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ORIGINAL PAPER

Biodegradation of 4-nitrotoluene with biosurfactant production by *Rhodococcus pyridinivorans* NT2: metabolic pathway, cell surface properties and toxicological characterization

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Abstract A novel 4-nitrotoluene-degrading bacterial strain was isolated from pesticides contaminated effluent-sediment and identified as *Rhodococcus pyridinivorans* NT2 based on morphological and biochemical properties and 16S rDNA sequencing. The strain NT2 degraded 4-NT (400 mg l^{-1}) with rapid growth at the end of 120 h, reduced surface tension of the media from 71 to 29 mN m^{-1} and produced glycolipidic biosurfactants (45 mg l^{-1}). The biosurfactant was purified and characterized as trehalose lipids. The biosurfactant was stable in high salinity (10 % w/v NaCl), elevated temperatures (120 °C for 15 min) and a wide pH range (2.0–10.0). The noticeable changes during biodegradation were decreased hydrophobicity; an increase in degree of fatty acid saturation, saturated/unsaturated ratio and cyclopropane fatty acid. Biodegradation of 4-NT was accompanied by the accumulation of ammonium (NH_4^+) and negligible amount of nitrite ion (NO_2^-). Product stoichiometry showed a carbon (C) and nitrogen (N) mass balance of 37 and 35 %, respectively. Biodegradation of 4-NT proceeded by oxidation at the methyl group to form 4-nitrobenzoate, followed by reduction and hydrolytic deamination yielding protocatechuate, which was metabolized through β-ketoadipate pathway. In vitro and in vivo acute toxicity assays in adult rat (*Rattus norvegicus*) showed sequential detoxification and the order of toxicity was 4-NT >4-nitrobenzyl alcohol >4-nitrobenzaldehyde >4-nitrobenzoate >> protocatechuate. Taken together, the strain NT2 could be used as a potential bioaugmentation candidate for the bioremediation of contaminated sites.

Keywords *Rhodococcus* · 4-Nitrotoluene · Biodegradation · Biosurfactant · Toxicity

Introduction

Among nitroaromatics, 4-nitrotoluene (4-NT) is a platform commercial chemical compound with an annual production volume of over 10 million pounds (Dunnick et al. 2003). 4-NT is extensively used as a chemical feedstock for synthesis of agricultural and rubber chemicals, explosives (di- and tri-nitrotoluene), azo and sulfur dyes, dyes for cotton, wool, silk, leather, paper and intermediate for plastic foams, dyestuffs, paints, optical brighteners (Ramalingam et al. 2010). Its widespread usage in various industries led to serious environment threat due to contamination of terrestrial and aquatic ecosystems and resulted in cytotoxicity, genotoxicity, necrosis, mutagenicity, and carcinogenicity in animals and humans (Snellinx et al.

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2002; Paden et al. 2011). Hence, 4-NT is recognized as priority pollutant by the U.S. Environmental Protection Agency (EPA) (Mulla et al. 2011a, b) with maximum permissible level of 0.51 μmol in discharge streams (40 CFR, Sect. 268.48, US EPA) and 2–5 mg m^{-3} in air. For these reasons, it is of considerable interest to examine the metabolic fate of nitroaromatic compounds in the environment.

While abiotic degradation of 4-NT involves non-specific reduction of nitro to amino derivatives, microbial removal of the nitro group has been reported to occur either by reductive or oxidative mechanisms prior to aromatic ring cleavage (Ju and Parales 2010). The oxidative degradation of 4-NT by several *Pseudomonas* strains involves formation of methylcatechols with concomitant release of nitrite, whereas reductive degradation leads to formation of toxic amino compounds with subsequent release of ammonia either through the partially reductive hydroxylaminolyase-mediated pathway or a mutase-catalyzed intramolecular conversion of the hydroxylamino compound to an ortho-aminophenol (Ju and Parales 2010). However, earlier reports pertinent to NT degradation products indicated higher toxicity than parent compound, while few reports reported reduced toxicity (Dodard et al. 1999) leading to an apparent controversy on toxicology of 4-NT. Moreover, no focus has yet been accorded to improve (i) degradation at higher concentrations, either as a function of nutritional supplements or physico-chemical parameters and (ii) dynamic changes in cell surface properties through biosurfactant augmentation (Cameotra and Singh 2009; Kundu et al. 2011).

Keeping in view these limitations, the objective of this study was to evaluate the biodegradation potential of a novel and indigenous *Rhodococcus* strain, the effect of its biosurfactant on cell surface properties and emulsification of 4-NT and lastly, to carry out an ecotoxicological risk assessment of parent and biodegradation intermediates to adequately reflect toxicity benchmarks.

Materials and methods

Bacterial strain

An indigenous bacterial strain was isolated from effluent-sediment collected from a pesticide manufacturing facility in Vapi, Gujarat Industrial Development Corporation (G.I.D.C., latitude 20°22'N, longitude 72°54′E), Gujarat, India. The strain NT2, used as 4-NT degrader, was able to grow utilizing 4-NT (100–400 mg l^{-1}) as the sole carbon and nitrogen source on the minimal medium. It was maintained on mineral salt basal (MSB) medium (pH 7.0 ± 0.2) containing 4-NT (100 mg l^{-1}) at 4 °C.

Chemicals

4-NT [$C_6H_4(CH_3)NO_2$, CAS#99-99-0] was purchased from Sigma–Aldrich (Steinheim, Germany) with 98 % purity. Acetone from Sigma–Aldrich (St. Louis, MO, USA) was used as the carrier vehicle for all experiments. All other chemicals were procured from Hi-media, Mumbai (India). All chemicals used were of analytical grade.

Medium and conditions for the growth of culture

The constituents of mineral salt basal (MSB) medium (pH 7.0 ± 0.2) were (g l^{-1}) K_2HPO_4, 0.75; KH_2PO_4, 0.2 and $MgSO_4.7H_2O$, 0.09. To prepare 4-NT-MSB medium (pH 7.0 ± 0.2), filter-sterilized (0.45 μm membrane filter, Septrane) 4-NT (from a concentrated stock solution in acetone) was delivered to the sterile empty Erlenmeyer flask to a final concentration of 400 mg l^{-1}. The acetone was allowed to evaporate in an operating fume hood. Then, sterile MSB solution was added. Before every experiment, cells of strain NT2 were induced at mid-log phase, harvested, washed with 100 ml MSB, and finally, resuspended in 4-NT supplemented MSB to get a final 0.5 OD units at 600 nm. It was then incubated in the orbital shaker at 120 rev min^{-1} and 30 °C.

Identification of the strain NT2

Morphological, physiological and biochemical characterization

The morphology of exponentially growing cells was examined by light microscopy and scanning electron microscopy (XL 30ESEM, Philips). Biochemical tests were performed as described elsewhere (Yoon et al. 2000).

Whole-cell fatty acids methyl ester (FAME) analysis

The strain NT2 was also identified based on whole-cell fatty acids, derivatized to methyl esters, and analyzed by gas chromatography (Agilent 6850 Series II) using the Sherlock microbial identification system (MIS-MIDI, USA) as described by Mrozik et al. (2011) and Kaczorek et al. (2013a, b).

Fatty acid compositional analysis of strain NT2 was also analyzed before and after biodegradation. For this, cells were harvested by centrifugation (8,000 g) at 4 °C for 20 min. Cell pellets were washed twice with 0.85 % NaCl to remove residual culture medium. Further fatty acid isolation and identification was conducted using the MIDI-MIS method as per Mrozik et al. (2011) and Kaczorek et al. (2013a, b).

16S ribotyping and phylogenetic analysis

Taxonomic characterization based on the nucleotide sequence of 16S rRNA gene was confirmed by Royal Life Science Pvt. Ltd., Hyderabad, India. DNA extraction from single colony was performed as per Ausubel et al. (2003). PCR amplification of the 16S rRNA gene was performed using B27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and Univ1517R (5′ ACG GCT ACC TTG TTA CGA CTT 3′) oligoprimer pairs on a GeneAMP PCR System 9700 thermal cycler in 25 µl reaction mixture. The PCR conditions comprised of (i) initial denaturation step (95 °C, 10 min), (ii) 25 cycles of (a) denaturation (95 °C, 1 min), (b) annealing (55 °C, 1 min) (c) extension (72 °C, 1.30 min) and (iii) final extension (72 °C, 10 min). PCR products were purified using Qiagen PCR product purification kit before being prepared for sequencing using the fluorescence-based ABI BigDyeTM terminator chemistry as per manufacturer's instructions. DNA sequencing was performed in automated ABI 3730XL DNA Analyzer (Applied Biosystems, USA) and basecalled using Sequence Scanner v1.0. Sequence similarities were inferred from NCBI GenBank database using BLAST (Altschul et al. 1997) for phylogenetic analysis using sequences of the related taxa. A neighbour joining tree (Saitou and Nei 1987) was constructed based on distance matrices calculated according to the Kimura two-parameter model (Kimura 1980) using MEGA v5.04 (Tamura et al. 2011).

Experimental set up for biodegradation studies by strain NT2

Sterile MSB medium (100 ml; pH 7.0 ± 0.2) containing 400 mg l^{-1} of 4-NT was prepared as described in 'Medium and conditions for the growth of culture'. Cultures were inoculated to a cell density of 0.5 OD units at 600 nm. The flasks were incubated on a rotary shaker (120 rpm) at 30 °C for 120 h. Samples were withdrawn at periodic intervals for analysis of cell growth (A_{600} and cells ml^{-1}), substrate utilization (residual 4-NT), release of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and surface tension.

