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Prevalence and screening of 2,4,6-Trinitrotoluene (TNT) degrading bacteria from nitroaromatics contaminated soil

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ABSTRACT

Explosive chemicals are energetic substance, which are essential for security and defence of the nation and hence their production continues. Among explosive TNT is commonly used explosive throughout the world, when their residues and transformed products released into environment it possess toxic hazards to both terrestrial and aquatic life. So the effective remediation of TNT is very important. In the present investigation potential TNT degrading bacterial consortia was isolated from nitroaromatic contaminated sites and it was identified as *Bacillus mannanilyticus* and *Staphylococcus saprophyticus* based on its 16S rRNA gene sequence. TNT degradation was monitored in shake flask assay, within a day TNT level was reduced 250ppm to 81ppm, thereafter gradually decrease was noted and complete degradation was resulted in 6th day of the study, meanwhile gradual increase of bacterial growth was observed. During the degradation of TNT level of nitrite, nitrate and ammonia was recorded. In initial day nitrite level was high and day by day it was reduced with the increase of ammonia level.

KEYWORDS: Isolation, Screening, TNT degradation, Bacterial consortium.

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INTRODUCTION

TNT is most commonly used explosive chemical in military as well as in private sectors like mining industries. TNT contaminated site includes manufacturing site, military shooting range and storage areas. Every year nearly 1000 tons of TNT was produced globally and 2 million L of TNT and nitroaromatic contaminated water was discarded every day[1]. TNT is highly toxic to human, animals as well as in environment and it is listed as possible human carcinogen. Remediation of TNT contaminated sites is crucial to reduce adverse effects on living organisms and environment[2]. Bioremediation offers a environmental friendly alternative approach and less expensive way to remove pollutants than engineering based technologies[3,4]. Number of bacteria and fungi are used to remove TNT and they can able to utilize TNT as a sole nitrogen source for their growth by removing nitrogen molecules under aerobic[5,6,7] and anaerobic conditions[8,9]. Synergistic participation of bacterial consortium is required for simultaneous partial degradation of several nitroaromatic compounds. For bioremediation process there is no report instances of *Bacillus mannanilyticus* and *Staphylococcus saprophyticus* isolated from nitroaromatic contaminated site and used for TNT biodegradation. Hence, the purpose of current study is to isolate potent TNT degrading bacterial consortia from nitroaromatic contaminated sites.

MATERIALS AND METHODS

Isolation and Identification:

There were 9 different soil samples were collected from nitroaromatic contaminated sites located in Salem (11.6643° N, 78.1460° E) and Sivakasi (9.4533° N, 77.8024° E) region of Tamil Nadu, India. Samples were collected at the depth of 2 - 10 cm from the surface using sterile polythene bag and transported to the laboratory in an ice box for further analysis. Soil texture was classified into silt loam with pH 6.0 - 7.0 in Salem region sample and deep red with pH of 6.5 to 8 in Sivakasi. The TNT concentration was not detectable. Isolation of bacterial strain was done by spread plate method. Morphologically different colonies were picked and purified by quadrant streaking. All bacterial strains were identified by their basic physiology and biochemical characteristics. The physiological observation includes colony colour, shape, size and elevations. The results were compared with Bergey's Manual of Determinative Bacteriology[10].

Screening of nitrate reducers:

In TNT there are 3 nitrogen molecules bonded with toluene ring. For biodegradation of TNT the main aim is to remove nitrogen molecules. So nitrate reduction test was done for the screening of bacterial strains which have an ability to reduce nitrogen molecules with the presence of nitrate

reductase enzyme. Potassium nitrate broth was prepared in test tube and all bacterial isolates were inoculated and incubated at 37°C for 48 hours. After incubation, 0.5ml of Nessler's reagent was added to the tube and results were noted based on the formation of deep orange or brown colour and intensity.

