

Aerobic degradation 2,4-dinitrotoluene: effect of raw organic wastes and nitrogen amendment

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Abstract

2,4-Dinitrotoluene (2,4-DNT), a major by-product of the synthesis of 2,4,6- trinitrotoluene, is widely used as a waterproofing, plasticizing and gelatinizing agent in propellants and explosives. Due to its toxicity, the compound is treated as a priority pollutant. Therefore, its removal from contaminated systems is a major focus of research and attention. Contaminated sites in Ibadan, Nigeria were screened for the presence of 2,4,-DNT degrading organisms. The technique of continual enrichment on NACs yielded bacterial isolates able to utilize 2,4-DNT as growth substrate. Based on phenotypic characteristics and 16S rRNA gene sequencing one of the isolates selected for further study was identified as *Proteus* sp. strain OSES2. Growth of the strain on 2,4-DNT resulted in exponential increase in biomass and complete substrate utilization within 72 h accompanied with NO₃ - elimination. Degradation competence enhanced in the presence of Corn steep liquor, molasses and Tween 80 compared to incubation without amendment. Conversely, amendment with nitrogen sources yielded no significant improvement in degradation. Use of this organism organic wastes as candidates in bioremediation strategy should be exploited. This would provide a cheaper organic source supplement for cleanup purposes with the ultimate aim of reducing the cost of bioremediation while reducing wastes intended for landfill.

Background

2,4-Dinitrotoluene, one of the several congeners of the dinitrotoluenes (DNTs) family is a major isomer generated from both the industrial synthesis of 2,4,6 Trinitrotoluene (TNT) and also a principal metabolite in the course of its microbial degradation [1,2]. They are not naturally present in the environment, but they are released into ground and surface water, and soils as a result of the widespread usage in ammunition manufacturing facilities and some chemical products [3]. These compounds are used by the military as an explosive intermediate, a component of gun powder and as a modifier of smokeless powders in moderate and high explosives. They are also used in the manufacturing of dyes, herbicides, polyurethane foam [4] and plasticizers. However, several national environmental regulators such as the U.S Environmental Protection Agency (USEPA) have described 2,4-DNT as a priority pollutant due to its carcinogenic and toxicological properties, widespread occurrence and recalcitrance to microbial attack which can potentially cause harm to public health [1, 5, 6, 7].

Among several methods available for decommissioning of environments laden with nitroaromatic compounds (NACs), the use of microbial metabolic potential offers the most environmental compatibility and cost effectiveness. It is therefore not surprising that microbial degradation of NACs has been a subject of research owing to rapid adaptability of microorganisms for degradation of these xenobiotics. This is because, the presence of these pollutants such as 2,4-DNT in the habitat creates a strong selection force due to exposure of these soil microorganism to varying levels of the xenobiotics, which invariably results in the development of soil microbiota capable of utilizing 2,4-DNT, as a singular source of carbon, nitrogen and energy [8– 10]. Interestingly, several organisms have been reported to metabolize 2,4-DNT and such organisms which have been reported to possess unique metabolic properties have been isolated from a polluted environmental matrix. For example, *Pseudomonas* sp., a bacterium strain

isolated from Waconda Bay close to an Army ammunition factory, was reported as the first organism to utilize 2,4-DNT as the sole carbon and energy source with a concomitant evolution of nitrite [11]. Since this initial report, an abundance of 2,4-DNT degrading bacteria including; *Burkholderia* sp. strain DNT [12], *Burkholderia cepacia* strain R34 [13], *Pseudomonas* sp. strain VM908 [14], *Bacillus* sp. [15], *Arthrobacter* strain K1 [16] and *Rhodococcus pyridinovorans* NT2 [17] have been documented. The elucidated 2, 4-DNT degradative pathway utilized by these organisms showed that the substrate was degraded through a dioxygenation reaction in which 2,4-DNT is oxidized to 4-methyl–5-nitrocatechol (MNC) with nitrite removal. The MNC was then sequentially reduced to 2-hydroxy–5-methylquinone in a reaction mediated by a monooxygenase, which is eventually converted to 2,4,5-trihydroxytoluene which is subsequently transformed by meta cleavage [10, 18].

Despite the isolation of organisms with inherent abilities to degrade NACs, the degradative competence of such strains can be improved or optimized in the presence of easily amenable cheap carbon sources that may sometimes constitute environmental nuisance. Remarkably, several investigators have reported the improved metabolism of contaminants when amended with additional carbon and nitrogen sources. For instance, Tharakan and Janice [19] documented enhancement of TNT degradation when fortified with 3000 mg L⁻¹ of glucose and citric; according to the authors, only 9% of the 30 ppm TNT substrate was degraded by the bacterial consortium. However, the same concentration of the substrate was completely consumed in 24 h upon citric and glucose fortification. In another report, the degradation capability of *Pseudomonas* sp. strain HK–6 on some NACs (TNT, royal demolition explosive [RDX], atrazine and simazine) showed significant improvement when amended with different carbon sources [20] while fortification with nitrogen sources revealed no enhancement. Amendment with peptone and yeast also significantly improved the biodegradation of 2,4,6-trinitrophenol (TNP) and biomass formation using *Rhodococcus pyridinivorans* strain NT2 as a source of inoculum compared with non-fortification of the culture fluid [21]. The same trend was also observed in biodegradation of TNT by an immobilized *Bacillus* sp. strain YRE1 when amended with Tween 80 [22]. Irrespective of the available reports in the literature, it is obvious that amendment with carbon and nitrogen sources most likely results in meaningful improvement in metabolic capabilities of microorganisms to varying degrees with few exceptions.

