



Novel *Bacillus* Consortium for Degradation of 2,4- Dinitrotoluene: A Xenobiotic Compound

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Authors' contributions

This work was carried out in collaboration between both authors. Author MSS managed the literature searches and wrote the first draft of the manuscript. Author RS designed the study, wrote the protocol, performed the analysis and supervised the subsequent drafts of the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/25837

Editor(s):

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 - (2) Ukiwe Lugard, Federal University of Technology, Owerri, Nigeria.
- Complete Peer review History: <http://sciencedomain.org/review-history/15146>

Original Research Article

Received 22nd March 2016
Accepted 9th June 2016
Published 24th June 2016

ABSTRACT

The xenobiotic compound 2,4-Dinitrotoluene (DNT) is used in the production of explosives (2,4,6-Trinitrotoluene, TNT), polyurethane/dyes, and in smokeless gunpowder. The cleanup of these compounds has gained much attention in the last decades due to hazardous nature of these compounds. Numerous bacterial strains capable of growing on DNT as the sole source of carbon, nitrogen and energy have been isolated by various scientists. Attempts to degrade DNT at high concentrations have never been found successful. The present study was conducted in Amity Institute of Microbial Biotechnology, Amity University between June 2010 and July 2011. About 18 bacterial cultures were isolated from the contaminated sites in the presence of 0.001% (w/v) 2,4-DNT. Isolated strains were further screened on the basis of their tolerance towards 2,4-DNT by growing them in the presence of 0.001% to 0.03% (w/v) 2,4-DNT. Out of 18 strains, eight tolerated varying concentration of 2,4-DNT and were mixed in different permutation & combination for preparation of microbial consortia. The best consortium (No.4 with strains RSE165, RSA32, RSB80 and RSD127) was selected and subjected to molecular characterization. Bacterial strains

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used in this study were identified as *Bacillus subtilis* RSE165 (NCBI accession no. JQ887982), *Bacillus megaterium* RSA32 (KR051485), *Bacillus cereus* RSB80 (JQ040533) and *Bacillus flexus* RSD127 (KR051486). The analysis of the 2,4-DNT degradation capabilities of the best four individual strains and their consortium by GC analysis shows that the spectral peak of 2,4-DNT is completely replaced by three small peaks which indicate its utilization and degradation by the bacterial strains as well as by their consortium.

Keywords: Dinitrotoluene compounds; bacillus; bioremediation; consortium.

1. INTRODUCTION

The xenobiotic compound 2,4-Dinitrotoluene (DNT) is used in the production of the explosives for which it serves as a gelatinizing and waterproofing agent [1,2]. It is also utilized as an intermediate in the synthesis of polyurethane/dyes and smokeless gunpowder [3,4]. Populations residing near hazardous waste sites are exposed to DNT (>10,000 µg/L in potable groundwater and ~100 mg/kg in soil) [5,6]. International Agency for Research on Cancer (IARC) has classified 2, 4- and 2,6-DNT as possibly carcinogenic to humans [7-9]. Due to its widespread environmental occurrence, it is listed as a priority pollutant by the U.S. Environmental Protection Agency and its mineralization along with other aromatics has been studied significantly [10].

No physical or chemical method has been reported till date to remove 2,4-DNT from contaminated sites. Bioremediation is a process of cleaning the harmful wastes with microbes or plants; it is one of the most common eco-friendly methods of clearing the pollutants [11]. Specific bacteria capable of using mono- and dinitroaromatic compounds as growth substrates have been detected at explosive-contaminated sites which can degrade 2,4-DNT [12]. Several scientists have isolated and characterized different bacterial strains which have the capability to degrade 2,4-DNT [13-17] and degradation pathway of 2,4-DNT has also been extensively studied biochemically and genetically in *Burkholderia* sp. strain DNT [18].

In nature, microorganisms do not live in isolation in a certain space and time, but are present as highly interactive communities called consortia. Consortia are defined by their diversity and seemingly coordinated effort to perform physiological functions such as chemical biotransformation and conduction of degradative pathways [19,20]. Microbial consortia have ability to perform functions in single step which

otherwise single bacterium either performs in multiple steps or would not be able to complete the process. This enables the consortia to perform complicated functions [21].

