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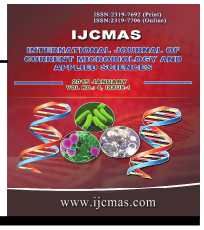

Original Research Article

Isolation, screening and assessment of microbial isolates for biodegradation of 2,4- and 2,6-dinitrotoluene

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Keywords

Nitrotoluene, Organic solvent, Substrate utilization, Biodegradation, *Rhodococcus pyridinivorans* NT2

A B S T R A C T

In this study, sixteen bacterial strains and two fungi (designated as NT1-18) were isolated from pollution impacted soil, water and effluent sludge samples. These isolates were subsequently screened on the basis of (i) broad substrate utilization (2- and 3-nitrotoluene, 2,4- and 2,6-dinitrotoluene, 3- and 4-nitroaniline, 2-, 3- and 4-nitrobenzoic acid, 4-nitrobenzaldehyde, and 4-nitrobenzyl alcohol) at 100 mg l^{-1}; (ii) tolerance to various organic solvents such as acetone, benzene, chloroform, cyclohexane, DMSO, dodecane, n-hexane, octane, p-xylene, and toluene; and (iii) minimum inhibitory concentration profiling of 2,4- and 2,6-dinitrotoluene. Of these, isolate *Rhodococcus pyridinivorans* NT2 could grow on 2,4- and 2,6-dinitrotoluene (100 mg l^{-1}) and 99% degradation was achieved within 48 h under aerobic conditions. From a biodegradation perspective, this strain may prove a promising source for bioremediation of nitrotoluenes at a commercial scale.

Introduction

The robust metabolic and biocatalytic repertoire of extremophilic microorganisms are of great industrial significance in biocatalysis, bioremediation, and other bioprocesses (Nicolaou et al., 2010; Pandey et al., 2014). Consequently, several previous studies have reported isolation and characterization of extremophilic microorganisms from ecological *niches* marked by extreme conditions of pressure, temperatures, pH, salinity and elevated concentrations of toxic contaminants (Pandey et al., 2014). However, studies on tolerance, growth and degradation of nitroaromatic compounds by organic solvent tolerant microorganisms from anthropogenic pollutant(s) impacted sites are relatively scarce. Amongst these, the majority is further limited to the genus *Pseudomonas* (Anokhina et al., 2006), *Arthrobacter* (Pandey et al., 2014), *Paracoccus* (Kirankumar et al., 2013) etc. The distinctive homeoviscous adaptations of these microorganisms to withstand the high concentration of toxic environmental toxicants are manifested via increased cell membrane rigidity (imparted by altered membrane fatty acid composition and alternation of cell morphology), changes in cell surface structure, active efflux of the toxic compounds (via energy-dependent

processes), thermostable membrane proteins, high turnover rates of various enzymes, and diverse bioactive metabolites (Alvarez, 2010). Of the recalcitrant nitroaromatics, 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are listed as a priority mutagenic and carcinogenic contaminants (Kulkarni and Chaudhari, 2007). Maximum permissible limits of 2,4-DNT and 2,6-DNT are 0.22-0.27 µM in potable water and 1.7-3.0 µM in industrial waste streams discharge under the US Environmental Protection Agency (EPA) and Code of Federal Regulations (CFR) (Han et al., 2011). Prominent electronegativity of the nitro group, highly electron deficient π-electron system and difficulty in the electrophilic attack impart greater stability, higher hydrophobicity and limited aerobic biodegradation of these xenobiotics (Kundu et al., 2011, 2014). Furthermore, microbes which could degrade 2,4-DNT are known to yield very small biomass, apparently because several intermediates of its catabolism are known uncouplers of respiration and oxidative phosphorylation (Hudcova et al., 2011). Although biodegradation of mono-NTs as sole source of carbon, nitrogen and energy is widely reported, only a few bacteria are capable of complete biomineralization of DNTs (Paca et al., 2005, 2008; Yang et al., 2008).