Although there is to some extent an existing body of knowledge on microbial metabolism of 2,4-DNT, there is dearth of information on the availability of such phenotypes in tropical Africa particularly in Nigeria where there is unabated and indiscriminate release of the pollutant into the environment. Floyd et al [23] whilst reviewing the captured prokaryotic diversity in American Type Culture Collection (ATCC) indicated that geographical locations and environmental types of indigenous organism exhibited a significant difference between both geographical locations and environment that were represented in the culture collection. Accordingly, North America accounted for 24.1% of all the entries followed by Europe (14.7%) and Asia (11.5%). Quite unfortunately, only 2.8% was attributed to Africa despite the huge landmass and unregulated inflow of environmental contaminants. Therefore, it is the intent of the present study to showcase for the first time, the occurrence of microbial strains with 2,4-DNT degradation

potential from a Nigerian polluted soil and to investigate the improvement of such metabolic capabilities by amendment with agro-allied waste such as molasses, corn steep liquor (CSL) and other nitrogen sources.

Results

Isolation and characterization of 2,4-DNT degrading bacteria

Thirty-four (34) distinct bacterial colonies were picked from the MSM agar plates after a preliminary enrichment on nitroaromatic mixtures. After screening of individual isolates for growth on 2, 4-DNT, five (5) isolates with unique metabolic ability to utilize this substrate as the singular carbon and energy source were obtained. Among them, strain OSES2, a rod-shaped Gram-negative bacterium with swarming motility on nutrient agar plate which was the most efficient degrader was subsequently selected for further study. A positive genotypic identification was conducted by sequencing of the 16S rDNA fragment. Using the BLAST algorithm, the sequence showed 96.86% identity with the type strain *P. mirabilis* PWN3A, as illustrated in Fig. 1. The 16S rRNA gene of *Archaeoglobus fulgidus* a clearly unrelated organism used as an out-group. Strain OSES2 clustered more cohesively with members of the genus *Proteus*, than with genera *Enterobacter* and *Achromobacter*. The organism was subsequently putatively identified as *Proteus* sp. strain OSES2.

Degradation of 2,4-DNT by Strain OSES2

Strain OSES2 grew readily on 2,4-DNT under aerobic batch conditions. Growth of the isolate was exponential; it commenced immediately without a display of lag phase (Fig. 2). The cell population density increased by eight orders of magnitude within 48 h, yielding a mean generation time of 25.14 h. Metabolism of the substrate is evidenced by gradual disappearance concomitant with increase in population densities and release of NO_3^- (Fig. 3). Lack of meaningful change in 2,4-DNT concentration in control flasks showed that the reduction observed in experimental flasks were essentially due to microbial action and not physiochemical factors. Since no carbon and nitrogen sources other than 2,4-DNT were introduced into the culture medium, the obtained data suggests that the organism could utilize the substrate both as the only source of carbon and nitrogen source. A critical examination of the results showed that nearly 65% of the 2,4-DNT substrate was utilized by the organism, at which time points biomass production was five folds even though the amount of NO_3^- recovered was less than 1 mg L^{-1} (Fig 3). By implication, only 28% of the substrate was utilized between 24 and 72 h of cultivation yielding a degradation rate of $0.032 \text{ mg L}^{-1} \text{ h}^{-1}$ as against $0.035 \text{ mg L}^{-1} \text{ h}^{-1}$. Overall, nearly 93% of the substrate was utilized in 72 h of incubation yielding a degradation rate of $0.035 \text{ mg L}^{-1} \text{ h}^{-1}$ assuming a constant rate of utilization of the substrate per time (Table 1). The amount of NO_2^- recovered throughout the incubation period was significantly low, whereas that of NO_3^- picked at 2.9 mg L^{-1} and declined slowly to 2.5 mg L^{-1} at the very end of the incubation. It is noteworthy, that incubation beyond 48 h produced no significant cell increase, in fact, the population density took a downward trend with a characteristic color

change of the medium from pale yellow to colorless. This observation notwithstanding, utilization of substrate did not cease, although the rate of consumption slowed considerably.

Effects of carbon and nitrogen sources on 2,4-DNT degradation

The effect of different carbon sources (molasses, CSL, glucose, glycerol, maleic acid, acetate, sucrose and aspartate) on biodegradation of 2,4-DNT was evaluated over a period of 48 h to determine the one that best enhanced degradation. The data obtained are summarized in Fig. 4. Addition of these co-substrates to the culture media resulted in a significant improvement in degradation competence of the organism, although to a varying degree. Amendment with all the carbon sources yielded over 80% degradation of 2,4-DNT except for aspartate. Interestingly, molasses and CSL produced the highest enhancement compared to other substrates utilized.

A more detail growth analysis of the isolates in MSM fortified with CSL and molasses is shown in Fig 5. Higher growth and degradation rates were recorded in the presence of these amendments in comparison with when 2,4-DNT was supplied alone. Growth and catabolic patterns of strain OSES2 in flask amended with CSL paralleled observations in flask containing molasses. In both cases, maximum cell densities of over fifteen orders of magnitude were observed within 36 h of incubation, which was three times higher than flasks without fortification. A detailed assessment of the data obtained indicated that between 60% - 70% of the initial NAC supplied was used by the organism within the first 12 h of cultivation cumulating in approximately $0.074\text{--}0.085\text{ mg L}^{-1}\text{ h}^{-1}$ as against $0.072\text{ to }0.087\text{ mg L}^{-1}\text{ h}^{-1}$ overall degradation rate (Table 1). 2,4-DNT utilization was relatively faster in CSL compared with molasses, nevertheless, complete degradation was accomplished in 36 h in both cases. When subjected to statistical analysis, both molasses and CSL amendments yielded lack of significant difference at $P < 0.05$ confidence level. Conversely, a significant difference was obtained when both incubations were compared with flask without amendment. By implication, both molasses and CSL significantly improved 2,4-DNT degradation competence of strain OSES2.