The OD value from cell growth was converted to dry cell mass and cells ml^{-1} using appropriate calibration curves. Ammonia was detected by Nessler's reagent (McClure and Venables 1986) and nitrate was analyzed using a spectrophotometric method at 275 nm following standard methods for the examination of water and wastewater (APHA 2001). Nitrite was assayed by Griess reaction as described by Montgomery and Dymock (1961). The equilibrium surface tension was measured using the du Nouy ring technique (DCA 315, Thermo Cahn, USA) at 21 ± 1 °C. Stabilization and equilibrium of each analysis was performed until the standard deviation of 10 successive measurements was below 0.4 N m^{-1}. Each result presented was the mean of three independent experiments. Maximum deviations from the average (error bars) are indicated.

To study the intermediates formed during the degradation of 4-NT (400 mg l^{-1}) by isolate NT2 at various time intervals, samples were periodically withdrawn from the spent culture (MSB) medium, centrifuged (6000 g, 15 min) and extracted twice with di-ethyl ether. The extracts were evaporated to dryness at 30 °C and redissolved in 0.5 ml methanol. Initial monitoring was done by TLC on silica gel G plates using toluene:ethyl acetate:acetic acid (60:30:10, v/v/v) as the mobile phase and were visualized under ultraviolet (UV) light (A_{254}). High performance thin layer chromatography (HPTLC) analyses of each sample were performed on a CAMAG system (CAMAG, Switzerland) as per Kulkarni and Chaudhari (2006). The intermediates were also analyzed by (i) HPLC following US EPA method 8330, (ii) GC-MS (Mulla et al. 2011b), (iii) ^{1}H NMR measurements by a Bruker 400 MHz NMR spectrometer using tetramethylsilane (TMS) as the internal standard, (iv) LC-MS using a

Finnigan MAT LCQ ion-trap mass spectrometer (Thermo-Fischer, MA, USA) with an electrospray ionization source coupled to a modular Spectrasystem LC system (Sagi-Ben Moshe et al. 2009) and (v) FT-IR recorded on FT-IR spectrometer (Nicolet 6700, Thermo Fisher, USA) at resolution of 0.5 cm^{-1} with an average of 32 scans. A ring cleavage inhibition study was carried out using an iron chelater, 2,2′-dipyridyl and samples were analyzed by HPLC (Pandey et al. 2011). To detect the presence of β-ketoadipate, Rothera's test was carried out. Briefly, 5.0 ml clear supernatant of periodically drawn aliquots was saturated with ammonium sulphate and a few drops of sodium nitroprusside solution were added. About 2.0 ml of liquid ammonia was carefully added to the tubes to form a layer on the top of the aqueous solution.

The cytotoxicity of the parent 4-NT and its biodegradation intermediates was evaluated by animal acute toxicity assay and are described in the Supplementary Material (Data S1).

Cell surface hydrophobicity (CSH)

Bacterial cell adhesion to 4-NT was measured by determining the changes in cell surface hydrophobicity during growth in MSB medium supplemented with 4-NT (400 mg l^{-1}) (Rosenberg et al. 1980). The cell surface hydrophobicity was expressed in terms of percentage of cells transferred to the NT-phase. Each experiment was repeated three times and the values of cell hydrophobicity were calculated as a mean value of three experiments.

Production, extraction and purification of the biosurfactant

Biosurfactant production medium and culture conditions were the same as described in biodegradation assay. Following incubation, the culture broth was centrifuged at 6000 g, 15 °C for 15 min. The cell free supernatant was extracted with equal volumes of chloroform/methanol (2:1 v/v), the organic fraction was pooled and dried. The biosurfactants thus obtained were weighed and expressed in mg l^{-1} of the cell free culture broth.

The crude extract was dissolved in dichloromethane/methanol and loaded to a silica gel 60 column at normal pressure (70–230 mesh, HiMedia, Mumbai, India). The column was equilibrated with *n*-heptane and washed with three column volumes of the *n*-heptane to remove the residual 4-NT and also compounds less polar than the biosurfactants. The surface-active compounds were eluted with dichloromethane/ethanol (CH_2Cl_2/C_2H_5OH) with increasing polarity from 8:2 to 1:1 (Tuleva et al. 2008). Collected fractions (5 ml) were then used to detect biosurfactants following alkaline hydrolysis based on the methods detailed by Peng et al. (2007) and Tuleva et al. (2008).

Structural and physicochemical characterizations of the biosurfactant

Fourier transform infrared (FT-IR) spectra of the purified biosurfactants (KBr pellet method) were recorded on Nicolet 6700 FT-IR spectrometer (Thermo Fisher, USA) at a resolution of 0.5 cm^{-1} with an average of 32 scans.

A Finnigan MAT LCQ Ion-Trap Mass Spectrometer (Thermo-Fischer, MA, USA) with an electrospray ionization (ESI) source coupled to a modular Spectrasystem LC system was used for identification of the biosurfactants as described earlier (Tuleva et al. 2008). Homologues of the biosurfactant were quantified from the molecular proportion of each of the pseudomolecular ions calculated by LC-ESI-MS.

The equilibrium surface tension and interfacial tension (against *n*-hexadecane) was measured using the du Nouy ring technique by tensiometer (DCA 315, Thermo Cahn, USA) at 21 ± 1 °C. The critical micelle concentration (CMC) of the biosurfactant was determined from the break point of the surface tension as a function of concentration curve. Stabilization and equilibrium of each analysis was performed until the standard deviation of 10 successive measurements was below 0.4 mN m^{-1}. Each result presented was the mean of three independent experiments.

The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Meylheuc et al. 2001). The capacity of the partially purified biosurfactant to emulsify 4-NT was examined as per Bouchez-Naïtali and Vandecasteele (2008). Briefly, 1.0 ml of biosurfactant solution (30 mg l^{-1}) was vortex-shaken (2 min) with 200 µl of 4-NT and absorbance (A_{600}) was taken directly after 2 and 24 h of settling. For measuring emulsification index,

hydrocarbons or oils were also added to aqueous phase containing the biosurfactant (30 mg l^{-1}), in a ratio of 3:2 (v/v), followed by vigorous agitation on a cyclo-mixer for 2 min. The oil, emulsion and aqueous layers were measured at every 24 h interval up to 240 h. The emulsification index was noted with respect to time and is represented accordingly, i.e. the emulsification indices after 24, 48, 72 h, etc. were denoted as E_{24}, E_{48}, E_{72}, respectively. Each measurement for a given sample was made thrice in separate tubes and each result was the mean of three measurements.

The effect of several environmental parameters on the surface activity of the biosurfactant was determined. NaCl at different concentrations, 0–10 % (w/v), was mixed with the purified biosurfactant (30 mg l^{-1}) and the surface tension (ST), CMD^{-1} and CMD^{-2} were then measured. The pH of the purified biosurfactant (30 mg l^{-1}) was adjusted to different values (2–10) using 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl. Afterward, the ST, CMD^{-1} and CMD^{-2} were measured. To determine the heat stability of the biosurfactant, 30 mg l^{-1} of biosurfactant was heated in an autoclave to 100 and 121 °C for 15 min and allowed to cool to room temperature. The biosurfactant properties including ST, EI_{24} (%), CMD^{-1} and CMD^{-2} were measured and compared to the corresponding values before heat treatment.

Statistical analyses

Data are reported as the mean ± S.E.M. of three independent experiments. For biodegradation assay and toxicity data, statistical analysis of differences was carried out by one-way analysis of variance (ANOVA). All analyses were performed using Minitab statistical software (release 16; Minitab Inc., State College, PA). $P < 0.05$ was considered to indicate significance.

Results

Isolation, identification and characterization of the 4-NT degrading strain NT2

An indigenous bacterial strain NT2 able to grow on 4-NT at concentrations approaching the solubility of 4-NT in water (400 mg l^{-1}) was isolated from effluent-sediment of a pesticide manufacturing facility. This isolate also grew on mononitrotoluenes (2- and 3-NT), two dinitrotoluenes (2,4-and 2,6-DNT) and other substrates (3- and 4-nitroaniline, 2-, 3- and 4-nitrobenzoic acid, 4-nitrobenzaldehyde and 4-nitrobenzyl alcohol) at 100 mg l^{-1} (Table S1). Growth of NT2 and depletion of concentration of each compound was found to be significant ($\alpha = 0.05$) with respect to the duration according to Duncan multiple range test (data not shown).