As single strain(s) are not able to accomplish the complete mineralization, the need of the hour is to use consortia for higher degree of degradation of 2,4-DNT. Two consortia have been reported that can degrade 2,4-DNT as their sole nitrogen source. One consortium consisted of *Variovorax paradoxus* VM685, *Pseudomonas* sp. VM908, *Pseudomonas marginalis* VM683, *Pseudomonas aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 and *P. viridiflava* VM907 was able to degrade 50 mg/L of 2,4-DNT [22].

In spite of the recent reports from different laboratories suggesting the degradation of 2,4-DNT using single bacterial species, to our knowledge there is no information on the mineralization of 2,4-DNT at higher concentration (0.3 mg/mL, maximum solubility of 2,4-DNT is 0.3 mg/mL) using microbial consortia. The present study investigates the degradation of 2,4-DNT using microbial consortia consisting of four different *Bacillus* species.

2. MATERIALS AND METHODS

2.1 Isolation of Microorganisms

Different hydrocarbon contaminated soil samples were collected from New Delhi and Noida, U.P. India. Soil sample were serially diluted and inoculated in nutrient broth in the presence of 2,4-DNT (0.001%) and incubated at 37°C under shaking (180 rpm) for 48 hrs. All the experiments were carried in duplicate. After two days of incubation, 0.1% inoculum (4.2×10^5 CFU/mL) was inoculated in nutrient agar plates and incubated overnight at 37°C. The strains showing growth were selected for further studies. Bacterial colonies were purified by repeated streaking on agar plates and pure bacterial

strains were maintained on nutrient agar slants at 4°C.

2.2 Media and Culture Conditions

Inoculums of the bacterial strains were prepared in nutrient broth (pH 7.5) by overnight incubation at 37°C and 180 rpm. Overnight grown bacterial culture 0.1% inoculum (4.2×10^5 CFU/mL) was used to inoculate 20 mL culture media overlaid with 0.001% w/v of 2,4-DNT in 100 mL sealed serum bottles. Inoculated serum bottles were incubated at 37°C with constant shaking at 180 rpm. The bacterial cultures without 2,4-DNT and un-inoculated media supplemented with 2,4-DNT were used as control under similar condition.

2.3 Determination of Tolerance Limit and Growth of Bacteria in Presence of 2,4-DNT

To determine the level of 2,4-DNT tolerance, 1% of the overnight grown and enriched bacterial cultures were inoculated in 20 mL nutrient broth supplemented with 0.001% to 0.03% w/v 2,4-DNT in 100 mL screw cap flasks. Inoculated flasks were incubated at 37°C under shaking (180 rpm) for 48 hrs. Bacterial growth was measured at 660 nm using UV-Vis spectrophotometer.

2.4 Preparation of the Consortium

To prepare an effective microbial consortium, bacterial cultures must be compatible with each other in order to concomitantly produce all the enzymes required for the degradation of 2,4-DNT. Different microbial consortia were prepared by inoculating four combinations of selected bacterial strains in 20 mL of nutrient broth. Eight different consortia were prepared and incubated overnight at 37°C and 120 rpm. The microbial consortium was treated with Tween-20 (0.001%). The Tween-20 prevents formation of aggregation-based artifacts and aligns themselves in their original arrangement. Individual bacterial consortium was selected on the basis of its maximum degradation capability.

2.5 Identification of the Consortium

Bacterial strains used for the preparation of consortium were isolated and characterized using morphological, biochemical characteristics and by 16S rRNA gene sequence analysis. The DNA was isolated using phenol-chloroform

procedure [23]. Isolated DNA was amplified using two sets of universal primers F [5' AGAGTTTGATCMTGGCTCAG 3'] and R [5' TACGGYTACCTTGTTACGACTT 3'] in 50 μ L reaction using PCR Master Mix (Fermentas Life Sciences, USA) in an automated thermocycler (Eppendorf AG 22331) with the following PCR Cycles: Initial denaturation at 94°C for 5 min followed by 40 cycles including denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute and final extension at 72°C for 5 minute. The PCR products along with 10kb DNA ladder (Merck millipore, India) were run on 1% agarose gel electrophoresis at 50 V for 2 hrs in 1x-Tris-acetate EDTA (TAE) buffer and visualized in UV transilluminator 2000 (BioRad). The amplified PCR product was purified using Qiagen DNA purification kit as per the manufacturer instructions and protocol.