In contrast to the enhanced degradation observed with carbon sources, amendment with nitrogen sources stimulated bacterial growth (Fig. 6), but there was relatively no effect on DNT degradation with reference to Fig. 2. Although, complete degradation of the substrate was not achieved until 72 h of incubation, the data was comparable with Fig 2 which lacked nitrogen sources. This may not be unconnected with the fact that the organism exhibited an ability to utilize 2,4-DNT both as the sole source of carbon, energy and nitrogen. Fortification with exogenous nitrogen sources appears to be insignificant. Interestingly, statistical analysis showed that the activity of strain OSES2 with or without alternative nitrogen sources yielded lack of significant differences.

Since amendment with nitrogen sources did not significantly improve degradation of 2,4-DNT, an experiment was launched to determine if the addition of surfactant with KNO_3 would result in meaningful enhancement. The findings from this investigation are summarized in Fig. 7. Typical kinetic data obtained are found in Table 1. While 2,4-DNT was completely utilized within 36 h in presence of

surfactant and KNO_3 , it took additional 12 h for surfactant alone to accomplish same feat. The implication of this study is that surfactant could also significantly improve mass transfer, ultimately enhancing the utilization of DNT by strain OSES2 as against fortification with nitrogen source. It is also noteworthy that combination of surfactant and KNO_3 resulted in similar enhancement with molasses or CSL. It is not surprising therefore that the degradation rate of surfactant and KNO_3 obtained is relatively similar to those obtained for CSL.

Discussion

Nitroaromatics-contaminated sites are known reservoir for many microorganisms with specific metabolic capabilities. It is therefore, not surprising that such sites are actively explored for isolation of competent degraders since prior exposure may enhance the catabolic potentials of such bacteria strains [8]. Using explosive contaminated soil samples from a quarry site in Ibadan, Nigeria, an enteric bacterium identified as *Proteus* sp. strain OSES2 was successfully isolated amongst other species following the technique described. Extensive study on the degradation of NACs and particularly, 2,4-DNT over the past two decades has culminated in the identification of a diverse collection of bacterial species, e.g., *Alcaligenes denitrificans* JS867 and JS871, *A. xylosoxidans*, and *Burkholderia cepacia* JS872 [24]; *Pseudomonas fluorescens* sp. [25]; *P. mandelii* HC88 [15] that can metabolize the compounds and utilize them as a sole carbon and energy source. The metabolic capability of an enteric organism to utilize 2, 4-DNT as a principal carbon and energy source could be regarded as a novel trend as there has been no previous documentation of such metabolic abilities by enteric bacterial strains. Enteric organisms are predominantly intestinal in origin and are not known for degradation of relatively recalcitrant xenobiotic organic compounds, although there are few exceptions. For instance, analysis of the *Escherichia coli* whole genome has shown that the bacterium harbor unknown genes that are suspected to be involved in the aerobic degradation and transformation of aromatic compounds [26]. Accordingly, *E. coli* cells was demonstrated to reduce two of the TNT nitro groups under aerobic and anaerobic conditions, with the formation of 2,4-diamino-6-nitrotoluene [27]. Likewise a strain of *E. coli* has been documented to cause the dechlorination of the 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) to 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD) [28] just as the *E. coli* mediated conversion of γ -hexachlorocyclohexane (lindane) to γ -pentachlorocyclohexene under aerobic condition was documented for the first time [29]. Adebusoye et al. [30] reported the isolation of an *Enterobacter* sp. SA2 from a PCB-contaminated soil with an attributed capability to metabolize a spectrum of PCB and chlorobenzene congeners. Recently, *Proteus vulgaris* strain CPY1 was unambiguously demonstrated to possess competent pyrene metabolic functions [31]. In contrast to strain OSES2, CPY1 was isolated from an animal waste, a clearly unusual place for sourcing of xenobiotic degraders. Since OSES2 was isolated from an explosive contaminated soil due to human activities, the presence of this recalcitrant compounds may have resulted in adaptability of the organism over time and development of its capabilities for utilization of 2,4-DNT. This suggests that some contaminated soils in sub-Saharan African environment may contain exotic bacterial strains whose metabolic capabilities and potentials are previously unknown and are yet to be discovered.

Strain OSES2 exhibited an unusual ability to utilize 2,4-DNT as a sole source of carbon, energy and nitrogen since the culture fluid contained no additional fortification with nitrogen or carbon sources. The percentage utilization of this substrate consumed by strain OSES2 was within the range previously reported for some 2,4-DNT degraders, and perhaps, superior to other phenotypes [16]. Mariela et al. [25] reported that 2,4-DNT was completely utilized in 90 h by *Pseudomonas fluorescens* when supplied as the only carbon, energy and nitrogen source. More recently, *Rhodococcus pyridinovorans* NT2 recovered from a pesticide polluted soil was shown to degrade both 2,4- and 2,6-DNT within 48 h of incubation [32]. It is noteworthy that, while the authors reported 70% degradation of 2,4-DNT in extended cultivation period of 10 days, strain OSES2 utilized over 90% of the same compound in 72 h. By implication, the catabolic repertoire in OSES2 may be unique and superior to that of K1 reinforcing its relevance and desirability as candidates for cleanup of contaminated systems. Aerobic metabolism of NACs is often accompanied by the release NO_3^- , NO_2^- and sometimes NH_4^+ [33, 34]. However, a thorough evaluation of the data obtained in this study showed a non-stoichiometric recovery of these metabolites. The reason for this observation is not farfetched. Since strain OSES2 depended on the DNT substrate both as sources of nitrogen and carbon, the difference in nitrogen mass balance could have been incorporated into the cellular architecture. In this case, the recovery of NO_3^- and NO_2^- in the culture fluid and the observable increase in biomass, undoubtedly indicate the metabolism of 2,4-DNT as previously documented by other workers [8, 17]. A plausible explanation for the extreme low level of NO_2^- could be due to its rapid conversion to NO_3^- [17].

