

# TNT biotransformation potential of the clinical isolate of *Salmonella typhimurium* - potential ecological implications

## Abstract

Out of fifty-six isolates screened three bacterial strains enriched with TNT either as sole source of nitrogen (for *Salmonella typhimurium*, *Klebsiella pneumoniae*) or along with co-substrate (for *Acinetobacter baumannii*), have been carried out nitro group reduction under aerobic conditions. During studies, *S. typhimurium* found to have high potential (100% of 50 mg l<sup>-1</sup>) in removal of TNT, than *K. pneumoniae* (70% of 20 mg l<sup>-1</sup>) and *A. baumannii* (52% of 40 mg l<sup>-1</sup>, in presence of co-substrate). Therefore studies were focused on *S. typhimurium*, which had shown good growth, and protein contents, with disappearance of TNT, and concomitantly release of nitrite over the period of time. Removal of TNT was analyzed by HPLC, and nitrite liberation was consistently found coincided with TNT disappearance from the medium. As compared to earlier reports, 100% disappearance of TNT within 30 h by *S. typhimurium* is encouraging, and may indicate its potential in bioremediation of TNT. This is the first report on *S. typhimurium*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* for transformation of TNT with nitrite release into the medium.

**Key Words:** *Acinetobacter baumannii*, Bioremediation, *Klebsiella pneumoniae*, *Salmonella typhimurium*, TNT

## INTRODUCTION

TNT (2, 4, 6-trinitrotoluene) is commonly used explosive, and its contamination is of a great concern because of the toxicity and mutagenicity.<sup>[1]</sup> TNT concentrations at manufacturing, processing and disposal sites can be as high as 100 mg/l in water and 12000 mg/kg in soil. Numerous cases of munitions workers reportedly developed liver damage and anaemia owing to TNT exposure.<sup>[2]</sup> Bioremediation offers as attractive and economic way of removal of explosives from contaminated sites.<sup>[3]</sup> Number of bacteria and fungi are able to utilize TNT<sup>[4]</sup> as a nitrogen source by removing nitrogen under either aerobic<sup>[5]</sup> or anaerobic conditions.<sup>[4,6]</sup> Traditionally, most of the research on TNT bioremediation was focused on anaerobic bacteria and fungi, and only recently studies begun to appear wherein aerobic bacteria degrade TNT. Under many typical setting the rates of degradation are limited,

mainly due to the accumulation of intermediate metabolites, and their toxic effects on biological systems.<sup>[2]</sup>

The investigators from various places have isolated several organisms, which degrade explosive aerobically. Most notable among them are *Stenotrophomonas*,<sup>[7]</sup> *Rhodococcus*,<sup>[8,9]</sup> *Enterobacter*,<sup>[5]</sup> *Pseudomonas*,<sup>[10]</sup> *Mycobacterium*,<sup>[11]</sup> *Bacillus*,<sup>[2]</sup> *Serratia*<sup>[12]</sup> and *Staphylococcus*.<sup>[2]</sup> Halasz *et al.*<sup>[13]</sup> have reported detection of degradation of explosives by soil indigenous organisms.

A present study demonstrates the removal of TNT from culture media by locally isolated aerobic species viz. *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*, which were successfully tolerated, sustained, removed, and grown in TNT enriched media. These organisms are not previously reported for his type of activity, and may prove to have potential to transform TNT. The study presented is focused *S. typhimurium*, which removed 100% TNT.

## MATERIALS AND METHODS

### Chemicals, reagents, and media

The stock solution of TNT (provided by Defence Research and Development Organization, Government of India) prepared in sterile de-ionized water, was filter (0.22 µm, Satorius, Germany) sterilized and used as 100 mg/l. A range of TNT (1 through 100 mg/l) concentration was used to add into mineral medium as the sole source of nitrogen. The mineral me-

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dium with some modification was used for evaluation of tolerance and degradation of TNT, the constituents of which were D-glucose (5mM),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.02%),  $\text{K}_2\text{HPO}_4$  (0.7%),  $\text{KH}_2\text{PO}_4$  (0.2%), sodium acetate (0.1%), tri-sodium citrate (0.05%), and  $(\text{NH}_4)_2\text{SO}_4$  (0.1%). For biodegradation studies nitrogen deficient mineral medium was used wherein  $(\text{NH}_4)_2\text{SO}_4$  was replaced with TNT. All chemicals were of analytical grade (Sigma Chemical Co., St. Louis, MO, USA) and solvents HPLC grade (Glaxo-Qualigen or Thomas Baker, India). Other culture media were obtained from either Difco (Detroit, MI, USA) or HiMedia (Bombay, India) Laboratories.