The purified PCR product was sent for 16S rDNA sequencing (Patron Life Sciences, Ghaziabad). The obtained sequence was subjected to nucleotide blast (blastn) at NCBI against the nucleotide database for the identification of bacterial strain. CLUSTAL Omega [24] was used for the multiple sequence alignments of partial 16S rDNA gene sequence. Phylogenetic tree was constructed using neighbor joining method through Neighbor (DNADIST) from PHYLIP 3.6 package and viewed using Tree View version 1.6.6 [25]. Bootstrap analysis was performed for the evaluation of phylogeny using 1000 iterations.

2.6 Measurement of 2, 4-DNT Degradation

The individual bacterial strains and their consortium were analyzed to determine rate of 2,4-DNT degradation by GC methods. Enriched bacterial consortium (2%) and each individual strains were inoculated into 20 mL of nutrient broth overlaid with 0.1% w/v of 2,4-DNT and incubated for 48 hrs at 37°C under 180 rpm. Degradation of 2,4-DNT was monitored by gas chromatography (Clarus500_APL/INS/730 analysis). The bacterial strains in absence of 2,4-DNT and uninoculated media enriched with 2,4-DNT were used as control under similar condition. Experiments were carried out in duplicate.

3. RESULTS AND DISCUSSION

About 18 bacterial cultures were isolated from the contaminated sites in the presence of

0.001 % (w/v) 2,4-DNT. Isolated strains were further screened on the basis of their tolerance towards 2,4-DNT by growing them in the presence of 0.001% to 0.03% (w/v) 2,4-DNT. The change in color of Nutrient media after 24 hrs of bacterial growth in presence of 2,4-DNT was observed which could be related to the degree of biodegradation of 2,4-DNT (Fig. 1).



**Fig. 1. A- Nutrient media with 2,4-Dinitrotoulene (0.3 mg/mL)
B- Nutrient media with bacterial consortium and 2,4-Dinitrotoulene (0.3 mg/mL)
C- Nutrient media with consortium without 2,4-Dinitrotoulene (0.3 mg/mL)**

Out of 18 strains, eight tolerated varying concentration of 2,4-DNT (0.001% to 0.03% w/v). These strains were further tested for their growth at higher concentration of 2,4-DNT (Table 1). During growth in the presence of 0.001% to 0.002% 2,4-DNT, all the strains exhibited high absorbance at 620 nm indicating microbial growth in the initial 8 hrs of incubation. However in the presence of 0.01% to 0.03% (w/v) 2,4-DNT, maximum growth was observed during 8-24 hrs of incubation.

Total eight 2,4-DNT tolerant bacterial strains were mixed in different permutation & combination for preparation of microbial consortia. Table 2 shows eight bacterial consortia made by these strains. Consortium no-4 with 4 bacterial strains (RSE165, RSA32, RSB80 and RSD127) exhibited good growth in the presence of 2,4-DNT (0.001-0.3 mg/mL) as compared to other consortia. Fig. 2 represents the growth of best four strains RSE165, RSA32, RSB80, RSD127 and their consortium in the presence of varied concentrations of 2,4-DNT.

Different scientists have reported the preference of selection of consortia as compared to single

strains with respect to biodegradation of several nitroaromatic compounds [13,26]. We have also developed 2,4-DNT degrading consortia because the results so far have suggested that nature does not select a single organism that is able to degrade the compound. The interaction between or among the microorganisms with diverse degradative properties is the key to solve the problem of pollution due to xenobiotic compounds. Interspecies metabolic interactions can be proposed for our consortium.