Microbial enzymatic activity is reliant on the organismal physiological nature as well as the physico-chemical characteristics the environment process [35, 36]. Nutritional consumption by microorganism is an important criterion for microbial activity which in turn can influence their enzymatic activity. Amendment with carbon source such as starch, molasses, pyruvate, sucrose, lactate, glucose, ethanol, and citric acid, does not only increase the population density of microorganisms, but also their effectiveness by producing enzymes, elimination of lag phases and ultimately shortening the degradation time [37–39]. Interestingly, strain OSES2 metabolic activity was greatly enhanced when amended with different carbon sources particularly, CSL and molasses resulting in complete DNT utilization in few hours as previously documented for other organisms [40–41]. Specifically, CSL was found to be the most effective carbon source. One possible reason for enhanced degradation of 2,4-DNT in CSL amended incubation was the increased biomass. This increased biomass resulting in higher growth rate may not be unconnected with the fact that CSL is rich in amino acids, minerals, co-factors and vitamins and other nutrients required by microorganisms. The composition of molasses on the other hand is very complex containing sugars (30% of glucose, 43% sucrose), organic nitrogen, vitamins, amino acids, proteins, vitamins and minerals [42]. The components of these carbon sources make them conducive for microbial growth and therefore, could have in turn stimulated enzymatic activity and biodegradation of the xenobiotic. When strain OSES2 was grown on 2,4-DNT alone, very little additional biomass could be produced because of the limited amount of carbon substrate available in the medium. Since CSL or other easily degradable substrate, was present, the organism grew on it while producing biomass for

degradation of the DNT. Amendment with CSL, molasses, surfactant and various supplemental sources have also been investigated to enhance the biodegradation rate of TNT (43–45).

It is noteworthy that CSL in addition to molasses and acetate are good supplemental carbon sources for enhanced DNT degradation more so, owing to the fact that they are among the inexpensive sources of carbon and are readily available as waste emanating agro-allied industries [42, 44]. Furthermore, since bioremediation is a sustainable waste management system that primarily details the usage of microorganisms or cheaper raw materials from waste as biostimulants to clean-up pollutants from a contaminated matrix; the use of CSL and molasses is likened to killing two birds with a stone. While reducing the overall cost of remediation on one hand, it is a means of waste management for industries generating them.

In contrast to carbon sources, amendment with nitrogen sources yielded no significant improvement in the metabolic capability of strain OSES2. It is noteworthy that fortification of the growth medium with yeast extract and KNO_3 awesomely supported proliferation of cells which, unfortunately, was not translated to 2,4-DNT consumption. Similar trend was previously documented by Cho et al. [20] and Shen et al. [21] while investigating conditions necessary for optimization of TNT degradation but not 2,4-DNT. By implication, amendment by exogenous nitrogen sources in systems inoculated with OSES2 may not be necessary since the organism could also utilize the substrate as a nitrogen source. Under this condition, the presence of additional nitrogen sources may present some metabolic bottlenecks to the organism thus impairing DNT degradation.

Considering the hydrophobic nature of 2,4-DNT, the addition of Tween 80 in combination with KNO_3 increased the rate of 2,4-DNT degradation, biomass production and decreased the degradation time by 12 h in comparison with when the surfactant was used alone. The effectiveness of Tween 80 in enhancing pollutant degradation by microorganisms was reported by Boopathy and Manning [46]. According to the authors, the surfactant can be utilized by microorganisms as an additional carbon source owing to the presence of long chain fatty acids, which include, oleic acid, myristic acid, palmitic acid, arachidic acid, linoleic acid and stearic acid while at the same time improving mass transfer and bioavailability of the pollutant to the degrading organisms. This inference was also reinforced by several researchers [47, 48]. Furthermore, it has now been established that both the growth and degradation rates are usually not dependent on a single substrate in a multi-substrate system.

Conclusions

The unique finding of this study lies in the demonstration of 2,4-DNT metabolic phenotypes for the first time in Nigerian contaminated system and the fact that this function is found in quite an unusual bacterium. It is therefore, reasonable to hypothesize that diversity of microbial populations may harbor bacterial strains with novel metabolic capabilities in unexplored tropical soils in other regions and that the genes responsible for metabolism of these compounds may be more widely distributed across different microbial genera than earlier documented. Also, the data presented here readily suggest that

CSL, molasses, acetate are potential stimulants for DNT degradation at least in strain OSES2. Therefore, the utilization of this organism as candidate in addition with the amended carbon sources in bioremediation strategy should be exploited. This will help reduce the amount of waste intended for landfill, thus reducing landfill gas emissions. It will also provide a cheaper source of organic supplement for cleanup purposes with the ultimate aim of reducing the cost of bioremediation. Nevertheless, further research into the biochemical attributes and genotypic profile of this isolate is necessary to determine its possible activity in the natural attenuation or overall engineered bioremediation of NACs.