Screening of toluene tolerance:

Toluene is the important primary molecule of TNT, so breakdown of toluene was necessary in the degradation of TNT. Toluene tolerance assay was done in mineral salt medium (MSM) incorporated with various concentrations of toluene such as 200, 400, 600, 800 and 1000ppm. Bacterial isolates were inoculated (1% of 1 OD) in various toluene concentrations and incubated at 37°C for 24 hours. Then bacterial growth rate was measured using UV visible spectrophotometer (Cyberlab UV-100). Based on highest growth rate bacterial isolates were selected for further study.

Antagonistic study for consortium formulation:

Synergistic activity of selected isolates was important for the formation of bacterial consortium. Screened bacterial isolates were subjected to antagonistic study by well diffusion assay. Nutrient agar (NA) was prepared and poured into sterile petridish. Selected bacterial isolates swabbed on the agar plates using sterile cotton swab and 6 mm wells were punched aseptically and filled with 60 µL of selected isolates. The plates were incubated at 30°C and clear inhibition zone around the wells were observed after 24 hours. The bacterial strains without antagonistic activity were selected for consortium formulation.

Molecular identification:

Molecular identification potent isolates SK16 and SV1 were determined using 16S rRNA gene sequencing. From the above screening and antagonistic test results SK16 and SV1 were selected as efficient TNT degrader and the species level identification was carried out by 16S rRNA gene sequencing. The 16S rRNA gene of selected bacterial stains SK16 and SV1 were amplified and sequenced by following condition. Add 5µL of template DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer 518F (CCAGCAGCCGCGGTAATACG) and Reverse Primer 800R (TACCAGGGTATCTAATCC), 5 µL of deionized water, and 12 µL of Taq Master Mix) and subjected to PCR following thermal cycling conditions, initial denaturation 95°C for 2 min, denaturation 95°C for 30 sec, annealing 55°C for 30 sec, extension 72°C for 2 min and final extension 72°C for 10 min, for total of 30 cycles. Sequencing of both PCR amplified fragment was subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The results were compared with the National Centre for Biotechnology Information (NCBI) public database using

BLAST similarity search tool for species identification. A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) 4.0 software.

Monitoring of TNT biodegradation:

In order to identify the efficiency of TNT degradation by bacterial consortium was investigated in aqueous medium. *Bacillus* SK16 and *Staphylococcus* SV1 was inoculated in mineral salt medium (units are in g/L) KH_2PO_4-0.1; K_2HPO_4-1; NH_4Cl-0.5; $CaCl_2$-0.004; $MgSO_4$-0.1; Na_2SiO_3-0.05; 250 ppm of TNT; pH 7.0 ± 0.2 and incubated at 35°C with agitation speed of 120 rpm. The amount of TNT in the medium was determined by high performance liquid chromatography (HPLC) according to US Environmental Protection Agency (USEPA) method 8330A. Aliquots were collected periodically from the each flask and TNT, metabolites and their concentrations were determined using HPLC (Shimadzu LC-20AP) as well as by UV-Vis spectrophotometer (Model: Cyberlab UV-100, USA) determination of nitrite, nitrate and ammonia concentration[7,11]. HPLC grade acetonitrile was blended with the culture supernatant of equal volume and vortexed for 5 min and the aliquot was filtered through membrane filter with the pore size of 0.2µm to eliminate the large size particles. The separation was conducted at 35°C using a C18 column, the mobile phase was methanol and water with the ratio of 50:50 and flow rate was adjusted to 1 ml/min and the sample injection volume was 20µL. Detection was performed by a photo diode array detector (PDA).

RESULTS AND DISCUSSION

Bacterial genera in nitroaromatics contaminated soil:

A total of 118 strains of bacteria were isolated from 9 different soil samples and they were identified according to the Bergey's Manual of Determinative Bacteriology. *Bacillus* was predominantly present, followed by *Clostridium*, *Pseudomonas*, *Micrococcus*, *Enterococcus*, *Cornyebacterium*, *Serratia*, *Alcaligens*, *Rhodococcus*, *Staphylococcus*, *E.coli*, *Flavobacterium* and *Sphingomonas* (Fig.1). The isolated colonies were restreaked in nutrient agar plates and stored at 4°C for further studies.