The genus *Rhodococcus* (Actinomycetales) thrive in a broad range of habitats, including tropical, arid and arctic soils, marine and deep sea sediments, water, and eukaryotic cells (Alvarez, 2010; Foster et al., 2014). Their suitable physiological adaptability and flexibility, along with genetic and catabolic diversity seemingly predestine actinobacteria for the degradation of aliphatic and aromatic hydrocarbons, oxygenates, halogenated compounds, nitroaromatics, heterocyclic compounds, nitriles, herbicides, and mycotoxins (Larkin et al., 2005; Martínková et al., 2009). Degradation of nitroaromatics by *Rhodococci* is mainly limited to 4-nitrophenol, 2,4-dinitrophenol and 2.4,6-trinitrophenol (Martínková et al., 2009). Recently, a 4-NT degrading *R. pyridinivorans* strain NT2 was isolated and identified (Kundu et al., 2013). Unfortunately, the use of acclimatized microorganisms especially adapted to metabolize contaminants like 2,4-DNT and 2,6-DNT at high concentration is yet to be achieved. With this perspective, this work reports the aerobic biodegradation of 2,4-DNT and 2,6-DNT by the previously isolated 4-NT degrading *R. pyridinivorans* strain NT2.

Materials and Methods

Chemicals

2,4-DNT [$CH_3C_6H_3(NO_2)_2$, CAS#121-14-2, 97%] and 2,6-DNT [$CH_3C_6H_3(NO_2)_2$, CAS#606-20-2, 98%] were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2-amino-4-NT, 2-amino-6-NT, 2,4-diaminotoluene, and 2,6-diaminotoluene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone from Sigma-Aldrich (St. Louis, MO, USA) was used as the carrier vehicle for substrate delivery in all experiments. All other chemicals were procured from HiMedia, Mumbai (India). All chemicals used were of analytical grade.

Enrichment, isolation and screening of NTs degrading isolates

Enrichment and isolation of NTs degrading microbes was carried out for each sample separately in the mineral salt basal (MSB) medium as described earlier (Kundu et al., 2013).

Metabolic versatility

Under sterile conditions, various nitroaromatic substrates (2- and 3-NT, 2,4- and 2,6-DNT, aniline, 3- and 4-nitroaniline, nitrobenzene, 2-, 3- and 4-nitrobenzoic acid, 4-nitrobenzaldehyde, 4-nitrobenzyl alcohol and o-chloronitrobenzene, 4-nitrophenol, 2,4-dinitrophenol) were supplied as sole carbon source at 100 mg l^{-1} in 250 ml flasks containing 100 ml MSB. Each flask was then inoculated with individual isolates (designated as NT1-18; cell density: OD_{600}=0.1) and incubated at 30 ^{o}C and 120 rev min^{-1} on a rotary shaker. All cultivations were performed in duplicate. A significant increase in OD_{600} was considered as positive growth. The purity of cultures showing signs of growth was checked microscopically.

Organic solvent tolerance assay

Isolates that were capable of utilizing nitroaromatic compounds such as mono-NTs (2-, 3-NT), two di-NTs (2,4- and 2,6-DNT) and other substrates (aniline, 3- and 4-nitroaniline, nitrobenzene, 2-, 3- and 4-nitrobenzoic acid, 4-nitrobenzaldehyde, 4-nitrobenzyl alcohol and o-chloronitrobenzene, 4-nitrophenol, 2,4-dinitrophenol) at 100 mg l^{-1} were analyzed for organic solvent tolerance assay as described previously (Ogino et al., 1995).

Bacterial cells were cultivated in 500-ml baffled Erlenmeyer flasks containing 50 ml of the liquid MSB medium and 15 ml of each organic solvent (acetone, dimethyl sulphoxide, chloroform, benzene, toluene, xylene, cyclohexane, n-hexane, octane and n-dodecane) at 30 ^{o}C on a rotary shaker (120 rev min^{-1}). All cultivation flasks were plugged with chloroprene rubber stoppers. The cell growth was studied by measuring the dry cell weight.

Determination of minimum inhibitory concentration (MIC)

To determine the tolerance level of the selected isolates screened from previous subsection (i.e., organic solvent tolerance assay) against 2,4- and 2,6-DNT (ranging from 100 mg l^{-1} to 400 mg l^{-1}), 100 ml MSB medium was autoclaved in 500 ml flask and then filter sterilized substrates (from a stock of saturated solution in acetone) and inoculum of OD_{600}=0.01 (from previously grown culture) was added. Cell growth was measured spectrophotometrically and substrate concentration inhibiting the microbial growth was recorded as MIC.