Methods

Chemicals

2,4-dinitrotoluene, acetone and hexane were acquired from Sigma Aldrich (St Louis, MO, USA) and these reagents were of high analytical grades (98–100%).

Microorganism and Culture Conditions

Top soils were sourced from a quarry site in Ibadan, Oyo State, Nigeria which have a history of contamination with explosive materials. The samples were kept in a clean plastic bag, conveyed to the laboratory immediately and stored in 4°C prior to analysis. Bacterial strain able to degrade 2,4-DNT were cultured on mineral salt medium (MSM) [49] with some modifications by continual enrichment technique. The medium contained the following composition in g/L of distilled water: NH_4SO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; KH_2PO_4 , 1.3 g; Na_2HPO_4 , 2.13 g; yeast extract, 0.2 g. The medium was amended with 10 ml of vitamin solution and 1 ml of trace element solution. One (1) gram of the contaminated soil sample was added to 250 ml Erlenmeyer flask containing 100 ml of MSM. The medium was supplemented to a final concentration of 100 mg L^{-1} with selected nitroaromatic mixtures of 2,4-DNT, 3-nitrotoluene, methyl 3-nitrobenzoate, 4-nitrophenol, 4-chloro-3-nitrotoluene in equal concentration as a sole source of carbon and energy. Enrichment was carried out by incubating in a rotatory shaker incubator (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm for 3–4 weeks in the dark at room temperature ($27 \pm 2^\circ\text{C}$). After twelve consecutive transfers, 2,4-DNT degraders were recovered by plating out aliquots of relevant dilutions onto MSM agar plate. The plate was sprayed after seeding with nitroaromatic mixtures and incubated for 5–7 d at room temperature. Typical Colonies surrounded by zones of clearance was selected, purified and subsequently screened for utilization of 2,4-DNT in MSM broth. Bacterial strain designated OSES2, which exhibited the highest DNT degradative ability was selected and maintained in glycerol: LB broth medium (50:50) at 20°C

Identification of Isolates

Bacterial DNA was isolated from an overnight culture of the isolate on LB broth using Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Germantown, MD, USA) and subjected to illumina sequencing. Illumina data sets in the FASTQ format were generated using MiSeq Reporter v2 (Illumina) and analyzed using

QIME (quantitative insight into microbial ecology) software and RDP classifier 16S rRNA gene sequence data base [50]. The partial sequence of 16S rRNA gene was compared with GenBank database using the BLAST algorithm and thereafter deposited with the NCBI under accession no. MH714749. Nucleotide sequence were aligned using mega X software, phylogenetic tree was drawn using Neighbor-joining algorithm and the branch points were determined using boot strap method in Mega X software.

Degradation of 2,4-DNT by Strain OSES2

Degradation of the substrate was conducted by inoculating 250 ml flask which contained 100 ml of modified MSM (without NH_4SO_4 , yeast extract) already supplemented with 2,4-DNT as the sole carbon, nitrogen and energy source at a concentration of 100 mg L^{-1} . The inoculum was prepared by growing cells overnight in LB broth. The culture was harvested by centrifugation for 20 min at 4°C . The pelleted cells were washed twice in phosphate buffer (pH 7.2) and resuspended in the same buffer to a final OD (600nm) of 0.2. Flasks were inoculated with 1% inoculum and subsequently incubated as described above. Flask seeded with heat inactivated cells served as control. At each sampling point, culture fluids were withdrawn for analysis while hexane was added to the culture fluids to stop metabolic reactions. Biodegradation indices including cell density ($(\text{OD}_{600 \text{ nm}})$), NO_3^- , NO_2^- and residual 2,4-DNT concentrations were monitored.

Nutrients and carbon sources amendment on 2,4-DNT degradation

Washed cells of strain OSES2 was seeded into a flask which contained modified MSM supplemented with 100 mg L^{-1} of 2,4-DNT. The Flasks were amended with 0.5% of different carbon sources (e.g., molasses, CSL, glucose, glycerol, maleic acid, acetate, sucrose and aspartate). Flask inoculated with heat inactivated cells served as control. Incubation and sampling were handled as previously described. A parallel experiment in which inoculated flasks were amended with 1% (w/v) either of yeast extract, KNO_3 or 1.5% (v/v) of surfactant (Tween 80) were set up to monitor the roles of nitrogen sources on 2,4-DNT degradation.

Analytical methods

The amounts of NO_2^- and NO_3^- released into the growth medium were assayed spectrophotometrically according to APHA [51].

Residual concentration of 2,4-DNT was quantified from the supernatant obtained after centrifugation. To this, was added hexane/Acetone solvent complex (ratio 1:1) in a 250-ml separating funnel. The organic layer of the mixture was carefully decanted, desiccated with sodium sulphate and concentrated using rotatory evaporator to 2 ml. Th extract was analyzed by Agilent 7820A gas chromatograph coupled to 5975C inert mass spectrometer (GC-MS) (with triple axis detector) with electron-impact source (Agilent Technologies Santa Clara, CA, USA) using a 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, $0.25 \mu\text{m}$). Helium was supplied as the carrier gas. The injector and detector temperatures were

both maintained at 250°C and 230°C, respectively. An initial temperature of 130°C was maintained for the oven for 2 min, then raised at 7°C min⁻¹ to 240°C and retained for 10 min.