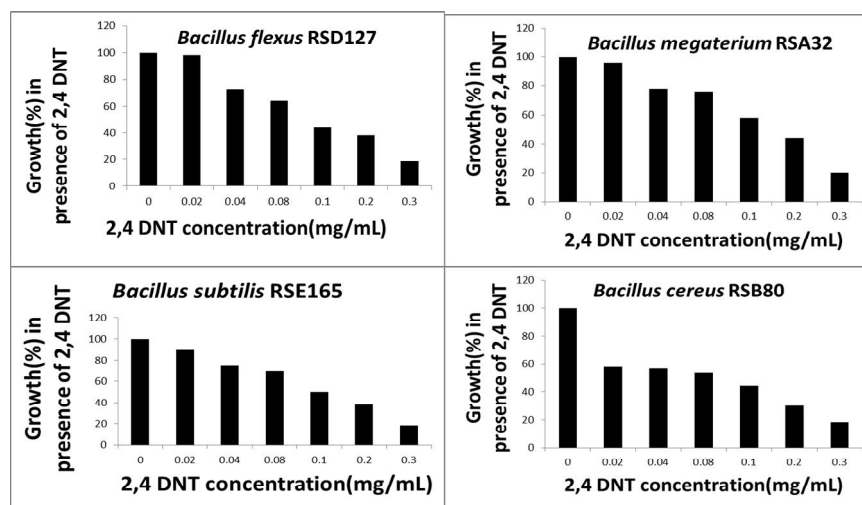
The selected strains were subjected to molecular characterization. Bacterial strains used in this study were identified as *Bacillus subtilis* RSE165 (NCBI accession no. JQ887982), *Bacillus megaterium* RSA32 (KR051485), *Bacillus cereus* RSB80 (JQ040533) and *Bacillus flexus* RSD127 (KR051486). Five phylogenetic trees were constructed using 16S rDNA sequence of four *Bacillus* species through Neighbor-Joining method using PHYLIP 3.6 package, to clarify the phylogenetic relationship among all the four *Bacillus* species and infer their evolutionary history. The trees represented the evolution of the four *Bacillus* species (Figs. 3a, 3b, 3c, 3d). Fig. 3a shows close proximity of *Bacillus subtilis* RSE165 with *Bacillus subtilis* KF475879. The phylogenetic tree (Fig. 3b) showed the detailed evolutionary relationships between the newly identified strain *Bacillus flexus* RSD127 with *Bacillus* sp KJ743304. The analysis further revealed that since the bootstrap value is high in Fig. 3c, this indicates that strain *Bacillus cereus* RSB80 (JQ040533) is showing close proximity with *Bacillus cereus* strain (KJ437489). The phylogenetic tree (Fig. 3d) showed the detailed evolutionary relationships among the newly identified strain *Bacillus megaterium* RSA32 (NCBI accession no. KR051485) and other closely related *Bacillus* species mainly *B. megaterium* and *B. arayabhattai* and demonstrated a distinct phylogenetic position of this strain within the genus.

The evolutionary history of the identified four *Bacillus* strains showed that out of the four strains *Bacillus megaterium* RSA32 is showing maximum evolution and *Bacillus cereus* RSB80 has evolved from *Bacillus megaterium* RSA32. *Bacillus subtilis* RSE165 and *Bacillus flexus* RSD127 are located on the same branch which indicates that *Bacillus subtilis* RSE165 and *Bacillus flexus* RSD127 are closely related. This evolutionary relationship is represented in the phylogenetic tree (Fig. 4).

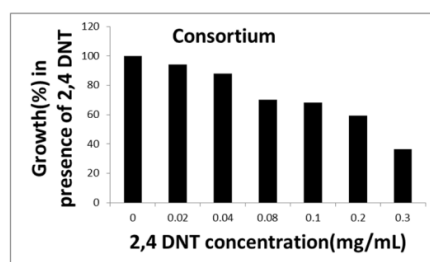
Table 1. Screening of 2,4-Dinitrotoluene tolerant bacteria

Bacterial strains	2,4-Dinitrotoluene				
	0.001% (0.01 mg/mL)	0.002% (0.02 mg/mL)	0.01% (0.1 mg/mL)	0.02% (0.2 mg/mL)	0.03% (0.3 mg/mL)
Bb5	+	+	++	+	+
Bb6	+	++	+	+	+
RSE165	+++++	++++	+++	++	+
Ab9	++	++	+	+	+
RSB80	++	++	+	+	+
RSA32	++	++++	++	++	+
RSD127	+++++	++ ++	++	+	+
A17	++	+	+	+	-

(+) denotes presence of bacterial growth
 (-) denotes absence of bacterial growth



(a)