### Isolation of bacterial strains, growth conditions and measurement

The random sample of soil and water were collected from the vicinity of ammunition plant, and its wastewater disposal stream meeting to river. In brief inoculum was prepared by suspending 1 g of soil or 1 ml of wastewater (collected from the vicinity of munitions plant) in 9 ml sterile distilled water, mixed thoroughly and allowed to settle down. The supernatant fluid was filtered through Whatman no. 1 filter and the filtrate was used as an inoculum. A loopful of the filtrate was streaked on nutrient agar (Difco) plates and incubated at an ambient temperature. The microbial colonies obtained were used to prepare secondary culture and identified according to standard methods described.<sup>[14,15]</sup> A single isolated colony from purified secondary culture plate was inoculated into 30 ml of nutrient broth to grow over night. One ml of this over night grown culture was then inoculated into 100 ml mineral medium containing TNT as nitrogen source in 500 ml flat bottom conical flask and incubated either keeping cultures stationary or on rotary shaker (400 rpm) at ambient temperature. The cell density of liquid culture was determined spectrophotometrically as optical density at 600 nm. The viable cell colonies were counted by serial dilutions plating on LB plates, whereas protein concentration estimated as described by French *et al.*<sup>[5]</sup> The incubation time for TNT experiments was up to 96 hr. The effect of 0.1% yeast extract (as growth enhancer) on initiation of growth of inoculated organism was also investigated. We isolated and screened 44 environmental, and 12 clinical isolates (laboratory strains originally collected for other clinical studies), for degradation activity of TNT. Abiotic cultures were performed for all the times as negative controls, and the cultures wherein DNT (Sigma) was limiting and sole source of nitrogen, as positive controls for the confirmation that these bacteria take aerobic path of transformation of TNT. Experiments were repeated minimum three times to reproduce the results, unless otherwise stated.

### HPLC monitoring for removal of TNT

The disappearance of TNT was monitored, and quantified by testing the culture supernatants (obtained after centrifugation of culture at 6000 rpm for 15 min.) using High Performance Liquid Chromatography (HPLC) during cultivation at

regular intervals. The HPLC analysis was performed with Perkin-Elmer system consisting of a biocompatible binary pump 250 and Lambda 3B UV/VIS spectrophotometer as detector. The Borwin version 1.21 (Jasco, Japan) software was used for data analysis. A  $\text{C}_{18}$  column (length 25cm, diameter 4mm (ID), and make VYDAC ( $\text{C}_{18}$ ), USA) was used to analyze supernatants, using 20 ml of sample injected for detection of TNT. An isocratic system was used for all the experiments. The mobile phase for TNT estimation was methanol: water (50:50 v/v), delivered at a flow rate of 1.0 ml/min. The TNT was monitored at 230 nm.

### Nitrite Assays

A demineralization of TNT in the form of release of nitrites during growth of organism was assayed colorimetrically as described previously.<sup>[5]</sup> In brief, to 1 ml sample aliquot, 0.4 ml N- (1-naphthyl) ethylenediamine-sulfonilamide solution containing 0.3 M sulfanilic acid and 0.5% N- (1-naphthyl) ethylenediamine-sulfanilamide dichloride were added. The reaction mixture was incubated for 20 min at room temperature and the OD at 543 nm was determined. Sodium nitrite was used as a standard.