Statistical analysis

Information about the specific growth rate of the isolate, mean generation time and reaction rate constant were derived from non-linear regression analysis using program Prism v7 (Graphpad software, San Diego, CA, USA).

Abbreviations

2,4-DNT: 2,4-Dinitrotoluene; TNT: 2,4,6 Trinitrotoluene; USEPA:U.S Environmental Protection Agency; NACs: Nitroaromatic compounds; ATCC: American Type Culture Collection; APHA: American public association; CSL: Corn steep liquor; MSM: mineral salt medium; GC-MS: Gas chromatography-mass spectrometry

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SAA conceptualized and designed the work. OEO, performed the experiments, and drafted the manuscript. SAA and OEO analyzed and interpreted the data. SAA and OSO supervised the work and edited the manuscript. All authors read and approved the final version of the manuscript.

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Tables

Table 1: Growth and degradation kinetics of strain OSES2 on 2,4-DNT with/without carbon and nitrogen sources

Substrate	T _g (h)	μ (h ⁻¹)	% degradation	D mg L ⁻¹ h ⁻¹
(i) 2,4-DNT	25.14	0.148	93	0.035 (0.054)
(ii) 2,4-DNT + CSL	9.03	0.244	100	0.085 (0.087)
(iii) 2,4-DNT + molasses	8.12	0.17	100	0.074 (0.072)
(iv) 2,4-DNT + Yeast	19.88	0.231	100	0.067 (0.032)
(v) 2,4-DNT + KNO ₃	20.97	0.192	100	0.057 (0.025)
(vi) 2,4-DNT + Surfactant	17.04	0.306	100	0.064 (0.072)
(vii) 2,4-DNT + Surfactant + KNO ₃	7.86	0.243	100	0.085 (0.087)

2,4-DNT was supplied at a concentration of 100 mg L⁻¹ and fortified with 0.5% of respective carbons, 1% (w/v) nitrogen sources as wells as 1.5% (v/v) Tween 80. Degradation rate (D) was calculated with the assumption that DNT utilization per sampling point was constant throughout the incubation period while values in parentheses are rates determined for the first 12 h of cultivation. Percent degradation values were evaluated with reference to the amount recovered from flasks inoculated with heat-inactivated cells. Specific growth rates (μ) and mean generation times (Tg) were determined from nonlinear regression of growth curves between 0 and 48 h for i, iv and v; 0 and 24 h for ii, iii and vii; 0 and 36 for vi during which growth rates were maximal.

Figures

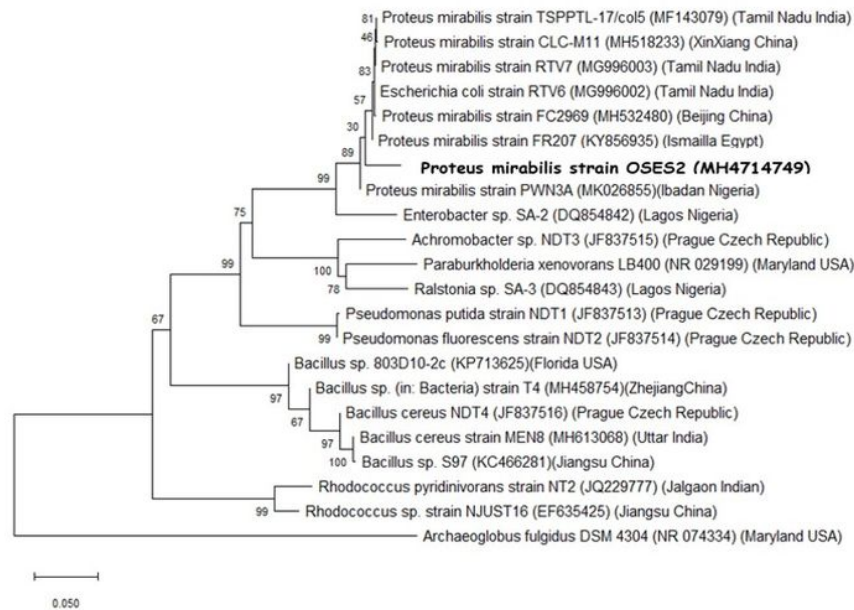


Figure 1

Phylogenetic tree with significant bootstrap values based on 16S rDNA sequences, showing the position of strain OSES2 relative to other selected related strains. The bootstrap values are expressed in percentage and the accession numbers are represented in parentheses.