The isolate NT2 was aerobic, Gram-positive, non-spore-forming and non-motile. Cells were irregular rods with a typical rod-coccus cycle (Fig. S1). This bacterium forms a short mycelium and long rods of variable length when grown on solid medium during the early growth phase. Mycelia fragment into short rods and cocci as the culture aged. These characteristics are consistent with earlier reports of the genus *Rhodococcus* (Yoon et al. 2000; Goodfellow et al. 2004). Neither aerial hyphae nor diffusible pigments were formed. Typical colonies of isolate NT2 on MSB agar (400 mg l^{-1}, 4-NT) were pale orange and flat. The isolate NT2 (i) possessed an ability to grow at 28, 30 and 45 °C, (ii) showed amylase, catalase, lipase, protease and urease activities and absence of oxidase, DNase and lecithinase, (iii) utilized and fermented glucose, (iv) showed growth at 6 % (w/v) NaCl and (v) lacked haemolysis, arginin dihydrolase and pyrrolidonyl arylamidase (Table S2). These data are in full agreement that strain NT2 may belong to the genus *Rhodococcus* (suborder *Corynebacterineae*) (Fazlurrahman Batra et al. 2009; Ghosh et al. 2010). Predominant fatty acids detected in FAME analysis were 14:0, 16:0, 18:0, 10Me16:0, 10Me18:0, 16:1*w*6*c*, 16:1*w*7*c*, 18:1*w*9c typical of the genus *Rhodococcus*. A similarity index of 0.807 to *Rhodococcus* was calculated based on guidelines suggested by Microbial ID, Inc.

The 16S ribotyping of the isolate NT2 revealed phylogenetic affiliation to the genus *Rhodococcus*. The rRNA gene sequence (1382 bp) shared 99 % sequence homology and query coverage with *R. pyridinivorans* strain SB 3094 (GU191923), PD7-2 (AB506120) and PDB9 (NR025033). The evolutionary relationships of the isolate NT2 determined by neighbor joining algorithm (Fig. 1) revealed highest degree of relatedness with GU191923 and AF459741. The results corroborated with the physiological characteristics of *R. pyridinivorans* as described by Yoon et al. (2000) except casein hydrolysis and production of protease, lipase and starch hydrolytic enzymes in contrast to NR025033. However, the strain showed a

strong pyridinolytic activity, a typical characteristic feature of the species. Based on these observations, it was inferred that this strain belongs to the genus *Rhodococcus* with *R. pyridinivorans* as its closest neighbor. The sequence was deposited to GenBank with an assigned accession number JQ229777. Several *Rhodococcus* sp. showed novel catalytic functions to metabolize nitroaromatics and other chemically diverse xenobiotics (Larkin et al. 2005).

Growth and biodegradation profile of strain NT2 at low and high concentration of 4-NT

Time course biodegradation studies were carried out with initial inocula of 0.5 OD units ($\sim 1.0 \times 10^7$ cells ml^{-1}). MSB medium containing 4-NT (100 mg l^{-1}) and pre-grown (pre-induced) cells of NT2 utilized 4-NT rapidly within 24 h under strictly aerobic conditions at 30 °C with a specific growth rate of 0.18 h^{-1}. When the 4-NT concentration was increased to 400 mg l^{-1}, the strain grew exponentially at $\mu = 0.12\ h^{-1}$ with a concomitant increase in biomass to 2.4 OD units ($\sim 2.4 \times 10^{10}$ cells ml^{-1}). Although the total biodegradation process was allowed to proceed upto 120 h, no significant change in growth, 4-NT utilization and surface tension reduction occurred after approximately 80 h. The extent of biodegradation of 4-NT was found to be 98 % (0.036 mmol h^{-1}) (Fig. 2a). There was a lag phase of about 12 h in the degradation of 4-NT because inocula were prepared with mid-log NT2 pre-cultures grown on 4-NT. There was a positive correlation between percentage of degradation and time, significant at the 0.01 level (2-tailed) according to the Pearson correlation of 0.985 (=1). The removal of 4-NT was accompanied by the formation of ammonium (NH_4^+) which attained a plateau of 90 mg l^{-1} (5 mmol) at 95 h whereas the nitrite (NO_2^-) concentration reached only 0.092 mg l^{-1} (2 µmol) (Fig. 2b) during the biodegradation period. Similar results were obtained when cells were induced by growth on 4-nitrobenzoate, centrifuged, washed and resuspended in minimal medium lacking any inorganic nitrogen but containing 4-nitrobenzoate. We did not observe any nitrate (NO_3^-) in the culture supernatant nor were any traces of NH_4^+, NO_2^-

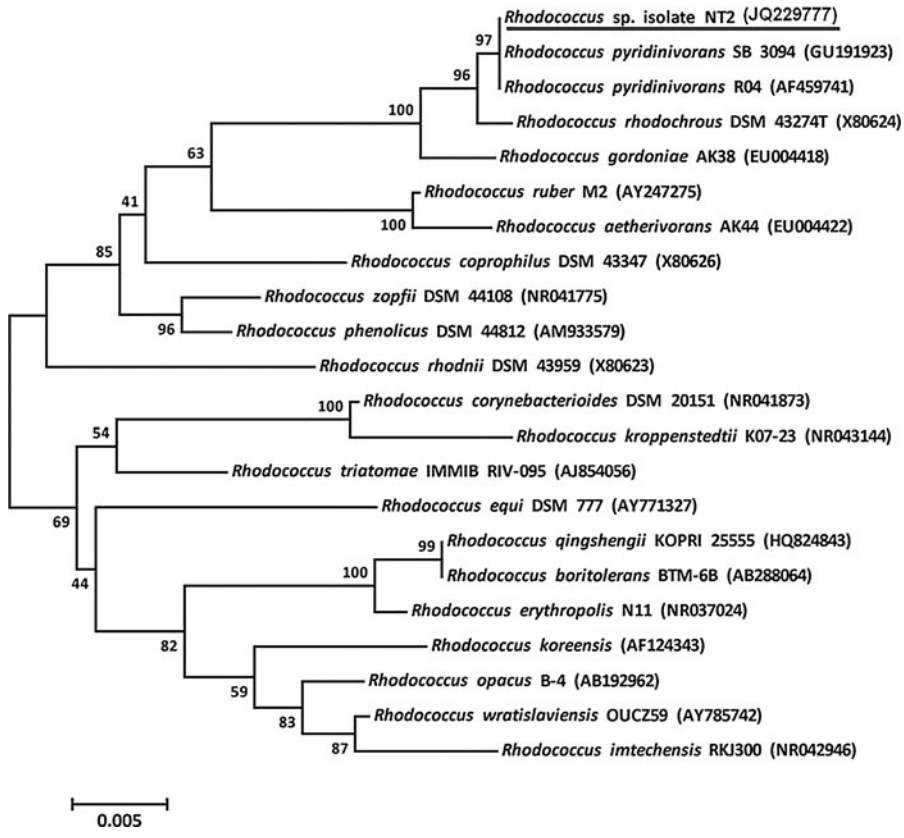

Fig. 1 Evolutionary relationships of *Rhodococcus* sp. isolate NT2 (JQ229777) with its relatives inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. *Figures* next to the *branches* represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The evolutionary distances are in the units of the number of base substitutions per site. *Text in the parenthesis* represents the GenBank accession numbers

and NO_3^- in controls containing 4-NT in the absence of *Rhodococcus* found.

The degradation of 4-NT at 400 mg l^{-1} by *R. pyridinivorans* NT2 was monitored on TLC, HPTLC, HPLC, GC-MS, LC-MS, FTIR and ^1H NMR as a function of time. TLC analysis of samples drawn at periodic intervals showed total disappearance of 4-NT at 48 h. Samples drawn at 0, 24, 48, 72, 96 and 120 h time-intervals showed R_f values identical to that of authentic 4-NT, 4-nitrobenyl alcohol, 4-nitrobenzaldehyde, 4-nitro benzoate and protocatechuate, respectively in HPTLC densitogram (Fig. 3). HPLC profile of isolated metabolites showed retention times of 15.0, 5.0, 6.0, 4.5 and 4.8 min and corroborated with standard 4-NT, 4-nitrobenyl alcohol, 4-nitrobenzaldehyde, 4-nitrobenzoate and protocatechuate, respectively (Fig. 4). These results were further validated by (i) R_t (min) values from total ion chromatogram (TIC) and mass spectra (Fig. S2 and S3) and (ii) molecular peaks and fragmentation patterns from GC-MS (Table S3). The MS spectrum of isolated metabolite from culture supernatant withdrawn at 120 h (after biodegradation) showed molecular peak M^+ at *m/z* 157 and is in good agreement with empirical formula $C_7H_6O_4$. The fragmentation pattern revealed base peaks at *m/z* 139.1 (M^+ –OH), 113 (M^+ –2OH) and 103.2 (M^+ –COOH) which cumulatively corroborated with protocatechuate (Table S3). The FTIR spectra of isolated