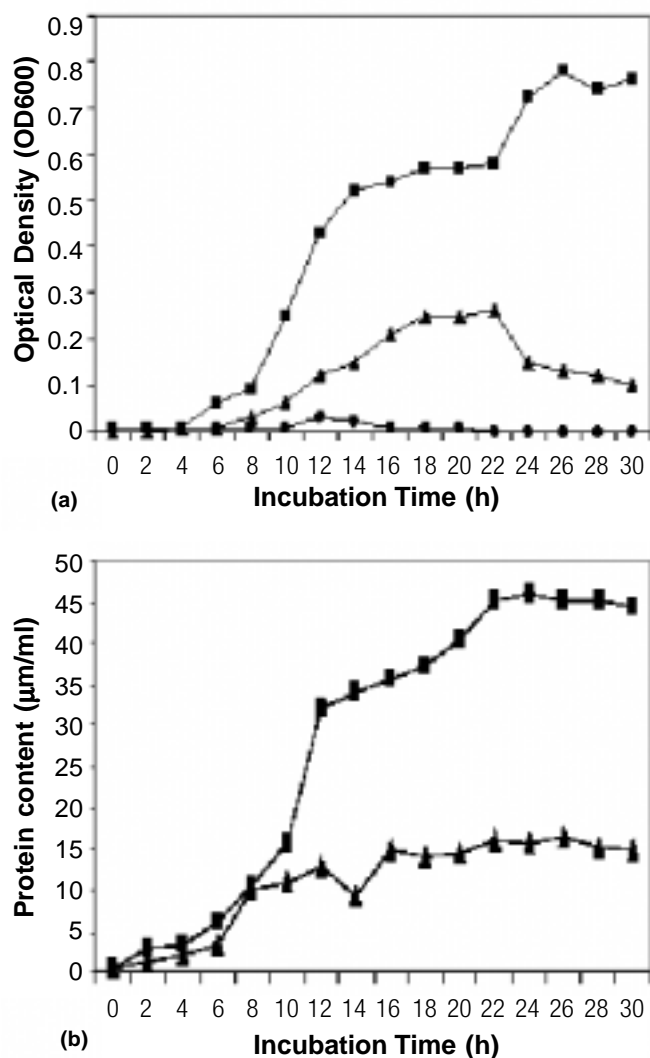
## RESULTS

### TNT as sole nitrogen source during growth of isolates

The organisms were inoculated in mineral medium containing either  $(\text{NH}_4)_2\text{SO}_4$  or TNT or DNT or no nitrogen source to observe their growth patterns and utilization of the nitrogen by aerobic pathway. The initial inoculi for all different organisms was about  $1.6 \times 10^6$  cells/l. Growth was monitored spectrometrically and measured as optical density at 600 nm ( $\text{OD}_{600}$ ). Parallel cultures of the same organisms were grown in known and easily available nitrogen source [ $(\text{NH}_4)_2\text{SO}_4$ ] or nitrogen deficient medium to compare the status of growth. Cultures grown in DNT were only for the purpose of monitoring its fate during incubation with bacteria under the similar conditions (that of TNT). In cultures containing variable initial concentrations of TNT, it is observed that TNT removal was associated with growth. The most promising growth was observed in two organisms, both of which were clinical isolates. The isolates of *S. typhimurium* and *K. pneumoniae* have removed respectively 100% (50 mg/l), and 70% (of 20 mg/l) of TNT very efficiently during growth, without any growth enhancer or growth promoting substances. Very few aerobic organisms able to use TNT as a sole nitrogen source have been reported and much less frequently about mineralization of this compound.<sup>[4]</sup> While testing the effect of 0.1% yeast extract (as co-substrate) on TNT biotransformation, we observed *A. baumannii* involved in significant transformation of TNT (52% of 40 mg/l), which it failed to do so in the absence of this co-substrate. These organisms could utilized DNT for their growth during cultivation, indicating that these

organisms take aerobic pathway (data not shown). None of these three species have earlier been reported to transform TNT. *S. typhimurium* has removed 100% of TNT (50 mg/l, highest among all tested strains), and therefore analysis of the results pertaining to this organism have been presented. Although *Acinetobacter johnsonii* and *Klebsiella* sp. have been reported to biotransform TNT (< 10 mg/l), our isolates are totally different species (*A. baumannii* and *K. pneumoniae*) which removed TNT > 10 mg/l.<sup>[16]</sup>

The growth and protein curves for *S. typhimurium* obtained during studies are shown in Figure 1a and 1b. Growth estimated by turbidity and proteins concentration was determined; in the presence of either TNT or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was pro-