Contact angle and zeta (ζ) potential

Contact angle measurement (CAM) assays were performed as per Neufeld et al. (1980). Three-phase contact angle between the aqueous drop, bacterial lawn, and non-aqueous aqueous phase liquid i.e., 2,4- and/or 2,6-DNT was measured using a goniometer (Model no. 200-F4, Rame´-Hart, Netcong, NJ). For measuring zeta (ζ) potentials, the washed cells were suspended in 2 mM phosphate-citrate buffer (pH 7.0) at a concentration of 1×10^{8} cells ml^{-1}. The ζ potentials were measured at 25 °C with a Zetasizer (Nano ZS90, Malvern Instruments, UK).

Growth and degradation studies of 2,4- and 2,6-DNT

Typical biodegradation time course experiment was carried out as per Kundu et al. (2013) with minor modification. Filter-sterilized (0.45 µmol, Septrane, USA) 2,4- or 2,6-DNT at 100 mg l^{-1} (from a concentrated acetone stock solution) dispensed in 500 ml Erlenmeyer flask was supplemented with 100 ml MSB medium (pH 7.0 ± 0.2) and was incubated on a rotary

shaker (120 rev min^{-1}) at 30 ^{o}C for 7 days. Acetone was removed by evaporation prior to the addition of the aqueous medium. The assay medium in all experimental sets was adjusted to cell density of OD_{600}=0.5 (mid-log phase; ~10^7 cells ml^{-1}) unless and otherwise mentioned. The culture samples (2 ml) were collected at different time intervals and subjected to quantification for growth, NO_2^- and NH_4^+ release, residual substrate, and identification of putative intermediates. The residual 2,4-DNT and 2,6-DNT in the culture medium was calculated using the formula:

$$\text{Residual NTs (\%)} = \left(\frac{Ct}{Co}\right) \times 100 \qquad (1)$$

where, C_0 is the initial concentration of NTs in the medium and C_t is the concentration at time *t*. Non-inoculated and inoculated flasks with heat killed cells were used as abiotic and negative controls, respectively.

Analyses of samples containing 2,4-DNT/2,6-DNT and their metabolic intermediates were conducted by HPLC (with UV detector and C-18 reverse phase column) following US EPA method 8330 (Hudcova et al., 2011). The mobile phase used was methanol/water (60:40, v/v) with 0.1% TFA in water. The flow rate was fixed at 1 ml min^{-1}. The detection limit was 0.05 mg l^{-1}. NO_2^- and NH_4^+ were detected as described earlier (Kundu et al., 2013).

Cell growth was monitored by measuring the optical density at 600 nm using a UV-visible spectrophotometer (model no. 1601, Shimadzu, Japan). OD_{600} values were then converted into dry cell mass (mg l^{-1}) using an appropriate calibration curve.

Statistical analysis

Data are reported as the mean ± S.E.M. of three independent experiments. For biodegradation assays, statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). All analyses were performed using Origin v6.1 (OriginLab Corporation, Northampton, MA). $P < 0.05$ was considered to indicate level of significance.

Result and Discussion

Screening and isolation of microbial isolates capable of degrading NTs

Enrichment cultures were obtained from five different soil and wastewater effluent samples after around 10 weeks of enrichment with 4-NT as an only carbon, nitrogen and energy source. After rigorous enrichment and acclimation, a total of 18 (16 bacteria and 02 fungus) morphologically distinct isolates from MSB agar plates (containing 100 mg l^{-1} of DNTs) were obtained out of a total of 119 screened colonies (Table 1). Previously, similar serial enrichment technique with successive transfers has proved to be effective in isolating microbes endowed with potential trait to degrade nitroaromatics (Kulkarni, 2005).