Screening of nitrate reducers and toluene tolerance strains:

All the bacterial strains were subjected to nitrate reduction test. Based on colour change among 118 bacterial isolates 5 were noted as high nitrate reducer (+++), 9 were noted as moderate nitrate reducer (++), 37 were noted as low nitrate reducer (+) and 67 were noted as non nitrate reducer (-) (Fig. 2a). In toluene tolerance test at toluene concnetration of 200 and 400 ppm all the

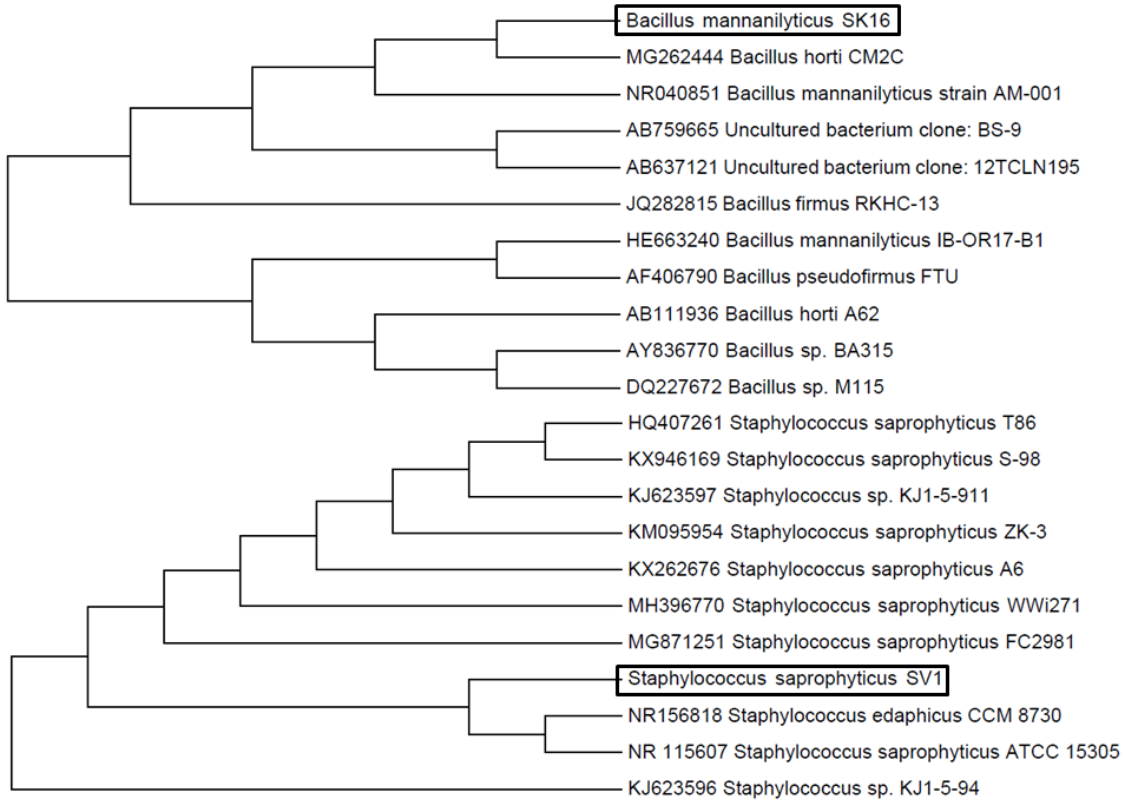
bacterial strains were grown, if the concentration was gradually increased to 600, 800 and 1000ppm the bacterial growth was reduced sharply and some bacterial strains were not able to grown at higher concentration of toluene. Out of 118, only 6 bacterial strains have the ability to tolerate the higher concentration of toluene (Fig. 2b). From the screening SI1, SK16, SS6, SW5, SV1 and SW12 were selected for further research.