**Figure 1:** The representative graphs showing evidence of growth of *S. typhimurium* in TNT (50 mg l<sup>-1</sup>) enrich and minimal mineral medium. (a) growth measured as optical density at 600 nm. (b) shows protein concentrations. The results shown are the mean of three independent experiments, and the error bar indicates standard error mean [ammonium sulphate (%), TNT (2%) or no nitrogen source (-)]

portional to the amount of nitrogen source present in the growth medium. Viable cell count was higher in growth medium containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> than TNT (data not shown). To monitor the influence of TNT and its metabolites on cell viability, *S. typhimurium* from the liquid cultures of variable time points during incubation was plated on LB. The analysis exhibited good growth, and maintained cell viability in the presence of TNT up to 36 hr, indicating that TNT or its metabolites do not inhibit growth, and have no toxic effect on this strain up to the given time points. It also indicated that the initial inoculum was sufficient to initiate growth in the presence of TNT. In enriched medium, the isolate observed to tolerate TNT up to 60 mg/l, and thereafter no tolerance, and rapid decreased in viable cell count, and inhibition of growth was observed. The optimal concentration of TNT was 50 mg/l. In Figure 1a, the lag phase appeared to increase up to 8 h, which corresponds with protein curve (Figure 1b). A change in color of medium was noted during cultivation. The medium started turning yellowish to slight orange around 4 h, and continued darkening until 8 h, around which the medium was pinkish red, which turned reddish brown when continued till 10 h. The color of the medium after 12 h again turned yellow. Once the growth was initiated, the growth rate, protein contents, and terminal cell densities were proportional. The results were consistent through out, and the experiments were repeated thrice to check their reproducibility.

#### Disappearance of TNT from culture medium

Data from HPLC analysis showed that TNT was removed from the mineral medium during growth (Figure 2a, 2b). Two prominent peaks were observed. The first one (retention time, ~3.4 min) corresponded with media composition (negative controls, which had only mineral medium), while the second peak (retention time, ~7.4 min) corresponded with TNT (when compared with mineral medium containing TNT). The area under the curve of TNT peak was reduced as incubation time advanced, but there was no appearance of any other new peaks at the above stated HPLC set up (Figure 2b). There was a complete disappearance of TNT peak during course of 30 h of incubation of culture. Figure 2b represents an overlay of the result of HPLC analysis of disappearance of TNT at various time points (0, 2, 10, 18, 24, 30h) during *S. typhimurium* cultivation in mineral medium.

#### Nitrite release from the TNT

*S. typhimurium* transformed TNT from mineral medium over 72% in 12 h, and >95% in 18h, and 100% in 30h (Figure 2a, 2b). Nitrite assays were performed to detect the release of -NO<sub>2</sub> group from TNT, a positive evidence of TNT demineralization. The results of nitrite assays are presented graphically in Figure 2c. The findings of nitrite release corresponded to disappearance of TNT from culture media with incubation time. The absence of nitrite in *S. typhimurium* cultures with-

out TNT, and in abiotic controls demonstrated that the presence of nitrite in medium was a result of bacterial transformation of TNT.

## DISCUSSION

Enrichment under nitrogen limiting condition facilitates the selection of bacterial isolates that release nitrite or ammonia by degradation of substrate such as TNT. *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*, all are opportunistic, and grow under variable growth conditions. Surprisingly the isolates which tolerated, and grown in TNT containing medium, utilized TNT without

use of any growth stimulant or enhancer, were from clinical specimens. The only clinical isolate of *A. baumannii* showed efficient biotransformation exclusively in the presence of 0.1% yeast extract. These findings are in agreement with earlier reports.<sup>[17,6]</sup> All environmental isolates other than two (which degraded <5 mg/l) that required growth enhancer (data not shown) for TNT transformation failed to transform TNT in either conditions. This study included focus on *S. typhimurium* because of its better ability to remove TNT by consuming it as sole nitrogen source during growth. The growth and viability was unaffected by 20 mg (for *K. pneumoniae*) or 40 mg (for *A. baumannii*) or 50 mg (for *S. typhimurium*) of TNT per liter. The concentrations of TNT beyond these points were inhibitory to isolates. An increase in bacterial growth and protein content is a positive evidence of TNT utilization from enriched medium (Figure 1).