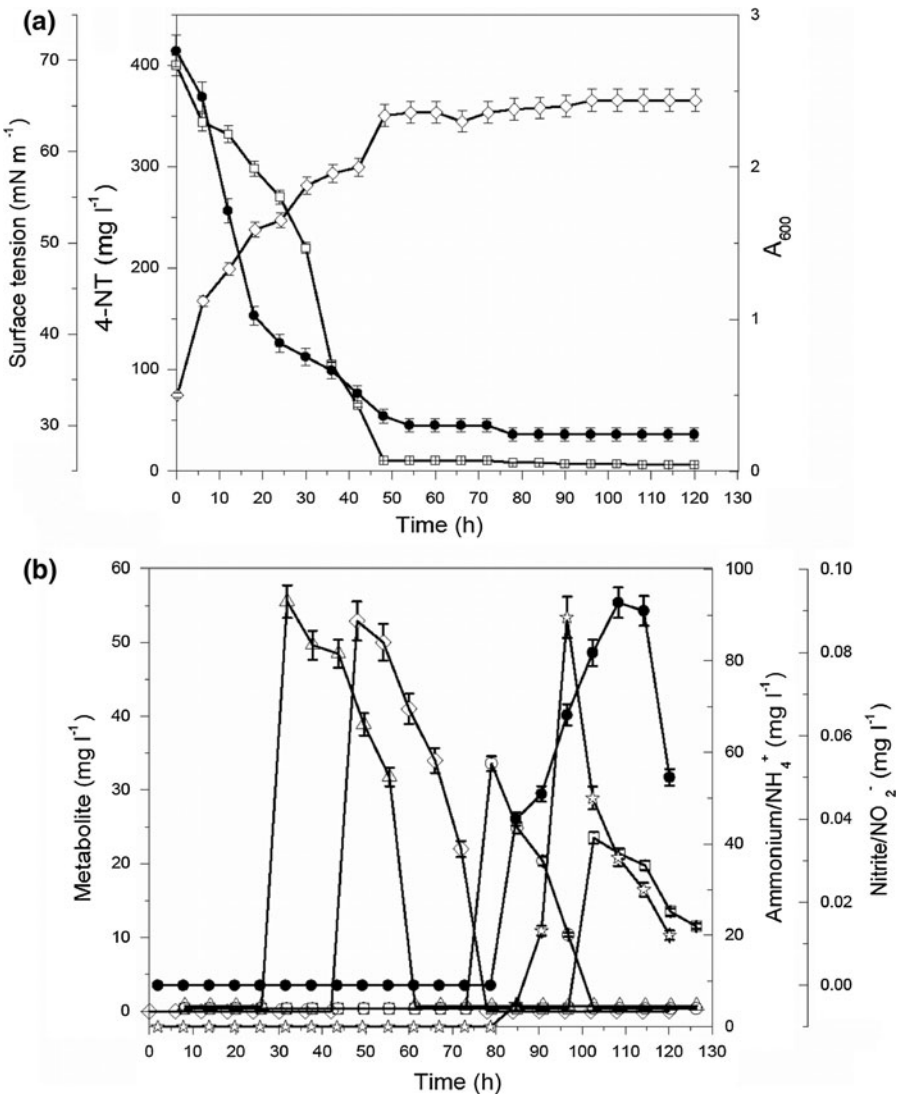

Fig. 2 **a** Degradation profile of 4-NT (400 mg l^{-1}) by *R. pyridinivorans* NT2 in MSB at 30 °C for 120 h (pH 7.0 ± 0.2): 4-NT (*open squares*); growth (*open diamonds*); and surface tension (*filled circles*). **b** Quantitative HPLC/GC analysis showing time course for evolution of intermediates during degradation of 4-NT (400 mg l^{-1}) by induced NT2 cells: 4-nitrobenyl alcohol (*open triangles*); 4-nitrobenzaldehyde (*open diamonds*); 4-nitrobenzoate (*open circles*); protocatechuate (*open squares*); NH_4^+ (*open stars*); and NO_2^- (*filled circles*). Data values represent mean ± standard deviation of triplicates. Small (non-visible) standard deviations are within the symbols

metabolites also showed characteristic absorption bands of –OH stretching at 3078 cm^{-1}, C=O stretching at 1674 cm^{-1}, aromatic CH stretching at 2900 cm^{-1}, C–O stretching at 1299 cm^{-1} and C=C stretching at 1400 cm^{-1}. The proton magnetic resonance spectrum of the intermediate metabolites demonstrated arrays of aromatic protons appearing as multiplets ranging from δ 6.7 to δ 7.3 ppm, a phenolic hydroxyl proton at δ 9.5 ppm and an acid proton at δ 12.3 ppm. Further, inhibition of the ring cleavage of protocatechuate performed using iron chelator 2,2′-dipyridyl indicated the inhibition of ring cleavage dioxygenase that needs Fe^{2+} to catalyze ring fission during degradation of 4-NT by *R. pyridinivorans* NT2. Thus, protocatechuate appeared to be a terminal aromatic compound in the catabolic degradation of 4-NT by *R. pyridinivorans* NT2. Further, Rothera's positive test confirmed ring fission and may possibly lead to the formation of keto acid.

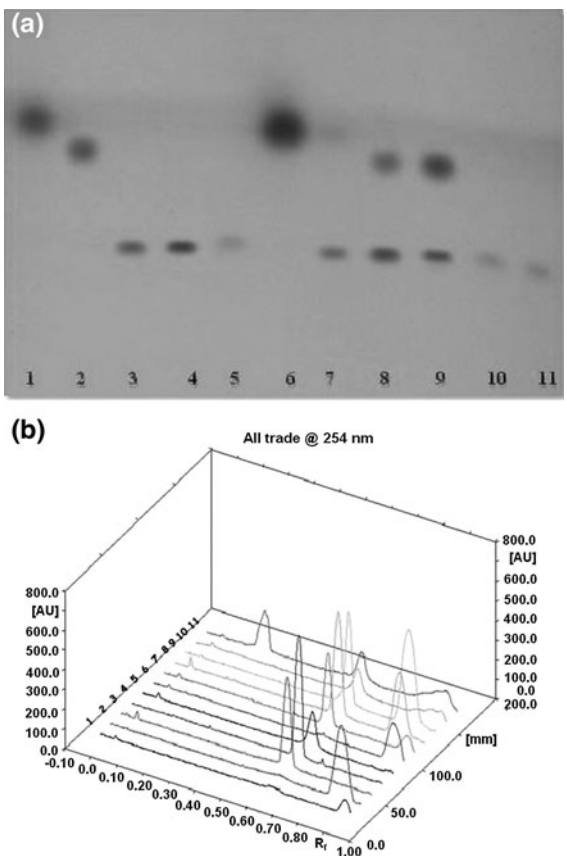

Fig. 3 Biodegradation of 4-NT (400 mg l^{-1}) monitored by HPTLC, **a** scanning chromatogram and **b** densitogram (A_{254}). 1–5: standard 4-NT, 4-nitrobenzaldehyde, 4-nitrobenyl alcohol, 4-nitro benzoate and protocatechuate, respectively; 6–11: samples withdrawn at 0, 24, 48, 72, 96 and 120 h time-intervals

We found an N-mass balance of 35 % distributed as follows: NH_3 measured as NH_4^+ (32 %) and NO_2^- (3 %). Regarding the carbon stoichiometry after 5 days of incubation, the total carbon recovery was 37 % and it was distributed as follows: 4-nitrobenzyl alcohol (12 %), 4-nitrobenzaldehyde (10 %), 4-nitrobezoic acid (7 %), protocatechuate (5 %) and β-ketoadipate (3 %). The remaining 65 % N and 63 % C were probably used for the production of new biomass and biosurfactants, or excreted as other mineralized products. Controls containing 4-NT without strain NT2 retained close to 99.5 % of unreacted 4-NT. This C and N stoichiometric mass balance seems plausible considering the fact that 4-NT was used as sole source of C and N.

Acute toxicity studies provide reliable primary quantitative and qualitative data to evaluate toxic characteristics of substances and to conduct environmental hazard risk assessments. In this study, in vitro 6 h-LD_{50} (95 % CI) were found to be 0.3, 0.03, 0.02, 0.01, 0.001 μg ml^{-1} for 4-NT, 4-nitrobenzyl alcohol, 4-nitrobenzaldehyde, 4-nitro benzoate and protocatechuate, respectively. MTT assay with 4-NT-treated primary rat hepatocyte cultures showed a time-dependent decrease in percentage viability of liver cells while samples drawn at 24, 48, 72, 96, and 120 h post degradation revealed no perceptible change in cell viability (Fig. S4). Moreover, parent 4-NT was found to induce higher reactive oxygen species (ROS) level as compared to biotreated samples drawn at periodic time-intervals (Fig. S5).

Hydrophobicity of *R. pyridinivorans* NT2 and biosurfactant production during biodegradation of 4-NT

In the initial growth phase, strain NT2 showed 78 % adhesion to 4-NT indicating high cell surface hydrophobicity (Fig. 5) in early-log phase. Subsequently, CSH reduced as time went on, reaching almost 50 % at the onset of stationary phase (78 h) and, remaining constant thereafter till 120 h. No change in CSH was observed in MSB medium without 4-NT.