Antagonistic study:

From the above screening study 6 strains were selected and subjected to antagonistic assay. For making bacterial consortium the synergistic activity of all they bacterial strains were important. Table 1 represent the antagonistic assay results. SI1, SS6, SW12 exhibit antagonistic activity against other bacterial strains (Fig.3). Based on the antagonistic study results, bacterial consortium was formulated using SK16 and SV1 and these two strains were subjected to species level identification and further TNT degradation study.

Molecular identification:

The bacterial strains SK16 and SV1 were selected as potent TNT degrader and subjected to molecular identification. The sequence of 16S rRNA genes of these bacterial strains were compared with NCBI public database. The strain SK16 sequence was 99% similar to *Bacillus mannanilyticus* and strain SV1 sequence was 99% similar to *Staphylococcus saprophyticus.* The nucleotide sequence was deposited in the Gene bank database. The nucleotide sequence accession numbers of the 16S rRNA genes of *Bacillus mannanilyticus* SK16 was MH244368 and *Staphylococcus saprophyticus* SV1 was MH244426 respectively.



Monitoring of TNT biodegradation:

The TNT level in the medium decreased sharply within a day from 250ppm to 81ppm and gradually decreased day by day to undetectable at 6th day (Fig.4), meanwhile in the same way bacterial growth was increased gradually (Fig.5). Correlation between increasing the biomass concentration and decreasing the TNT level in medium proves that the bacterial strains utilize TNT as nutrient for their growth. This result is agreement with those who obtained previously by many researchers[12,13]. During the TNT removal by bacterial consortium a visible colour change was observed in the culture medium. The colour of the medium was changed to brown at the third day of the incubation and it turned to deep red or deep brown colour after 4th day of study. Previous studies reported biotransformation of TNT by bacteria via elimination of nitro groups, were responsible for colour change of these medium[14,15]. The amount of TNT present into the bacterial cell was measured by the amount of nitrogenous compound present in the medium by bacterial consortium. The major TNT degradation intermediates including nitrite, nitrate and ammonia produced via denigration pathway[16]. The detection nitrite, nitrate and ammonia in the culture medium was the direct indicator of TNT catabolism. NO_2 and NO_3 produced probably at the initial stage of TNT biotransformation and its subsequently converted to other products like NH_4. The highest nitrite level was 0.426mg/L. The nitrite level was sharply increased in the first day, thereafter the level of nitrite was gradually

decreased to 0.022mg/L on the final day of the study its due to the formation of amino groups from nitro groups. The ammonia level gradually increased due to the breakdown of nitrite and nitrate.

CONCLUSION

This study shows bacterial consortium of *Bacillus mannanilyticus* SK16 and *Staphylococcus saprophyticus* SV1 could be the promising agent for the effective transformation of TNT in aqueous medium. Further studies are required to gain detailed understanding of enzymatic activity of bacterial consortium, optimum conditions required for the enhancement of TNT degradation and field application.

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Table 1.Antagonistic test of selected bacterial strains

Strains in well	Zone of inhibition (mm)					
	SI1	**SK16**	**SS6**	**SW5**	**SV1**	**SW12**
SI1		+ (12)	-	+ (15)	+(9)	-
SK16	-		-	-	-	-
SS6	-	-		+ (5)	+ (8)	+ (7)
SW5	-	-	-		-	-
SV1	-	-	-	-		-
SW12	-	+ (7)	-	+ (8)	+ (8)	