Amongst all isolates, prevalence of Gram negative rods (*n*=15) was observed (83%). Although 56% (*n*=10) of the total isolates (*n*=18) exhibited tolerance to all of the twelve different substrates (100 mg l^{-1}), isolate NT2 utilized all the substrates with prolific growth as shown in Table 2. A similar strategy for assessment of metabolic versatility as outlined in the present work was employed by Mulla (2011) for 2-NT degrading bacterial consortium from pesticide-contaminated agricultural soil samples collected around Dharwad, Karnataka. Based on the results obtained, isolate NT1, 2, 3, 4, 7, 8, 11, 12, 16 and 18 were selected. The effect of various organic

solvents on the growth of these selected isolates (*n*=10) was subsequently investigated. Several aromatic and aliphatic hydrocarbons with varying log *P* (logarithm of the partition coefficient of a particular solvent between n-octanol and water; ranging from -0.23 to 6.6) were incorporated in the culture medium and growth patterns of these isolates were examined (Fig. 1). It was found that except isolate NT2, viability decay decreased significantly in all other strains against the organic solvents with a lower log *P*; prominently at log *P* <2.5. Although isolate NT2 was tolerant to log *P* values of 2.5 or higher, dry cell weight reached to 0.23, 0.22 and 0.22 g l^{-1} when grown on n-dodecane (log *P*=6.6), n-octane (log *P*=4.5) and toluene (log *P*=2.5), respectively.

Further, from the organic solvent tolerant assay, NT1, 2, 7, 11 and 16 were screened. To determine the tolerance level of these selected isolates (*n*=05) for 2,4- and 2,6-DNT, MSB medium supplemented with 1-3 mmol l^{-1} (mM) of each substrate was inoculated separately with overnight grown culture (OD_{600}= 0.01) in 100 ml flasks and the results are depicted in Fig. 2. Evidently, isolate NT2 could tolerate 2.1 and 1.6 mM l^{-1} of 2,4- and 2,6-DNT, respectively.

Besides, contact angle measurement (CAM) and cell surface charge using ζ potential was assessed for isolate NT1, 2, 7, 11 and 16 (Table 3). Of these, NT2 showed a high degree of hydrophobicity in CAM assay. The ζ potentials of NT 1 and 2 hovered around zero (0.18 and -1.68 mV, respectively), whereas NT 7, 11 and 16 strains showed distinctly negative ζ potentials (-14.5, -12.5 and -11.5 mV, respectively).

Based on the foregoing results, isolate NT2 was selected for further studies as it was able to (i) grow well in MSB containing maximum number of nitroaromatics; (ii) relatively high growth when grown on 2,4- and 2,6-DNT; and (iii) show high hydrophobicity as measured from contact angle and ζ potential. This strain was identified as *R. pyridinivorans* NT2 based on 16S rRNA gene sequencing (Kundu et al., 2013).

Tolerance to and degradation of 2,4- and 2,6-DNT by *R. pyridinivorans* NT2

The growth of strain NT2 fitted well according to the logistic model at an initial concentration of 100 mg l^{-1} (R^2= 0.99 and 0.97 for 2,4- DNT and 2,6-DNT, respectively). The Gompertz model, Richards model, von Bertalanffy model and Weibull model determines the specific growth rate and other parameters through nonlinear regression. However, parameter determination through nonlinear regression was not feasible in this study as (i) growth profile was determined using multiple batch flasks even for a fixed concentration; (ii) the number of data points in each growth profile was limited; and (iii) the growth curve was characterized by greater variability. Hence, no attempt was made to fit any other growth model to the culture growth versus time data. Similar observation was made by Ghosh et al. (2014) during biodegradation of pyrene.

Complete removal of 2,4- and 2,6-DNT (100 mg l^{-1}) was achieved within 48 h of incubation (Fig. 3). The temporal course of substrate degradation correlated well with cell growth. The growth yield of strain NT2 was found to be 0.68 (± 0.04) and 0.65 (±0.02) g of cells g^{-1} of 2,4- and 2,6-DNT, respectively. The standard error in the parentheses is the propagated error calculated based on three replications.