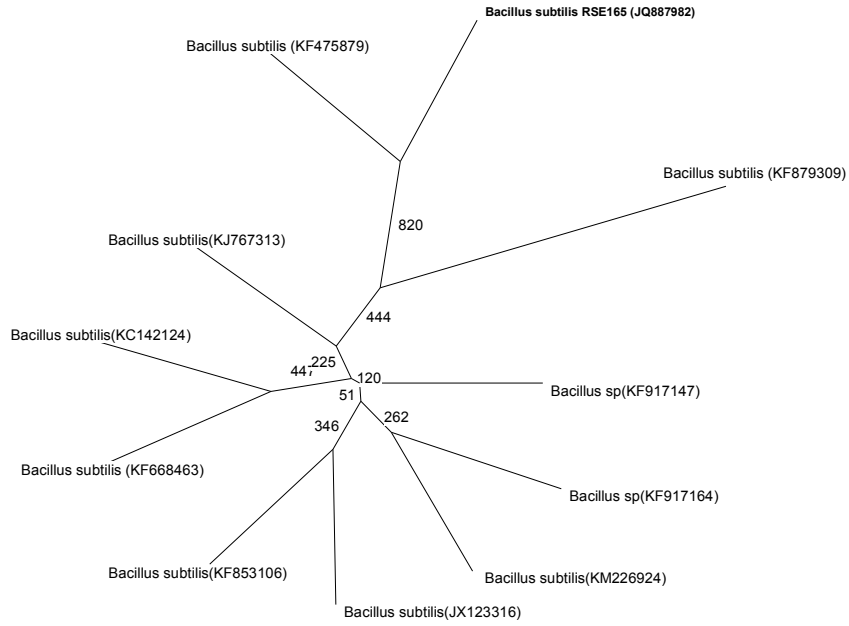


(b)

Fig. 2. The growth in presence of 2,4-Dinitrotoluene at different concentrations in 24 hrs of:-(a) Four bacterial strains (*Bacillus flexus* RSD127, *Bacillus megaterium* RSA32, *Bacillus subtilis* RSE165 and *Bacillus cereus* RSB80) & (b) Developed consortium

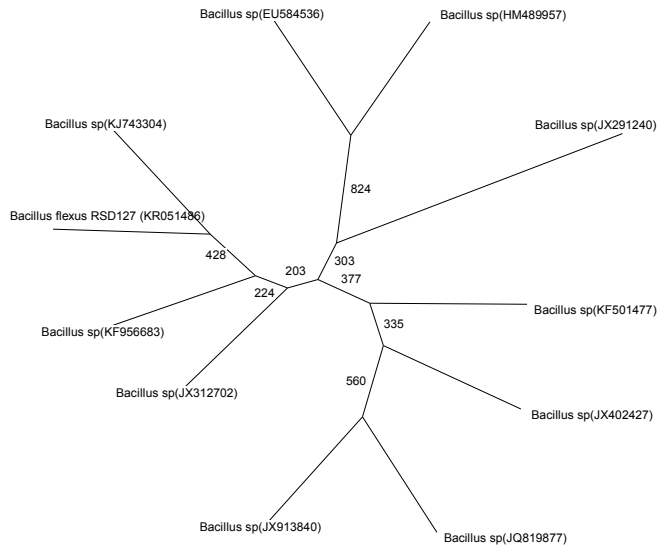
The analysis of the 2,4-DNT degradation capabilities of the best four individual strains and their consortium by GC analysis shows that the spectral peak of 2,4-DNT is completely replaced

by three small peaks which indicate its utilization and degradation by the bacterial strains as well as by their consortium (Fig. 5).



_100

Fig. 3(a)



_100

Fig. 3(b)

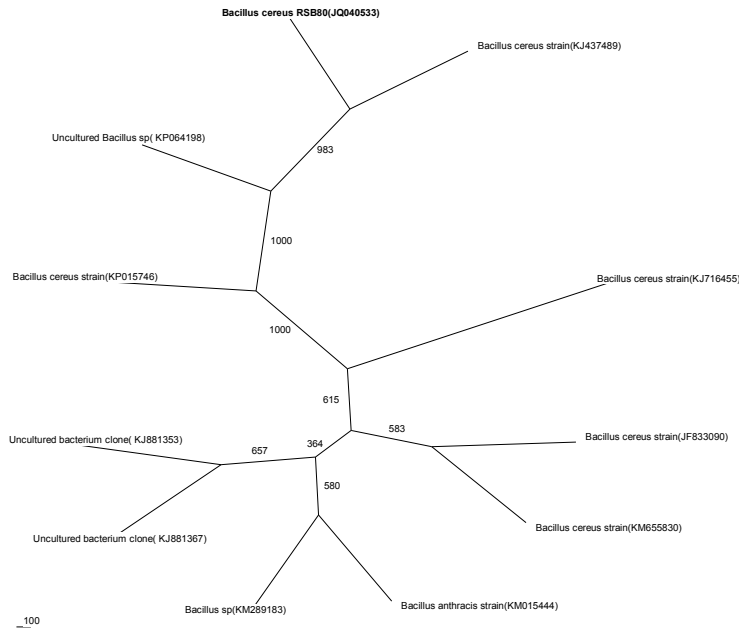


Fig. 3(c)