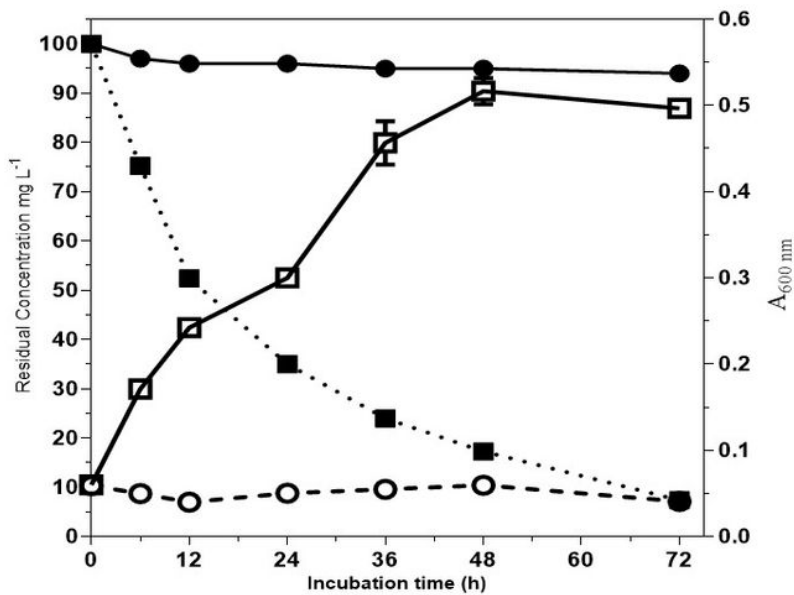


Figure 2

Biodegradation of 2,4-DNT by *Proteus* sp strain OSES2 in mineral salt medium (pH 7.2) supplemented with 100 of 2,4-DNT (□) 2,4-DNT concentration in experimental flask; (■) 2,4-DNT concentration in control flask; (●) OD₆₀₀nm growth; (○) OD₆₀₀nm control. The substrate was supplied at a concentration of 100 mg L⁻¹. In the control flask, 2,4-DNT was not utilized and minimal abiotic loss occurred. Data points are average of three replicate determinations, with error bars above and below the mean. Values were determined with reference to 2,4-DNT recovered from heat activated cells.

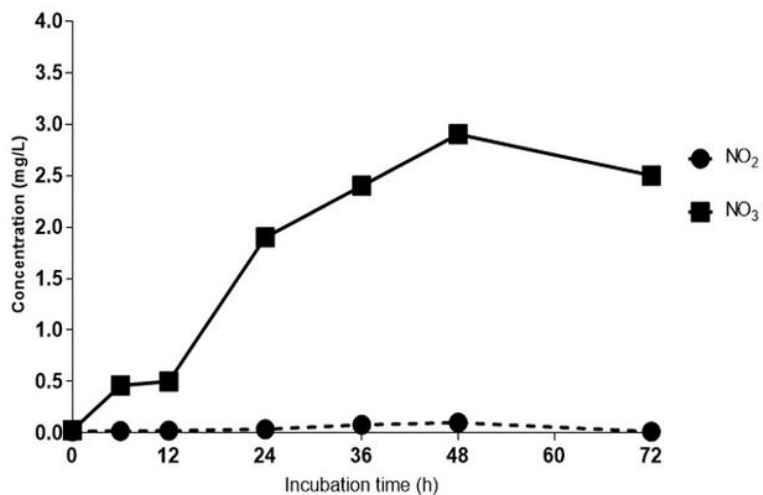


Figure 3

Time course for 2,4-DNT metabolism and accumulation of nitrite and nitrate by *Proteus* sp. on experimental tubes. (●) release of nitrite; (■) release of nitrate

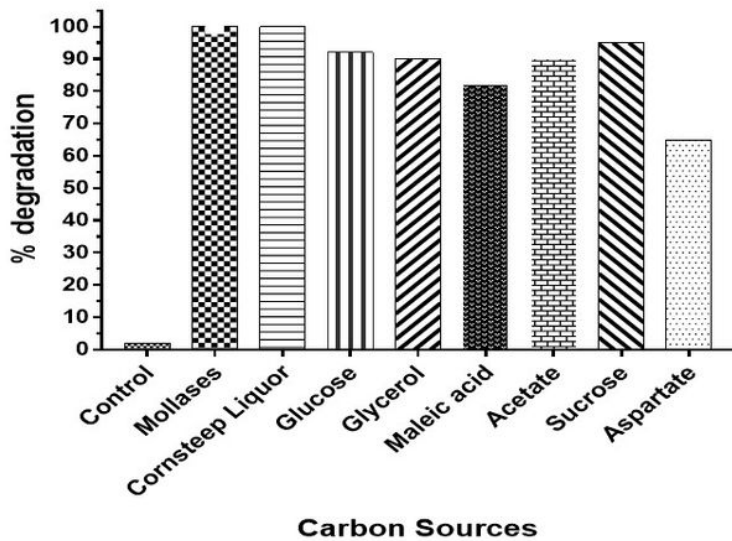


Figure 4

Performance of *Proteus* sp. strain OSES2 with different supplemental carbon sources on biodegradation of 2,4-DNT.

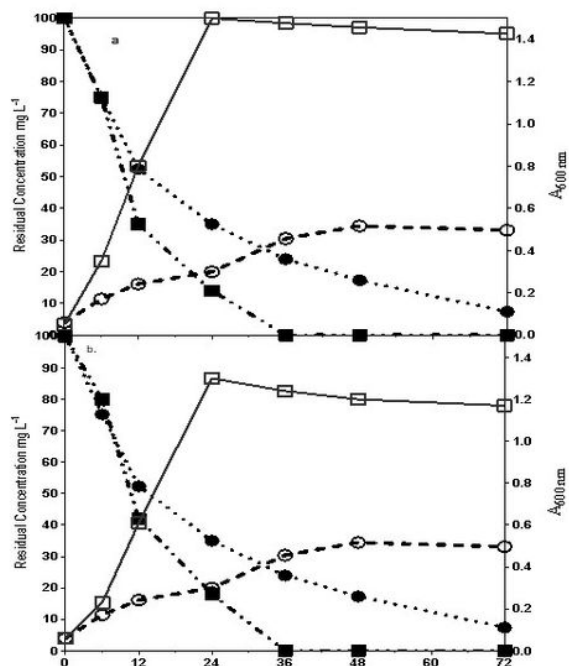


Figure 5

Effect of various carbon sources on degradation of 2,4-DNT by *Proteus* sp. at initial concentration of 100 mg L⁻¹. CSL (a), Molasses(b). \blacksquare , 2,4-DNT concentration in experimental tubes with amendment; \square , 2,4-DNT concentration in non-amendment controls; \blacklozenge , OD600nm with amendment; \lozenge , OD600nm; Control. Data are represented in replicates; error bars represent the means \pm standard deviation of the three replicates flask.