Table.1 DNTs (100 mg l^{-1}) degrading microbial isolates and their origin

Eco-habitat	Origin	Geographical location	Site coordinates	No. of potential isolates *	Gram character and cell morphology	
Soil and effluent	Pesticide industries	Gujarat Industrial Development Corporation (G.I.D.C.), Vapi, Gujarat	20°22'N, 72°54'E	06	NT1	Gram negative rod
					NT2	Gram positive R-C[a]
					NT3	Gram negative rod
					NT4	Gram negative rod
					NT5	Gram negative rod
					NT6	Gram negative rod
		Maharashtra Industrial Development Corporation (M.I.D.C.), Jalgaon, Maharashtra	20°59'03.15"N, 75°35'40.63"E	05	NT7	Gram negative rod
					NT8	Gram negative rod
					NT9	Gram negative rod
					NT10	Gram negative rod
					NT11	Gram negative rod
Sewage wastewater	Synthetic wet lab	University Institute of Chemical Technology (U.I.C.T.), Jalgaon, Maharashtra	21°00'56.91"N, 75°29'20.63"E	03	NT12	Gram positive cocci
					NT13	Gram negative rod
					NT14	Gram negative rod
		Department of Chemical Science, North Maharashtra University, Jalgaon, Maharashtra	21°00'56.91"N, 75°29'20.63"E	01	NT15	Gram negative rod
Soil and effluent	Waste treatment unit	Varangaon Ordnance Factory, Maharashtra	21°01'24.56"N, 75°56'27.87"E	03	NT16	Gram negative rod
					NT17	-[b]
					NT18	-[b]

Key: [a]rod-coccus growth cycle; [b]fungus; * Screened on the basis of growth ($O.D._{600}$>1.0) in MSB medium containing 100 mg l^{-1} of DNTs as sole carbon, nitrogen and energy source under shaking conditions (120 rpm) at 30 °C for 72 h.

Table.2 Utilization profile of broad spectrum of nitroaromatics by the isolates

Substrates (100 mg l^{-1})	Isolates																	
	NT1	NT2	NT3	NT4	NT5	NT6	NT7	NT8	NT9	NT10	NT11	NT12	NT13	NT14	NT15	NT16	NT17	NT18
2-NT	+++	+++	++	++	+	+	+++	++	+	+	+	++	+	+	+	+++	+	+
3-NT	+++	+++	++	++	+	+	+++	++	+	+	+	++	+	+	+	+++	+	+
2,4-DNT	++	+++	+	+	-	-	++	+	-	-	+	+	-	-	-	++	-	+
2,6-DNT	++	+++	+	+	-	-	++	+	-	-	+	+	-	-	-	++	-	+
Aniline	+++	+++	+++	++	+	-	++	++	-	+	++	++	-	+	-	+++	-	++
3-nitroaniline	+++	+++	+++	+++	+	-	++	++	-	+	+++	+++	-	+	-	++	-	+
4-nitroaniline	+++	+++	+++	+++	+	-	++	++	-	-	+++	+++	-	+	-	++	-	+
Nitrobenzene	++	+++	++	+	+	+	++	+++	+	+	+	+	-	+	-	+	++	+
2-nitrobenzoic acid	++	+++	+	+	+	+	++	++	+	+	+	+	-	+	-	++	+	+
3-nitrobenzoic acid	++	+++	+	+	-	-	++	++	-	-	+	+	-	+	-	+	+	+
4-nitrobenzoic acid	++	+++	+	+	+	+	++	+	+	+	+	+	-	+	-	+	+	+
4-nitrobenzaldehyde	++	+++	+	+	-	-	++	+	-	-	+	+	-	-	-	+	-	+
4-nitrobenzyl alcohol	++	+++	+	+	-	-	++	+	-	-	+	+	-	-	-	+	-	+
***o*-chloronitrobenzene**	++	+++	++	+	-	-	++	+	+	-	+	+	-	+	+	++	+	-
***p*-nitrophenol**	+++	+++	+++	+++	++	+	++	+	+	+	++	++	-+	+	-	++	-	++
2,4-dinitrophenol	++	+++	++	++	++	+	++	++	++	+	+	+	-	+	-	+	++	+

Key: (-), $O.D._{600} < 0.1$; (+), $0.2 \leq O.D._{600} \geq 0.5$; (++), $0.5 \leq O.D._{600} \geq 0.7$; (+++), $0.8 \leq O.D._{600} \geq 1.0$ after 72 h of growth.
The experiment was carried out in triplicates and the difference in the individual results was < 5%.