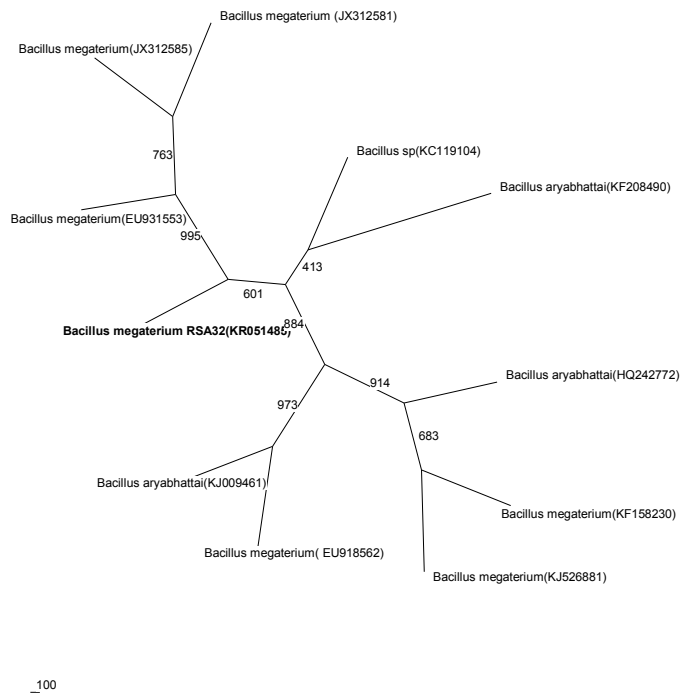


Fig. 3(d)

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences of (a) *Bacillus subtilis* RSE165 (b) *Bacillus flexus* RSD127 (c) *Bacillus cereus* RSB80 (d) *Bacillus megaterium* RSA32: Neighbor-joining analysis and bootstrap support was performed on the gene sequences. Bootstrap values are given at nodes. Values in parentheses are accession numbers