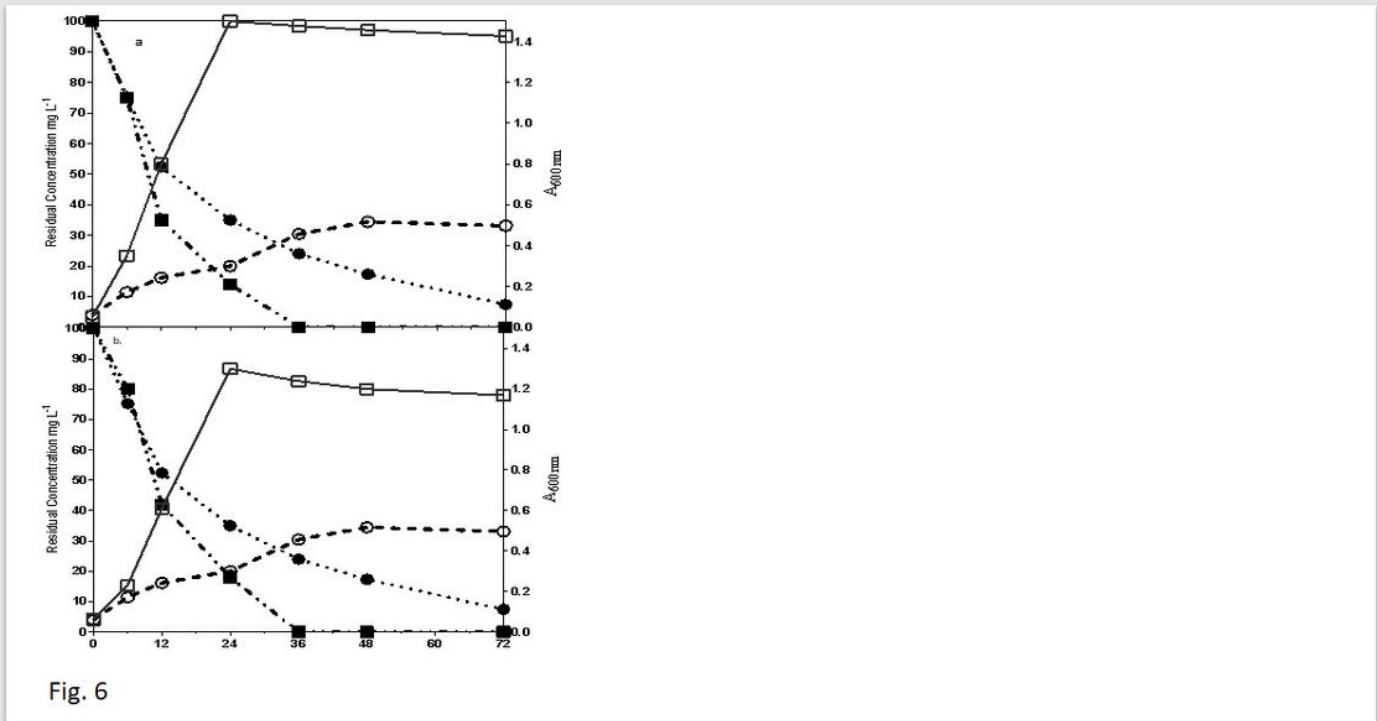


Fig. 6

Figure 6

Effect of various nitrogen sources on degradation of 2,4-DNT by *Proteus* sp. at initial concentration of 100 mg L⁻¹. Yeast (a), KNO₃ (b). □, 2,4-DNT concentration in experimental tubes with amendment; ○, 2,4-DNT concentration in non-amendment controls; □, OD600nm with amendment; ○, OD600nm; Control. Data are represented in replicates; error bars represent the means ± standard deviation of the three replicates flask.

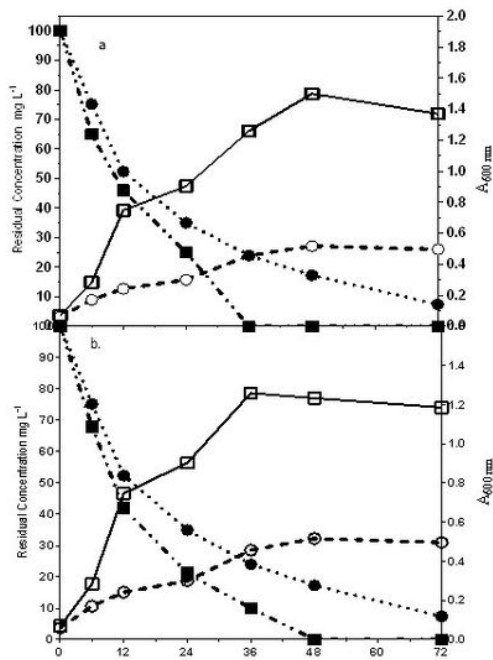


Fig. 7

Figure 7

Effect of surfactants on degradation of 2,4-DNT by *Proteus* sp. at initial concentration of 100 mg L⁻¹. Surfactant + KNO₃ (a), Surfactant (b). □, 2,4-DNT concentration in experimental tubes with amendment; ▤, 2,4-DNT concentration in non-amendment controls; ●, OD_{600nm} with amendment; ○, OD_{600nm}; Control. Data are represented in replicates; error bars represent the means ± standard deviation of the three replicates flask.