Table.3 Determination of water contact angle and zeta potential of the selected isolates

Isolates	CAM (^oC) (*n*=10)		Zeta potential (mV)	
	2,4-DNT	2,6-DNT	2,4-DNT	2,6-DNT
NT1	19.6±2.3	19.1±2.5	0.18±2.7	0.15±2.1
NT2	116±2.8	113±3.1	-1.68±0.9	-1.63±0.7
NT7	25.3±0.7	28.5±1.4	-14.5±1.6	-13.2±1.1
NT11	39.3±3.5	39.1±2.7	-12.5±3.8	-14.5±3.3
NT16	68.3±1.8	64.6±1.2	-11.5±0.6	-10.8±1.4

Fig.1 Comparative growth of the selected isolates in the presence of volatile organic solvents of varying log *P* values (in parentheses). Data values represent mean ± standard deviation of triplicates. Small (non-visible) standard deviations are within the symbols. Control, without solvent

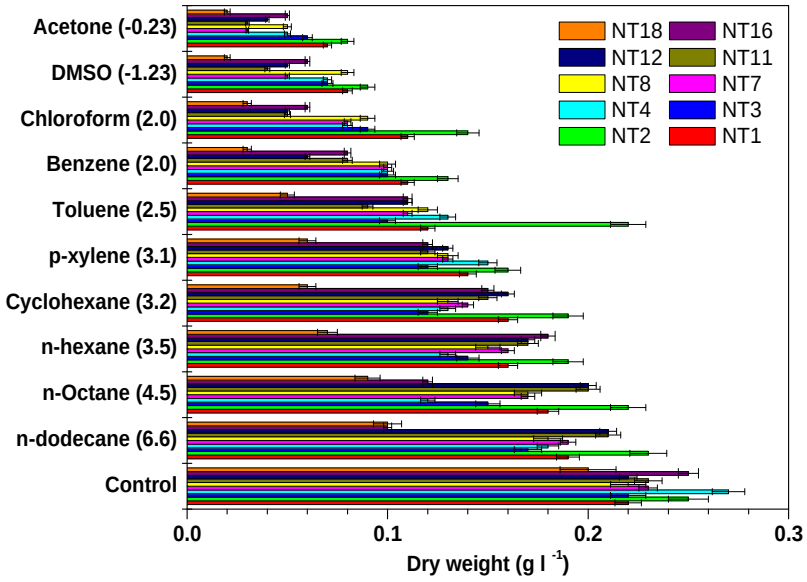
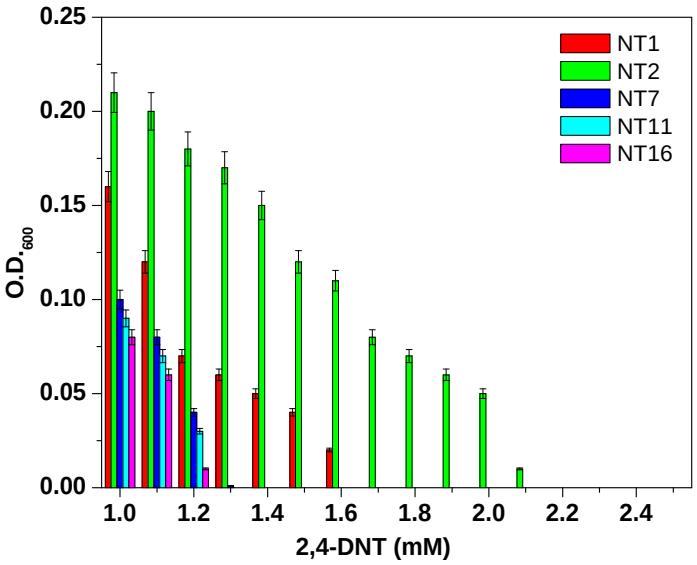


Fig.2 MIC profile of the selected isolates for various concentrations of (a) 2,4-DNT and (b) 2,6-DNT as a function of growth in MSB medium inoculated with 0.01 OD at 30 ^{o}C. Data values are mean ± standard deviation of triplicates. Small (non-visible) standard deviations are within the symbols