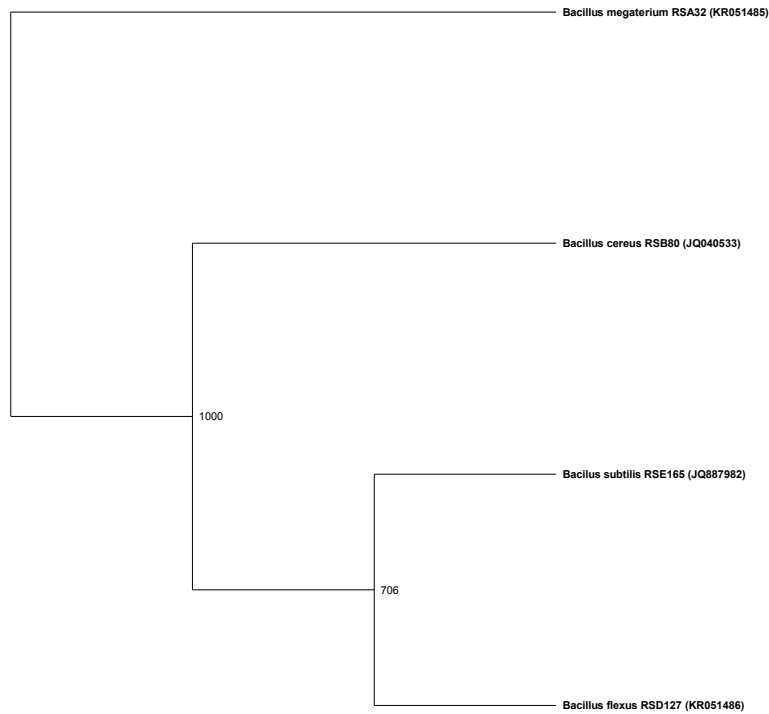
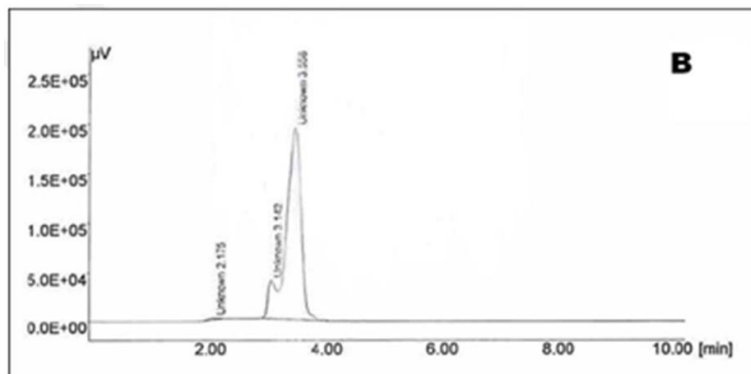
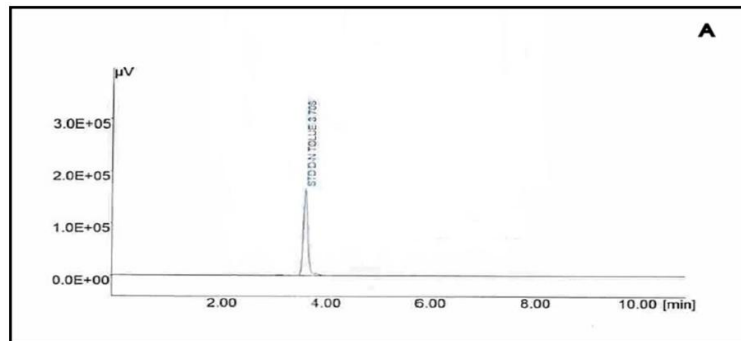
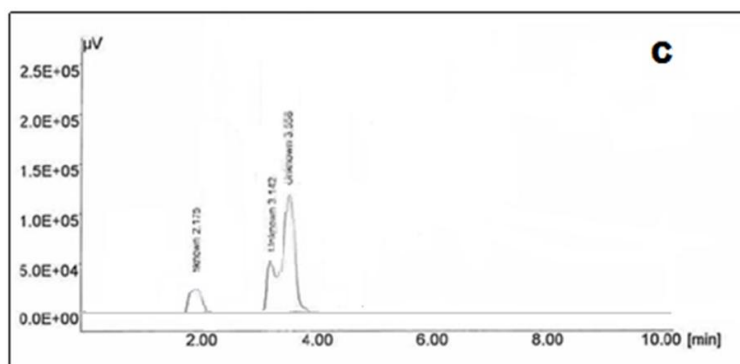


Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences from four *Bacillus* species: Neighbor-joining analysis and bootstrap support was performed on the gene sequences. Bootstrap values are given at nodes, 1000 bootstrap replicates were run. Values in parentheses are accession numbers





**Fig. 5. A- GC Chromatogram of 2,4-Dinitrotoluene Control
 B-GC Chromatogram of Degradation products of 2,4-Dinitrotoluene (0.3 mg/mL) in presence of Consortium after 8 hours
 C-GC Chromatogram of Degradation products of 2,4-Dinitrotoluene (0.3 mg/mL) in presence of Consortium after 48 hours**

Table 2. Different composition of the bacterial consortia

Consortia no	Composition
1	Bb5, Bb6, RSE165, Ab9
2	Bb6, RSE165, Ab9, RSB80
3	RSE165, Ab9, RSB80, RSA32
4	RSE165, RSB80, RSA32, RSD127
5	Ab9, RSB80, RSA32, A17
6	A17, Bb5, Bb6, RSE165
7	Bb6, A17, Ab9, RSB80
8	Bb5, RSB80, RSA32, A17

4. CONCLUSION

Degradation of 2,4-DNT is a difficult phenomenon, due to which the disposal and presence of these compounds in the surroundings poses environmental hazard. The bacterial consortia prepared in the present study can tolerate high concentration of 2,4-DNT along with its degradation.

ACKNOWLEDGEMENTS

The authors would like to thank Centre for Fire, Explosive and Environment Safety (CFEES/ATEG/CARS/001/09-10), DRDO for the financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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