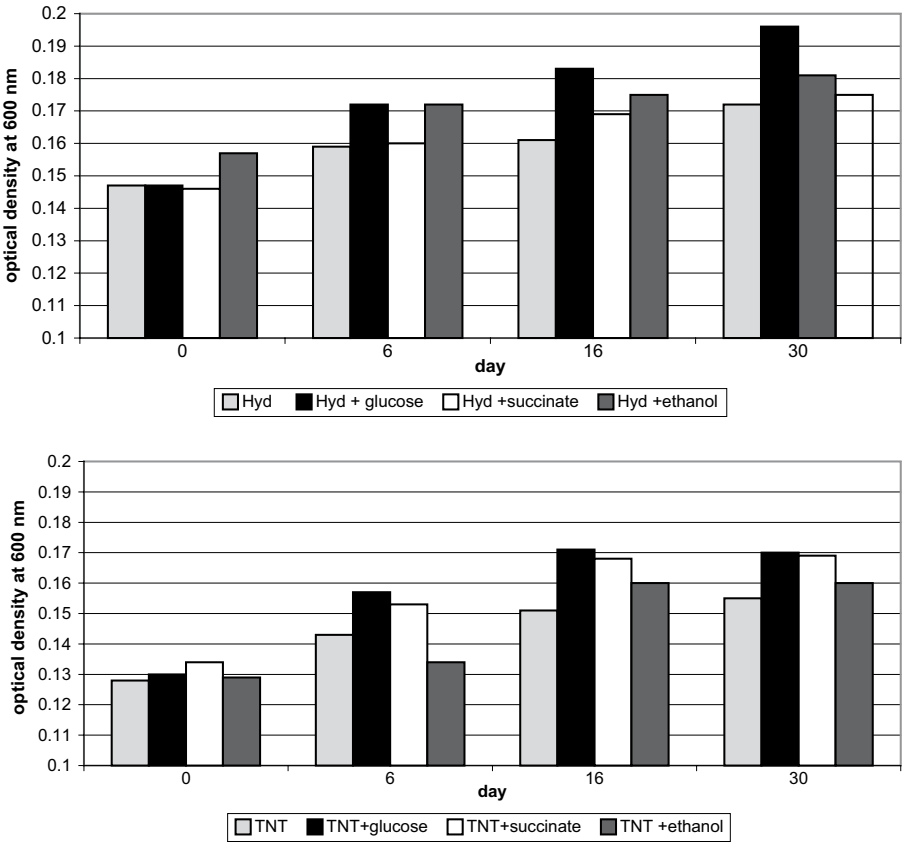

Figure 1. Growth of *Burkholderia cepacia* strain SH-1 in BSM containing TNT or TNT-hydrolysate (designated Hyd) either alone or supplemented with different carbon sources.

Growth of all the microbes over a shorter time (48 hrs) in TNT and TNT-hydrolysate supplemented with 4 g L^{-1} glucose is presented in Figure 3. In agreement with long term growth data of SH-1 cells presented in Figure 1, the SH-1 cells grew very poorly in the short-term experiments as well. The other *Pseudomonas* strains grew well compared to the *B. cepacia* SH-1 and generally resulted in considerably higher cell density (based on culture absorbance) in TNT-hydrolysate than in TNT.

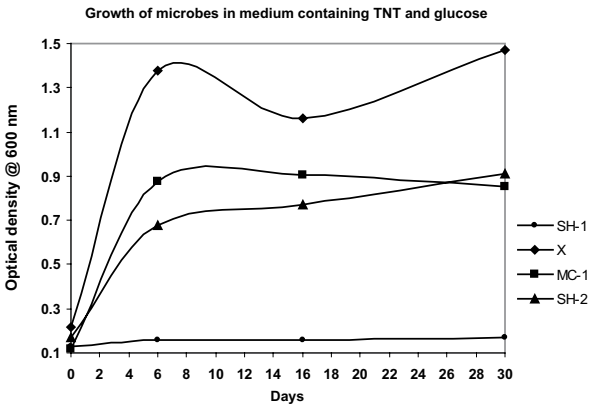

Figure 2. Growth of microorganisms in BSM containing 8.2 ppm TNT and 4 g L^{-1} glucose.