Similarly, the surface tension of the medium dropped from 71 to 53·5 mN m^{-1} at 12 h, then to 31 mN m^{-1} at 48 h and thereafter remained stable

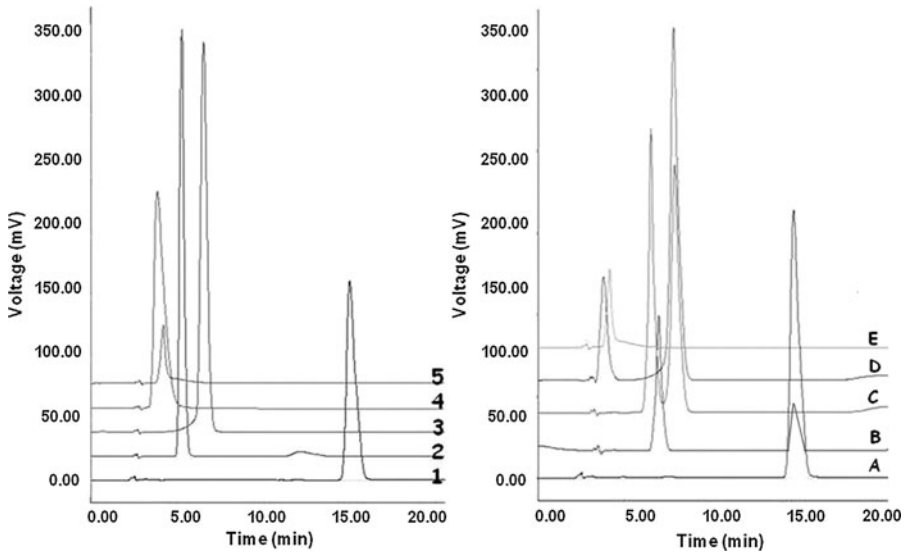

Fig. 4 HPLC analysis of (1–5) standard 4-NT, 4-nitrobenyl alcohol, 4-nitrobenzaldehyde, 4-nitro benzoate and protocatechuate, respectively and (*A–E*) samples withdrawn at 0, 24, 48, 72, and 120 h time-intervals

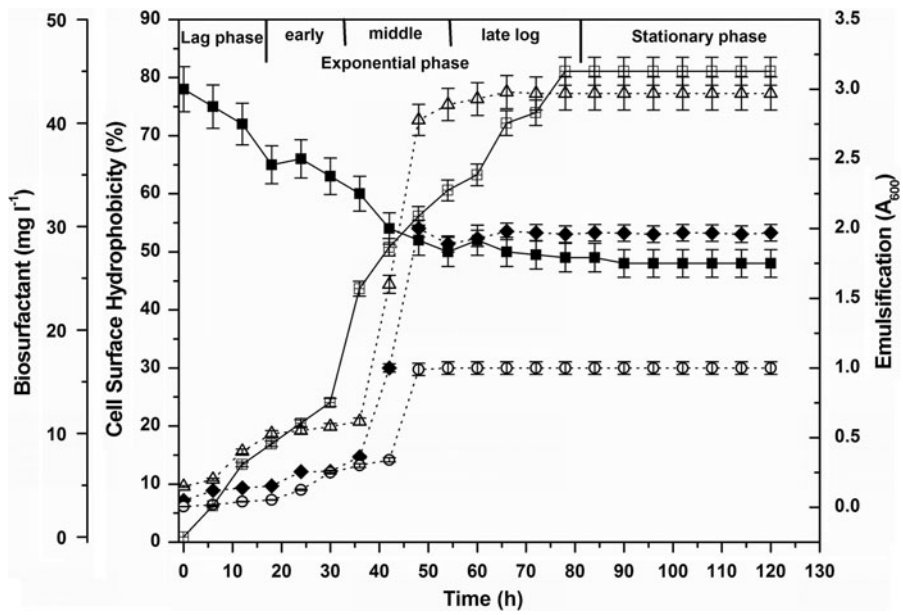

Fig. 5 Growth characteristics of *R. pyridinivorans* NT2 using 4-NT (400 mg l^{-1}) in MSB at 30 °C for 120 h (pH 7.0 ± 0.2): CSH (*filled squares*); emulsification after 30 s (*open triangles*), 2 h (*filled diamonds*) and 24 h (*open circles*); biosurfactant (*open squares*). Data are mean ± standard deviation of triplicates. Small (non-visible) standard deviations are within the symbols

(29 mN m^{-1}) till 120 h. The surface tension remained at around 71.12 mN m^{-1} in MSB medium devoid of 4-NT. When strain NT2 was grown on 400 mg l^{-1} of 4-NT, the biosurfactant production was low for the initial 30 h, after which it increased steadily and reached to 45 mg l^{-1} at 78 h (Fig. 5). Beyond this point, biosurfactant concentration was same indicating that biosurfactant synthesis was cell-growth associated. Interestingly, the biosurfactants produced by strain NT2 were able to emulsify 4-NT (Fig. 5) in the stationary phase (78–120 h).

The influence of 4-NT and biosurfactant on fatty acid profiles

To assess how biodegradation and production of biosurfactant affect whole-cell-derived fatty acid profiles of *R. pyridinivorans* NT2, a MSB medium

was supplemented with 4-NT at 400 mg l^{-1} and whole-cell derived fatty acid profiles was compared at 0 h (before biodegradation) and at 120 h (at the end of biodegradation). For the interpretation of results, identified fatty acids were categorized into two major groups: saturated and unsaturated fatty acids. The first group included: straight-chain, hydroxyl-, cyclopropane and branched fatty acids. As indicated in Table 1, significant changes in the proportion of total saturated and unsaturated fatty acids were observed. In particular, it was found that the 0 h sample contained mainly saturated fatty acids: tetradecanoic (myristic, 14:0); hexadecanoic (palmitic, 16:0); and octadecanoic (stearic, 18:0) and these components were absent in the 120 h sample. The total amount of lipids in the 0 h sample (15 mg g^{-1} wet weight) was slightly higher compared to the 120 h sample (13.5 mg g^{-1}) while the CSH of 0 h sample was considerably higher compared to the hydrophobicity of the 120 h sample (78 vs. 50 %). At the end of biodegradation, saturated fatty acid was more (58 %) than at the beginning (54 %). There was a decrease in the level of unsaturated fatty acids from 46 % (0 h) to 41 % (120 h). These changes were expressed as the saturated/unsaturated (sat/unsat) ratio and showed an increasing trend from 1.17 to 1.38. This increase in the sat/unsat ratio observed in 120 h sample, compared to 0 h sample, was mainly effected by an increased abundance of 10:0 3OH (from 2.11 to 14.38 %), 15:0 *anteiso* (from 2.22 to 12.28 %), 16:0 *anteiso* (from 1.35 to 8.72 %) and 17:0 *anteiso* (from 0 to 5.40 %). It is reasoned that biosurfactant induced removal of 3-hydroxy fatty acids, primarily of carbon chain lengths C_{12}, C_{14}, C_{16}, and C_{18} from the cell surface for increase in the solubility and uptake of 4-NT. It may be hypothesized that these changes in quantity of hydroxylated, branched and straight-chain fatty acids might give the optimum hydrophilic-lipophilic balance required for optimum uptake of 4-NT. If this hypothesis is appropriate, it could explain the marked absence of tetradecanoic (myristic, 14:0); hexadecanoic (palmitic, 16:0); and octadecanoic (stearic, 18:0) in 120 h sample.

A polyunsaturated fatty acid (18:2*w*6,9c), a rarely occurring fatty acid in *Rhodococcus* sp., was observed in strain NT2 upon exposure to 4-NT in the medium (Table 1). Besides, the biosurfactant production in the medium during biodegradation resulted in an increase in cyclopropane fatty acids (19:0 cyclo *w*8c) (Table 1).

Table 1 Changes in total fatty acid compositional profile of *R. pyridinivorans* NT2 grown on MSB medium supplemented with 4-NT (400 mg l^{-1}). Data shown are the average of three independent trials ± standard deviation

Fatty acids	% of total fatty acids	
	Before biodegradation (0 h)	After biodegradation (120 h)
Saturated		
10:0 3OH	2.11 ± 2.17	14.38 ± 0.67
12:0	6.1 ± 0.80	4.56 ± 0.02
12:0 3OH	3.34 ± 0.98	4.26 ± 2.08
14:0	2.05 ± 0.61	–
15:0 *iso*	2.69 ± 2.30	–
15:0 *anteiso*	2.22 ± 0.04	12.28 ± 0.55
16:0	28.33 ± 3.05	–
16:0 *iso*	0.52 ± 0.02	2.61 ± 0.05
16:0 *anteiso*	1.35 ± 0.38	8.72 ± 0.28
17:0 *anteiso*	–	5.40 ± 0.73
18:0	4.05 ± 0.01	–
19:0 *cyclo w*8*c*	1.20 ± 0.16	5.80 ± 0.27
Unsaturated		
16:1*w*7*c*/16:1*w*6*c*	18.23 ± 2.86	11.27 ± 1.94
18:1*w*7*c*	19.85 ± 0.53	15.64 ± 0.79
18:1*w*9c	5.14 ± 0.21	8.02 ± 2.11
18:2*w*6,9c	3.08 ± 0.46	7.15 ± 0.33
Sat./unsat. ratio	1.17 ± 0.26	1.38 ± 0.04

Characterizations of the purified biosurfactant

Structural elucidation

TLC analysis of solvent extracted spent culture sample run on the solvent system $CH_2Cl_2/CH_3OH/H_2O$ (2.6:0.6:0.02) revealed a single spot with a R_f value of 0.4 suggesting that the biosurfactant is trehalose lipid, a type of glycolipid. A positive reaction with orcinol assumes a presence of sugar-containing compounds in a surfactant (Fig. S6; panel a). Yellowish spots with the same R_f value was detected with iodine vapours demonstrating a lipid moiety in the compound (Fig. S6; panel b). This is in accordance with previous reports (Rapp and Gabriel-Jurgens 2003; Tuleva et al. 2008). Staining the TLC plate with ninhydrin indicated no protein or amino acids moiety in NT2 biosurfactant (Fig. S6; panel c) and this is consistent with earlier observation of the resistance of glycolipid