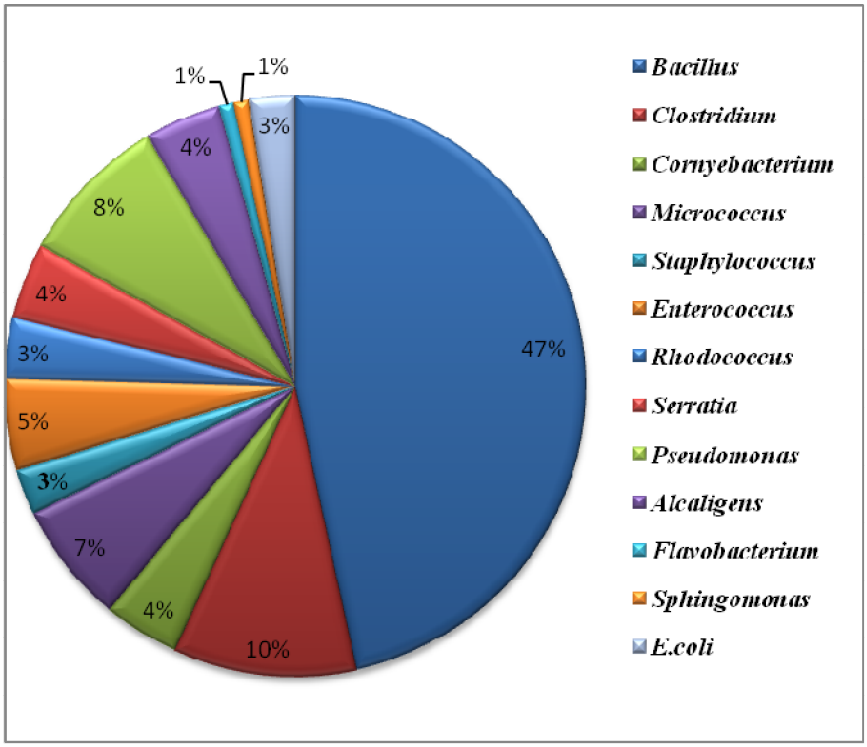

Fig. 1 Bacterial diversity of nitroaromatic contaminated soil

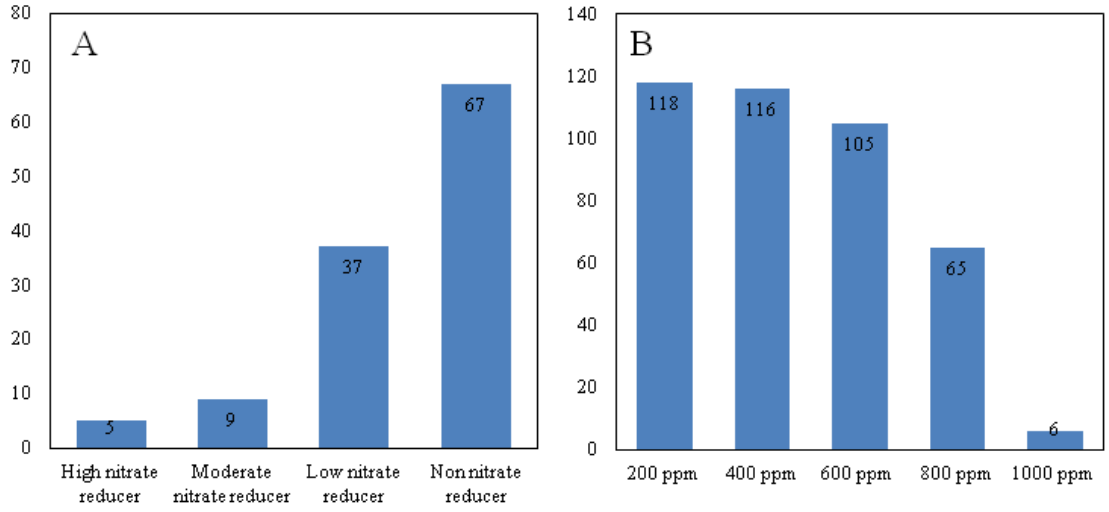

Fig. 2 Screening of TNT