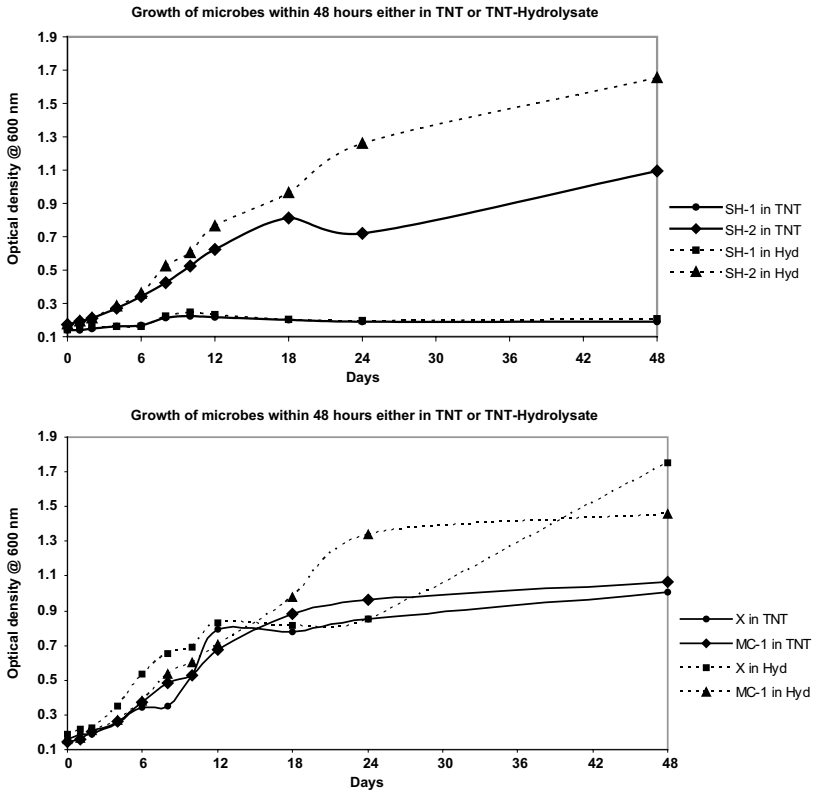

Figure 3. Short-term growth data for the different strains in BSM containing TNT or TNT-hydrolysate and 4 g L^{-1} glucose.

Disappearance of TNT from broth

Reduction in concentration of TNT after 6-day incubation of *B. cepacia* SH-1 cells in basal salt medium with or without supplementation with additional carbon sources is presented in Figure 4. More than 80% decrease in TNT concentration was observed even when TNT was the only carbon source present in the medium. Addition of 4 g L^{-1} glucose or equivalent amount of succinate to the medium resulted in almost complete removal of TNT from broth, but addition of the same amount of ethanol to the medium decreased the extent of TNT-removal. Recalling that all the flasks were shielded from light to prevent photodegradation of TNT and since TNT has a low vapor pressure, all of this reduction in TNT concentration can be attributed to biotransformation of TNT by cells.

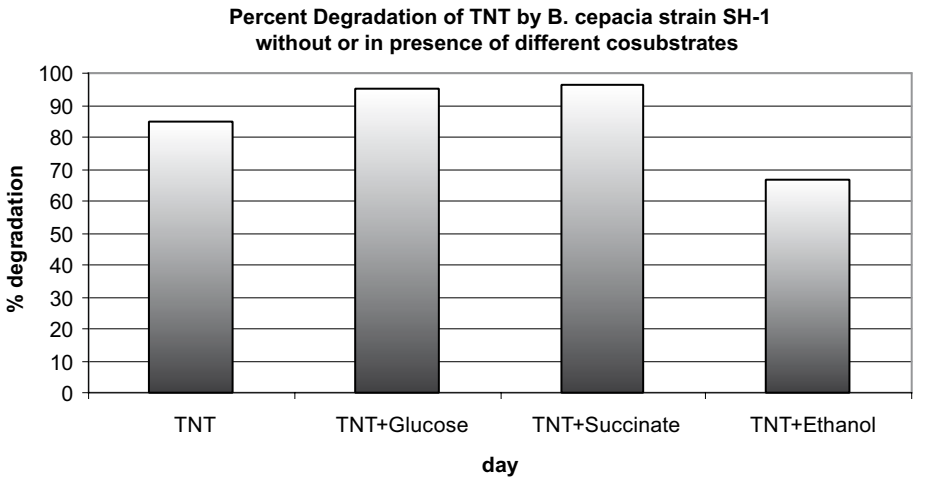

Figure 4. TNT transformation by *Burkholderia cepacia* strain SH-1 within 6 days.

Biodegradation of TNT by all the cells when incubated in basal salt medium supplemented with glucose is shown in Figure 5. All the four cell-lines were very effective in transforming TNT with almost complete transformation taking place within first six days, except for strain SH-1. Strain SH-1 was the slowest which perhaps is due to little growth of these cells (Figure 2). Even with SH-1, 80% reduction in TNT concentration took place in the first six days and TNT-degradation was almost complete by day 16. Results of short-term biodegradation experiment are presented in Figure 6. Strain SH-2 was the most active strain, followed by strain MC-1, strain X, and strain SH-1 (in decreasing order).

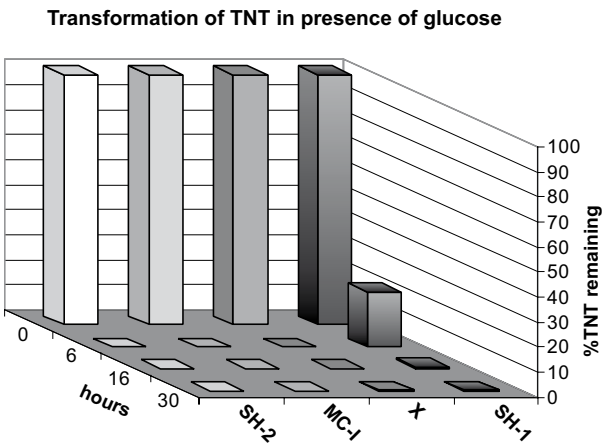

Figure 5. TNT transformation by the different strains in BSM containing 8.2 ppm TNT and 4 g L^{-1} glucose.