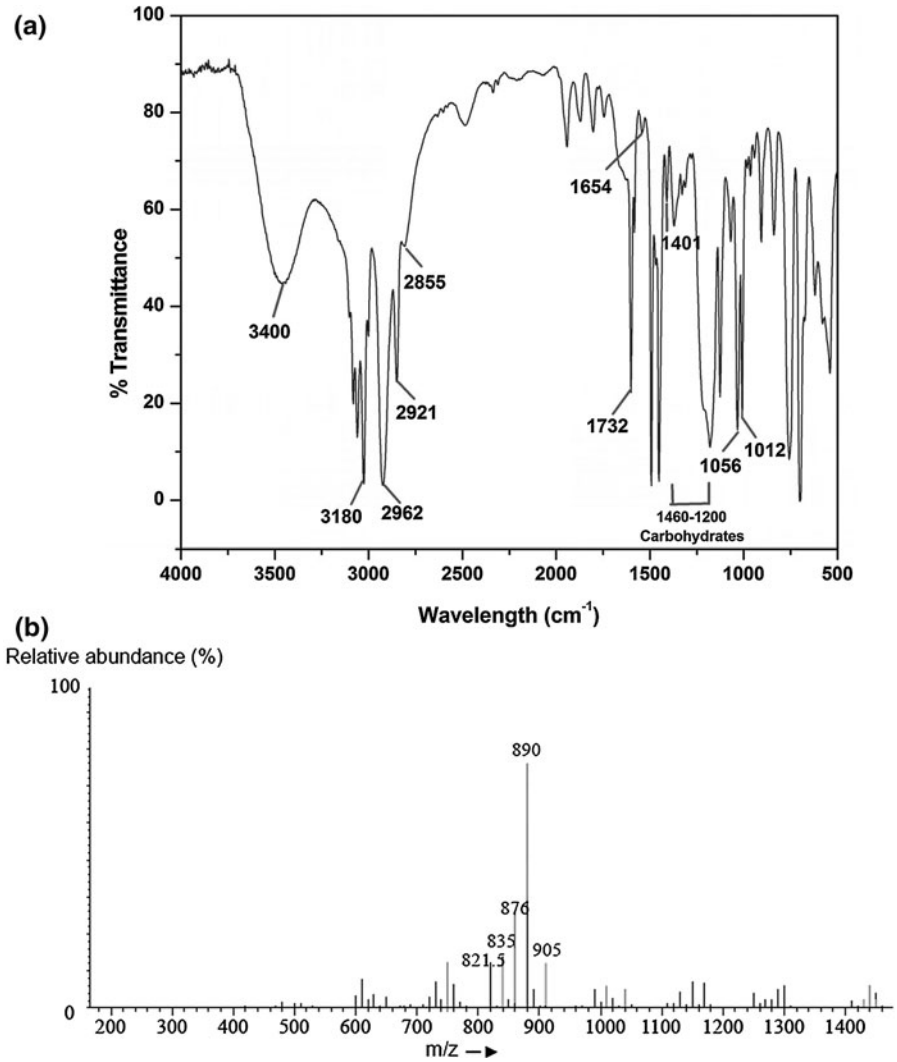

Fig. 6 **a** FTIR spectrum and **b** LC-ESI-MS of the purified biosurfactant isolated from *R. pyridinivorans* NT2 grown on MSB medium supplemented with 4-NT (400 mg l^{-1})

surface activity to the proteinase treatment (Shavandi et al. 2011).

From the FTIR data (Fig. 6a), the dominant absorbance bands of the biosurfactant were correlated to functional group absorbance frequencies. The broad negative bands at about 3,400 cm^{-1} and at 3,180 and 1,654 cm^{-1} are attributed to O–H stretching (for O–H bonds) confirming the presence of glycolipid moieties. The double band at 2,921 and 2,962 cm^{-1} derived from CH_2–CH_3 stretching and those at 2,855 and 1,401 cm^{-1} are assigned to (–CH_3) symmetric deformation vibrations, (C–H) bending vibrations of CH_3 and CH_2 groups and CH_2–CH_3 stretching vibrations which are characteristic of polysaccharides. A C=O stretching band at 1,732 cm^{-1} is characteristic of ester bonds and carboxylic acid groups while characteristic frequencies at 1012–1056 cm^{-1} represent C–O–C ethereal vibration. In the fingerprint region of the spectrum, the area between 1,200–1,460 cm^{-1} represents C–H and O–H deformation vibrations, typical for carbohydrates as in the trehalose units of the molecule. The lower range of the fingerprint region below 1,200 cm^{-1} represents different kinds of C–H, C–O and CH_3 vibrations which cannot be allocated more closely.

The chemical structure of the purified biosurfactant from strain NT2 grown with 4-NT as the carbon source was studied by liquid chromatography (LC) coupled to mass spectrometry (MS). This analysis revealed that this surfactant is a mixture of at least five components, with the pseudomolecular ions being between *m/z* 905 and 821 (Fig. 6b). The retention time, the main pseudo-ions, the relative abundance and the possible fatty acid structure are shown in Table S4. The most abundant component was the pseudomolecular ion 890, which reached an abundance of 59.7 %. This molecular weight may correspond to either trehalose-succinic acid-C_7–C_{11}–C_{11} or trehalose-succinic acid-C_9–C_{10}–C_{10} or trehalose-succinic acid-C_9–C_9–C_{11} (Marqués et al. 2009). The other pseudomolecular ion at *m/z* 876, of lower concentration, may also be attributed to 2,3,4,2′-trehalose tetraester, where C-2, C-3 or C-4 is esterified with two octanoic acids (Tuleva et al. 2008). Further MS^n fragmentation and ^1H, ^{13}C, ^1H COSY NMR-spectroscopy is needed to exactly deduce the chemical composition and model structure of these trehalose lipids.

Physicochemical and tensioactive properties

The purified biosurfactant was whitish in appearance, readily dissolved in water and methanol with a broad pH range (2–10) and produced clear solutions. Some commercial emulsifiers, particularly those with fatty acid components, tend to form clumpy solutions that limit their applications. Consistent with earlier reports (Ferhat et al. 2011), the biosurfactant from strain NT2 exhibited a high foaming power.

The CMC value of the biosurfactant was determined from a semilog plot of surface tension against different concentrations of biosurfactant (Fig. 7a). A linear function described a reasonably good fit ($r^2 = 0.97$) for the data at <5 and >15 mg l^{-1}. The close fit of this linear function implies that there are different behaviors for the biosurfactant in solution. The CMC (30 mg l^{-1}) obtained in this study is in agreement with rhamnolipids, surfactin, trehalose tetraesters, SDS, Triton X- 100, Brij 35, Brij 30, Tween 20, and Tween 80. The interfacial tension at the water/hexadecane interface decreased to 1.2 mN m^{-1}. Agar double diffusion tests revealed the anionic character of the biosurfactant.

The purified biosurfactant efficiently emulsified with aliphatic, aromatic hydrocarbons and oils (Table S5). Higher emulsification (90–95 %) was obtained with long-chain hydrocarbons like diesel, liquid paraffin, motor oil, groundnut oil and soybean oil while shorter-chain alkanes resulted in less emulsification (50–80 %) by this biosurfactant.

Figure 7b presents the effects of NaCl concentrations on surface tension and emulsification capacity of NT2 biosurfactant. A slight tendency of reducing the surface tension proportional to the increase of the salt concentration was observed, whereas the emulsifying capacity was not strongly affected by the presence of salt. While 2–3 % salt is sufficient to deactivate chemical surfactants, glycolipidic biosurfactant from NT2 was not precipitated or salted out even at 10 % NaCl (2.5 mol l^{-1}). This unique ionic strength tolerance renders the biosurfactant more suitable for oil-related applications most of which are in high saline conditions.

The effects of different pH levels on the surface tension and CMD^{-1} were insignificant, while emulsification index was very sensitive to the pH changes (Fig. 7c). There was an increase in the emulsification index and stability with the decrease in H^+ ions in the solution.

Table S6 shows the effects of heat treatment on the surface and emulsification activities of the purified biosurfactant from NT2, demonstrating that no appreciable change would occur if the biosurfactant solution is heated. The biosurfactant surface properties (surface tension, CMD and EI) remained stable even after autoclaving at 120 °C for 15 min.

Discussion

The main objective of this study was to assess the potential of a novel and indigenous biosurfactant producing *Rhodococcus pyridinivorans* strain NT2 for biodegradation of 4-NT. Besides purifying and characterizing the extracellular biosurfactant from strain NT2, we investigated if and how this biosurfactant can influence the cell surface properties and whether it is correlated with biodegradation of 4-NT. Finally, toxicity of parent and biodegradation intermediates was probed in order to ascertain effective biodegradation benchmarks.