degraders. A. Nitrate reducers B. Toluene tolerance

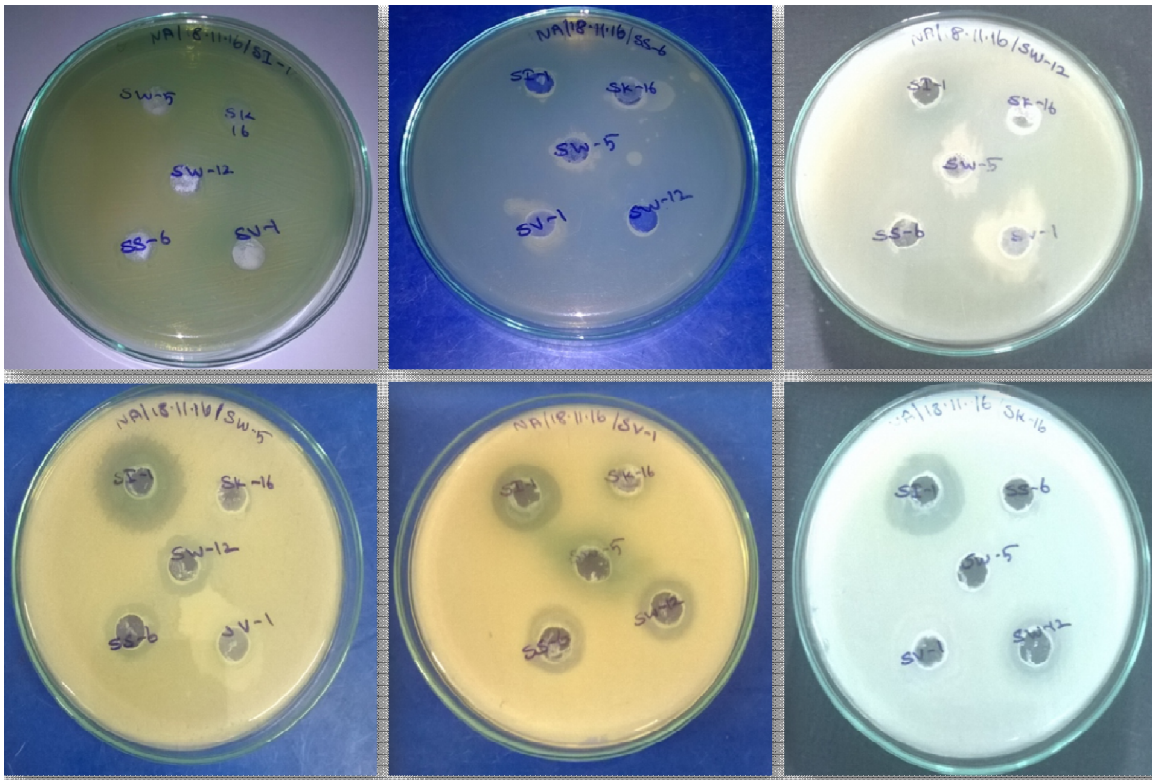

Fig. 3 Antagonistic test of selected bacterial strains

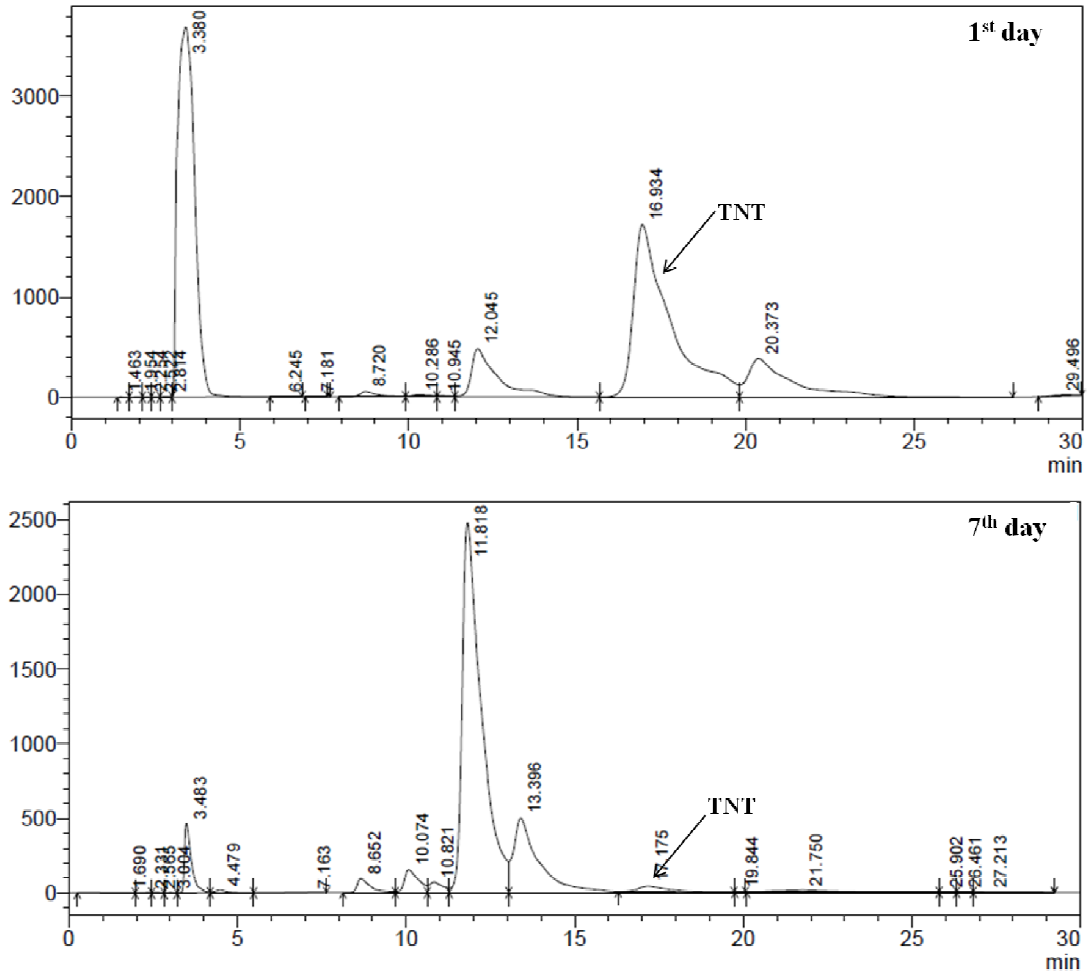