During degradation process of TNT by *S. typhimurium*, a pinkish red to brown color appeared, which gradually darkened around 10 h of incubation, but disappeared after further incubation. From previous reports of TNT degradation, the hydride-Meisenheimer complex of TNT ( $H^-$ -TNT) showed a red-brown color by aerobic bacteria, and some of the fungi.<sup>[11,18-20]</sup> Vorbeck *et al.*<sup>[20]</sup> also reported the reduction of TNT to  $H^-$ -TNT, which subsequently gave yellow product by whole cells of *Rhodococcus erythropolis* and *Mycobacterium* sp. The yellow products were identified as the protonated and dissociated forms of the C-3, C-5 dihydride-Meisenheimer complex of TNT (2H-TNT). In our studies both these color changes were noted. Nitroreductase-like activity is not surprising, since the aromatic nitro groups of TNT are extremely susceptible to reduction and can be reduced by a variety of

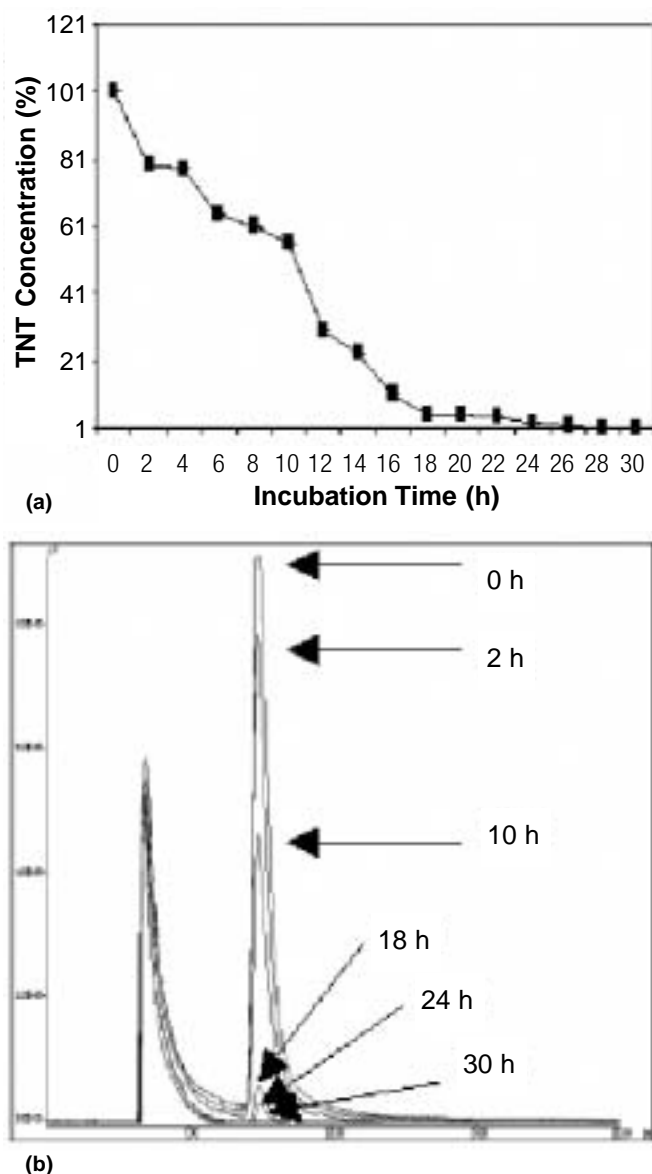


Figure 2: A graphical presentation of evidence, showing utilization of TNT as sole and limiting source of nitrogen during growth of *S. typhimurium* in rich mineral medium. (a) is graphical presentation of HPLC data, showing a percentage of TNT presented in mineral medium during growth at given time points. (b) is HPLC graphical overlay of crucial time points during growth. A second peak (retention time, 7.4 min) corresponds with TNT, while the first with mineral medium (retention time, 3.4 min) at  $\lambda_{max}$  230 nm. (c) is a graph shows release of nitrite during TNT degradation by this strain. There was a high amount of nitrite present in mineral medium during growth. (Error bar is standard error mean)

oxidoreductases.<sup>[5]</sup> Nitrite and Dinitrotoluenes (DNTs) are the products of transformation of dihydride-Meisenheimer complex.<sup>[19]</sup> In our studies we proposed that a H-TNT like metabolite might have formed as a red-brown colored intermediate products, which probably led to a yellowish 2H-TNT-like metabolite, during TNT degradation by *S. typhimurium*, with a concomitant release of nitrite and disappearance of TNT from mineral medium (Figure 2), suggests the involvement of denitration pathway.