The xenobiotic compounds metabolized by *Rhodococci* cover a wide range of structural groups, including aliphatic and aromatic hydrocarbons,

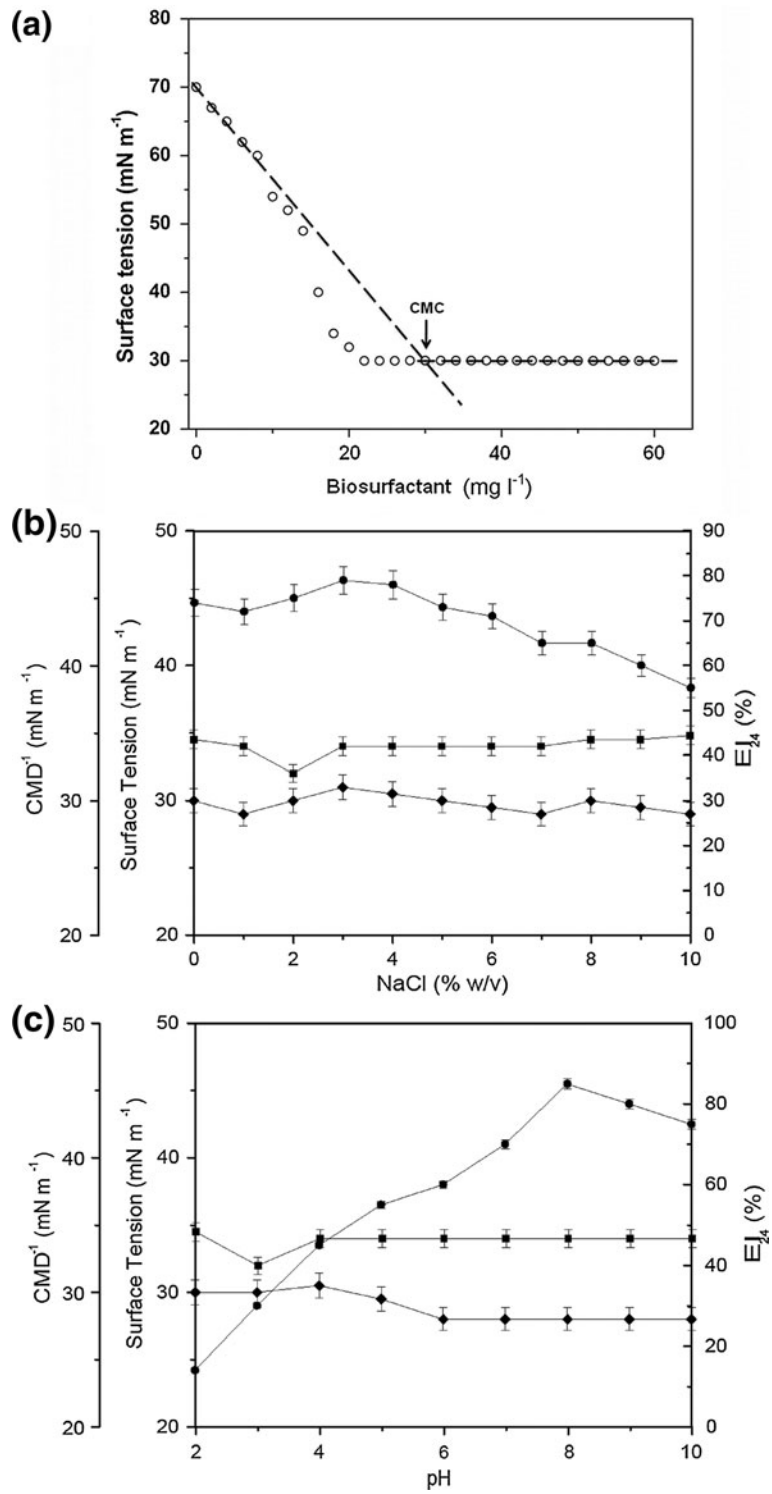

Fig. 7 **a** Surface activity of the crude biosurfactant using du Nouy ring method. Symbols: *open circle*, experimental data; *dotted line*, best-fit logarithm or constant function. The *arrow* shows the CMC as calculated from the intercept of the regression lines. Effect of **b** salinity and **c** pH variation on the ST (*filled diamonds*), CMD^{-1} (*filled squares*) and EI_{24} (%) (*filled circles*) of the purified biosurfactant from *R. pyridinivorans* NT2

oxygenates, halogenated compounds, polychlorinated biphenyls, nitroaromatics, heterocyclic compounds, nitriles and various herbicides (Martínková et al. 2009). To date, *R. pyridinivorans* is known to degrade pyridine, bezothiazole, biphenyls, styrene, BTEX and aromatic mycotoxins (Larkin et al. 2005; Martínková et al. 2009). Underscoring the inherent problems with 4-NT biodegradation, only five strains have been isolated so far by their growth on 4-NT: *Pseudomonas* sp. 4-NT, *P. putida* TW3, *Mycobacterium* sp. strain HL 4-NT-1, *P. putida* A1 and *Acidovorax* sp. strain JS42 which degraded 0.18, 2.0, 0.5, 0.04 and 1 mmol of 4-NT, respectively. However, *R. pyridinivorans* strain NT2, isolated from pesticides contaminated effluent-sediment has been found to degrade 400 mg l^{-1} (2.91 mmol) of 4-NT in MSB media. As of now, three routes are known for biodegradation of 4-NT: (i) an oxidative pathway via methylcatechols with release of nitrite as found in several *Pseudomonas* strains and *Acidovorax* sp. strain JS42 (Ju and Parales 2010); (ii) reduction of nitro into amino group and subsequent release of ammonia either via the partially reductive hydroxylaminolyase-mediated pathway in *P. aeruginosa*, *P. putida* and *P. fluorescens* (Rhys-Williams et al. 1993; He and Spain 2000; Nishino et al. 2000) and (iii) reductive mutase-catalyzed intramolecular conversion of the hydroxylamino compound to an ortho-aminophenol in *Mycobacterium* sp. (He and Spain 2000). *Rhodococcus* spp. are known to effectively degrade 4-nitrocatechol, 4-nitrophenol, 2-chloro-4-nitrophenol, 2,4-dinitrophenol and 2,4,6- trinitrophenol, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and MNX (Larkin et al. 2005; Martínková et al. 2009). To the best of our knowledge, this is the first report of biodegradation of 4-NT by a *R. pyridinivorans* at 400 mg l^{-1}.

Based on the comparison between the parent molecule (4-NT) and detected intermediates, the biodegradation in strain NT2 is postulated to follow reduction of the nitro-group and release of ammonia, rather than oxidation of the aromatic ring and release of nitrite. This was ascertained from these observations: (i) no significant levels of nitrite were detected in supernatants, whereas ammonium was released stoichiometrically which is in agreement with other reductive 4-NT degradation pathways (Haigler and Spain 1993; Rhys-Williams et al. 1993); and (ii) reaction intermediates included 4-nitrobenzyl alcohol, 4-nitrobenzaldehyde, 4-nitrobenzoic acid, protocatechuate and β-ketoadipate. These results are congruent with the hypothesis that strain NT2 followed partial reductive pathway for degrading 4-NT. Previous studies in *P. putida* TW3 and *Pseudomonas* sp. strain 4NT have also shown that partial reduction of the nitro group of 4-NT under aerobic degradation conditions is initiated by oxidative reaction of the methyl group to the corresponding alcohol (Haigler and Spain 1993; Rhys-Williams et al. 1993). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase then convert 4-nitrobenzyl alcohol to 4-nitrobenzoate, followed by further conversion to protocatechuate (Ju and Parales 2010). Based on these findings, a putative outline of biodegradation of 4-NT by *R. pyridinivorans* NT2 is shown in Fig. 8. Continuing the above trend, samples withdrawn at periodic time-intervals during biodegradation revealed limited cytotoxicity and ROS levels compared to control samples (with 4-NT). This suggests oxygenolytic degradation of 4-NT by the strain NT2 led to sequential detoxification process.

During growth on 4-NT, strain NT2 itself possessed a hydrophobic cell surface which is consistent with previous reports (Chang et al. 2009; Liu and Liu 2011). *Rhodococcus* cells are hydrophobic due to the presence of aliphatic chain of mycolic acid, glycolipids, fatty acids, and polysaccharides in the cell surface. This makes cells contact to hydrophobic substrates more easily (Peng et al. 2007; Chang et al. 2009). As the extracellular biosurfactant peaked in late-log to early stationary phase, hydrophobicity decreased. The results could be explained by the orientation of the amphiphilic structure of biosurfactant in relation with the hydrophobic and hydrophilic character of the bacterial surface. Adsorption of produced biosurfactant onto the hydrophobic surface of NT2 might take place through the hydrophobic interaction between the hydrophobic regions on the cell surface and the hydrophobic tails of the surfactant. Consequently, the hydrophilic parts of the biosurfactant may have been exposed to the aqueous phase leading to a significant reduction of the CSH of NT2. It is noteworthy that the decrease in CSH of NT2 induced by the secreted biosurfactant was irreversible as indicated by the fact that pre-washing of cells did not recover the loss of CSH. The irreversible change might be due to the removal of extracellular hydrophobic substance(s) from the cell surface by the biosurfactant.