Fig. 4 HPLC chromatogram of TNT during biodegradation

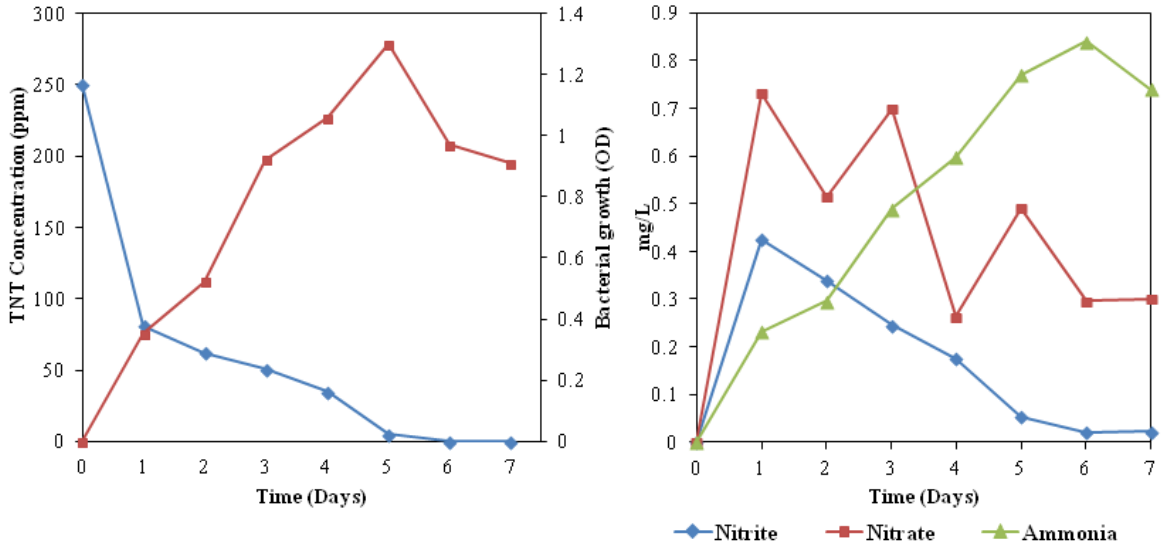

Fig. 5 Degradation of TNT in aqueous medium using bacterial consortium