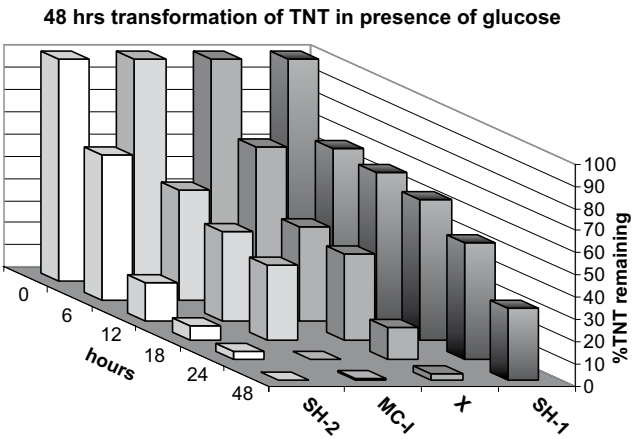

Figure 6. Transformation of 8.2 ppm TNT by the different strains in short-term experiments in BSM supplemented with glucose.

Effect of alkali hydrolysis of TNT

Cell growth data on alkali hydrolysate of TNT have also been presented in Figures 1 and 3. For these experiments, TNT-hydrolysate was prepared 24 hours ahead of time and the hydrolysate was added to the medium instead of TNT. These results show that hydrolysis of TNT results in higher growth of strains MC-1 and SH-2. However, there is no effect on growth of strains X and SH-1. It is clear that hydrolysis of TNT does not result in formation of any compound toxic to growth of any of the cell lines.

Effect of alkali hydrolysis on production of carbon dioxide was measured by adding known quantities of radioactive TNT to the TNT stock before it was added to the medium or before it was hydrolyzed. These experiments were conducted in radiometric flasks where carbon dioxide evolved was trapped in KOH solution. The KOH solution was removed from the traps regularly using a syringe, followed by addition of equal amount of fresh KOH. These solutions were used in a scintillation counter to count radioactivity in KOH traps. At the end of 30 days, the medium in the flasks was acidified to release dissolved carbon dioxide from solution. This carbon dioxide was also trapped and counted in a scintillation counter. The mineralization studies were conducted in parallel with the short and long-term growth and degradation studies and strain SH-1 was randomly chosen for this study.

Radioactivity was also measured in the biomass (solid-phase) and in the acidified medium (liquid phase) for the sake of making a balance of radioactivity in the system.

Table 2. Total ^{14}C activity measured different phases over 30 days of incubation of SH-1 in BSM containing either ^{14}C-TNT or alkali hydrolysate of ^{14}C-TNT when supplemented with 4 g L^{-1} glucose

Fate of ^{14}C %	^{14}C-TNT	Alkali hydrolysate of ^{14}C-TNT
In CO_2 (Mineralization)	0.15	0.44
In Biomass	4.88	6.90
In Solution	71.89	22.53
Missing	23.08	70.13
Total	100.00	100.00

Total radioactivity measured as carbon dioxide evolved, biomass, and compounds in solution has been presented in Table 2 as a percentage of activity added to medium at the start of the mineralization experiment. The percent mineralization increased three fold as a result of alkali hydrolysis, although it was still exceedingly small. Incorporation of radioactive TNT into biomass also increased slightly; the largest fraction of ^{14}C activity was measured in cell-free broth in both the cases. However, the biggest difference observed in the radioactivity balance was in the percentage of missing radioactivity. 23% and 70% of ^{14}C introduced was unaccounted for in the flasks containing TNT

and TNT-hydrolysate, respectively. Since such a large loss cannot be accounted for by the normal variations in scintillation counting, this is very likely due to formation of gaseous products that were not trapped by KOH. One such product might be methane but this was not measured.

Conclusions

All the four Gram-negative microbial strains (*Pseudomonas* sp. strain X, isolated in USAERDC from a TNT contaminated soil; *Pseudomonas putida* strain MC-1, isolated from a mixed culture enriched from a contaminated soil from Pardubice, Czech Republic using a mixture of mono- and di-nitrotoluenes; *Burkholderia cepacia* strain SH-1, isolated from the aqueous solution of TNT; *Pseudomonas aeruginosa* strain SH-2, also isolated from the aqueous solution of TNT) transformed TNT rapidly from 8.2 ppm to non-detect levels in BSM medium when glucose was present as a co-substrate. The cells were able to grow equally well or better in alkali hydrolysate of TNT than on TNT itself. The cell line SH-1 used for mineralization studies showed a three-fold improvement in the amount of ^{14}C-CO_2 production and a slight increase in assimilation of TNT-carbon in cell mass when grown on alkali hydrolysate. The main difference between TNT and TNT-hydrolysate was in the amount of ^{14}C-intermendiates in aqueous solution and in the unaccounted for radioactivity. This needs to be explored further.

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