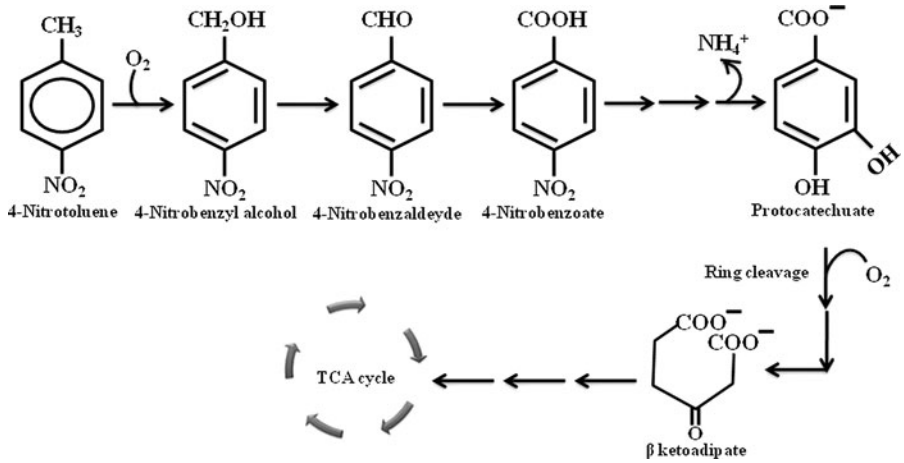

Fig. 8 Proposed biodegradation pathway of 4-NT by *R. pyridinivorans* NT2

The effective production of biosurfactants is usually viewed as the obvious criterion for the existence of biosurfactant-mediated hydrocarbon transfer. Suggested mechanisms for the uptake of hydrophobic contaminants by degrading bacteria include direct contact of substrates with microorganisms having a high CSH and biosurfactant-mediated uptake by microorganisms capable of producing biosurfactant (Gorna et al. 2011; Kaczorek et al. 2011; Zhao et al. 2011). Our results indicated that both direct contact and the biosurfactant-mediated uptake took place in the 4-NT removal by *R. pyridinivorans* strain NT2. This is in line with the observation that biosurfactant of 4-NT-grown strain NT2 was found to produce emulsions that remained stable at least upto 24 h. In our case, the role of biosurfactant in 4-NT uptake is further reinforced by following points: (i) we did not observe any bioflocculation phenomena like other *Rhodococcus* species which affects the cell growth and access to substrate during biodegradation through direct contact (Chang et al. 2009; Liu and Liu 2011); and (ii) extracellular biosurfactant did not prolong the initial lag period for biodegradation nor did they inhibit biodegradation as noticed by others (Zhang and Miller 1994; Zhong et al. 2008). The possible utilization of secreted biosurfactant prior to 4-NT by NT2 may also be ruled out since that could have led to inhibition of biodegradation. Still, further studies are required to elucidate the exact mechanism. The hydrophobic nature of this bacterium is highly suitable for the biotransformation of water-immiscible chemicals in petroleum fractions, as hydrophobic substrates dissolved in the organic phase are directly accessible by cells present at the oil-water interface (Torres et al. 2011).

Biodegradation of 4-NT, simultaneous production of biosurfactant and subsequent shift in CSH were strongly correlated with changes in the cellular FAME profiles before and after biodegradation. The mechanisms involved in the adaptation of cell membrane modifications in Gram-positive strains remain scarce (de Carvalho 2010), although many factors are similar to those of Gram-negative ones (Torres et al. 2011). In general, *Rhodococcus* strains responded to the presence of aromatic substrates by changing the degree of saturation of the fatty acids of the cellular membrane, by changing the length of the fatty acids and mycolic acids according to the chain length of the carbon source, by altering the percentage of branched fatty acids and by increased lipophilicity (de Carvalho 2010). We incline to explain that *R. pyridinivorans* NT2 cells adapted to 4-NT by increasing the saturated/unsaturated ratio of fatty acids and saturated *anteiso*/*iso* ratio, and thus, decreased membrane fluidity. These data are similar to those of *Staphylococcus haemolyticus* in response to toluene (Nielsen et al. 2005). Echoing the same view, a considerable *de novo* synthesis of saturated *anteiso*-branched C_{15} and C_{17} fatty acids, and concomitant decrease in corresponding *iso*-branched fatty acids, had been observed during *Bacillus subtilis* adaptation to low temperatures (Klein et al. 1999). On the other hand, *Arthrobacter chlorophenolicus* responded to the presence of phenol, 4-chlorophenol, and 4-nitrophenol by decreasing the *anteiso*/*iso* ratio, and thus, decreasing membrane fluidity (Unell et al. 2007). *R. erythropolis* cells also

adapted to toluene by increasing the proportion of saturated *iso*-branched fatty acids and decreasing the amount of straight-chain fatty acids (de Carvalho et al. 2007). The *anteiso*-branched fatty acids present a significantly larger cross sectional area than the *iso*-branched fatty acids. By increasing the content of the *anteiso*-branched fatty acids, NT2 cells alter the fluidity or flexibility of the cellular membrane. A noticeable abundance of cyclopropane fatty acid (19:0 cyclo *w*8c) in bacterial cells grown on 4-NT (120 h) was observed as compared to 0 h sample. This confirms earlier observations that high levels of saturation and high degrees of sat/unsat ratio come from high contents of cyclopropane fatty acids in bacteria upon exposure to aromatic compounds (Mrozik et al. 2007, 2011). Cyclopropane fatty acids are known as compounds that stabilize membrane lipids and assist in stress tolerance in the genus *Pseudomonas*. The exact physiological role(s) of cyclopropane fatty acids is presently unknown, but formation of these fatty acids in bacterial cellular membranes may be a protective mechanism to counteract toxic compounds (Mrozik et al. 2007, 2011; Kaczorek et al. 2013a).

In order to explore the interplay between biodegradation, CSH, cell membrane modifications and biosurfactant production, extracellular biosurfactant from NT2 was further purified and characterized. Several microorganisms produce growth-dependent extracellular biosurfactants to modulate cell surface hydrophobicity (Hua and Wang 2011; Mnif et al. 2011). During biodegradation of 4-NT, CSH correlated with biosurfactant produced by strain NT2. The production of biosurfactant was related directly to cell growth and reached a maximum at late-log phase and remained unchanged throughout the stationary phase (Fig. 5). This growth-associated production of biosurfactant by strain NT2 is similar with earlier reports (Chen et al. 2012). LC-ESI-MS showed that the main product was trehalose-succinic acid of molecular mass 890 g mol^{-1}. Another structural isoform or homologue of molecular mass 876 g mol^{-1} was found as 2,3,4,2′-trehalose tetraester. These data are in correspondence with published literature (Rapp and Gabriel-Jurgens 2003; Peng et al. 2007; Tuleva et al. 2008; Marqués et al. 2009). Trehalose tetraesters and succinoyl trehalose lipids are known as anionic biosurfactant from *Rhodococci* (Kuyukina and Ivshina 2010). The purified glycolipid from NT2 reduced surface and interfacial tensions to 29 and 1.2 mN m^{-1} at a CMC of 30 mg l^{-1}. To date, glycolipid biosurfactants reduced the surface tension of water in the range of 29–38 mN m^{-1} and interfacial tension from <1 to 17 mN m^{-1} (Tuleva et al. 2008). Moreover, it efficiently emulsified with aliphatic, aromatic hydrocarbons and oils. The water-oil emulsions showed to be compact and remained stable for nearly one month at room temperature, suggesting that the addition of such biosurfactant into a remediation process may enhance the availability of the recalcitrant hydrocarbon(s). On the other hand, the ability to emulsify the vegetable oils suggests a potential application in the pharmaceutical and cosmetic industries. This observation was corroborated with other microbial glycolipids (Shavandi et al. 2011). This purified biosurfactant also showed tolerance in the presence of 10 % NaCl (2.5 mol l^{-1}) without compromising its properties. The interpretation for this behaviour stems from the fact that trehalose lipids contain a free carboxylic group from the succinic acid (Marqués et al. 2009). The addition of an electrolyte to a surfactant solution causes a decrease in the repulsive forces between similar charges. Thus, for ionic surfactants in general, the surface activity increases when an electrolyte is added and both micelle formation and micellar growth are enhanced. Besides, stability of this biosurfactant to the environmental stresses prevalent in the oil reservoirs such as high temperature, salinity and different pH strengths reveals its suitability for oil well injection and enhanced oil recovery (Shavandi et al. 2011). It should be highlighted that among 30 valid *Rhodococcus* species, only six species are reported to produce biosurfactants so far (Kuyukina and Ivshina 2010) and this is the first report of a glycolipid biosurfactant from a strain of *R. pyridinivorans*.

To summarize, *R. pyridinivorans* NT2 isolated from pesticide contaminated habitat remarkably achieved catabolism of higher concentration (400 mg l^{-1}) of 4-NT as carbon and nitrogen source with the assistance of extracellular biosurfactant production which is strongly related to the cell surface properties. Our results demonstrated that changes in the CSH and the emulsification degree mediated by biosurfactants are prerequisites for the removal of hydrophobic substrates by this strain. Production of glycolipid biosurfactant resulted in decreased hydrophobicity as time went on. This was accompanied by

an increase in degree of fatty acid saturation, sat/unsat ratio, saturated *anteiso*/*iso* ratio and presence of an unusual cyclopropane fatty acid (19:0 cyclo *w*8c). Despite having similar 4-NT degradation pathway in the bacterial isolates characterized earlier, strain NT2 has a unique ability to degrade very high concentrations of 4-NT (400 mg l^{-1}) through partial reduction of the nitro group under aerobic condition with a sequential detoxification process. The *R. pyridinivorans* NT2 may either be potentially useful for *in situ* bioremediation of soils and sites contaminated with NTs or could be established as a model bioaugmented system for remediation of the contaminated sites.

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