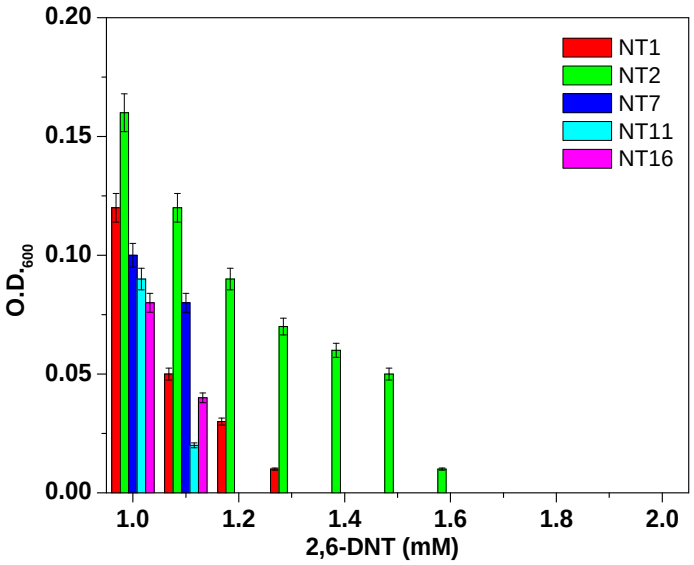
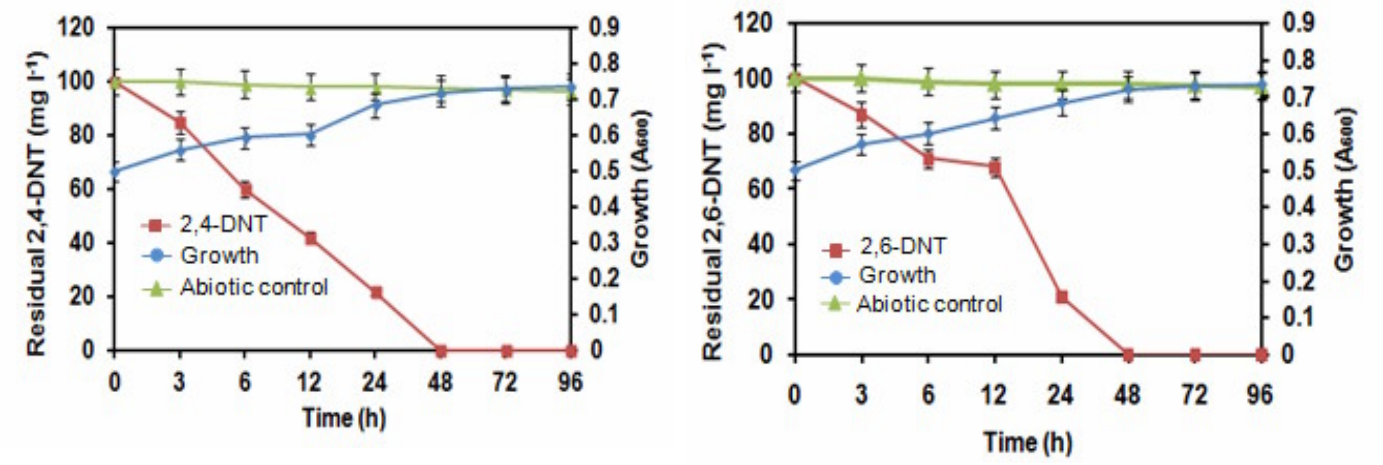


Fig.3 Time course profile for degradation of (a) 2,4-DNT and (b) 2,6-DNT by *R. pyridinivorans* NT2 at 100 mg l^{-1}. Data values are mean ± standard deviation of triplicates. Small (non-visible) standard deviations are within the symbols



Similarly, the rate of 2,4- and 2,6-DNT degradation reached 1.38 and 2.08 mg l^{-1} h^{-1}, respectively. Active biodegradation of DNTs increased with the increase in cell count exponentially in the test samples, while no degradation was observed during the stationary phase.

In all the studies, abiotic loss of DNTs determined in the un-inoculated control flasks was in the range of 0-10%. Therefore, the overall degradation of DNTs obtained over the study period in the inoculated flasks was primarily due to biodegradation by strain NT2. The contribution of adsorption of 2,4- or 2,6-DNT to bacterial biomass to the overall loss may be ignored since the extraction solvent was added directly to the culture broth. Thus, the solvent is expected to solubilize both residual DNTs remaining in solution and DNTs adsorbed on to the biomass.

To conclude, a mesophilic organic-solvent tolerant actinobacterium *Rhodococcus pyridinivorans* strain NT2 has the ability to degrade 2,4- and 2,6-DNT as sole carbon, nitrogen and energy source. Given the unique metabolic capabilities and distinctive adaptive responses described here, *R. pyridinivorans* strain NT2 could potentially be exploited as a potential candidate for bioremediation of the DNTs-containing waste or industrial discharge.

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Conflict of Interest

None declared

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