The utilization of TNT as sole and limiting source of nitrogen was observed through HPLC data analysis. Figure 2b is an overlay of selected incubation time points' HPLC peaks for TNT (retention time 7.4 min), which got reduced as incubation time for culture advanced. By the end of 12 h about 72% TNT, and 18h >95% TNT was transformed, and at 30 h of incubation almost 100% TNT was removed from culture medium (Figure 2a). The disappearance of TNT and concurrently appearance of nitrite is highly indicative of denitration. Under these experimental conditions there was no appearance of any new peaks corresponding to intermediate metabolites. DNT was also efficiently degraded by these organisms under similar conditions, is indicative of possible involvement of mineralization pathway during TNT degradation, and one of the intermediates could be DNT. Although there was growth and increasing concentrations of protein contents, the amount nitrite release was increasing over the period of time during incubation, shows that the nitrite release was much higher than proportionate depletion (Figure 2c).

Several researchers have reported TNT degradation by aerobic bacteria, which use denitration pathway<sup>[2,4,20,21]</sup> and showed that TNT denitration is profitable to bacteria.<sup>[22,23]</sup> The level of denitration was substantially higher in static cultures than in orbital shaken cultures (data not shown). In fact, orbital agitation of TNT enriched culture was detrimental to TNT degradation, in contrary to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-supplemented cultures. This showed that static culturing is required for TNT transformation to H-TNT to nitrite in our set up. The formation of red-brown color compound was also not seen during orbital agitation supports this explanation. Although we did not estimate the accumulated compounds of TNT or its intermediate products associated with the whole cells of *S. typhimurium*, a release of high amounts nitrite, and corresponding increased in bacterial growth as well as their protein contents are sufficient to correlate the use of TNT as sole N source under limiting conditions.

Although the intermediate products of biotransformation have not been defined, an increase in bacterial growth and protein contents, and the release and accumulation of nitrite in mineral medium as TNT is degraded, is a strong indication that this organism has an enzymatic machinery to degrade TNT, and utilize the nitrogen, released out of denitration. In clas-

sical pathway most aerobic bacteria follow that degrade TNT, dihydride-Meisenheimer complex (2H-TNT) + hydride-Meisenheimer complex (H-TNT) + protonated 2H-TNT (2H-TNT.H<sup>+</sup>) are formed, which subsequently undergo 2e<sup>-</sup> reduction followed by re-aromatization to form DNTs with concomitant release of nitrite. The isomers of DNTs undergo either hydroxylation or dioxygenation to further mineralize into methylcatechol and nitrite release. The methylcatechol is suitable for further ring cleavage by aerobes and utilize as C and N source during growth. Vorbeck *et al.*<sup>[20]</sup> showed that the ability to utilize TNT diminished with successive subculturing, suggesting sustained use of TNT as N source might not occur. But during our studies we noticed that the TNT degrading ability is sustained for more than 12 successive passages, as well as prolonged storage at -80°C (for 2 year), did not affect this ability. The growth and viability of *S. typhimurium* was also not affected by intermediate metabolites during TNT degradation, suggests that they have been rapidly cleaved further into non-toxic products during growth of this strain. Therefore this strain may prove to have good TNT transforming potential. To best of our knowledge this is the first report on *Salmonella* species transforming 100% (of 50 mg/l) TNT aerobically, and its possible usefulness in the bioremediation of TNT.

## CONCLUSION

TNT is an environmental toxin, which needs to be removed from contaminated sites to prevent its toxicity on ecosystem. Bioremediation is an effective and eco-friendly method of removal of TNT from natural occupational and environmental sites. Bacterial degradation of TNT by aerobic method is easy to maintain than anaerobic system of cultivation. *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* have not been reported earlier for using TNT as sole source of nitrogen with concomitant release of nitrites and therefore it is logical that these isolates may be having potential for TNT degradation.

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