Biodegradation of 2,4,6-trinitrophenol by *Rhodococcus* sp. isolated from a picric acid-contaminated soil

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ABSTRACT

A picric acid-degrading bacterium, strain NJUST16, was isolated from a soil contaminated by picric acid and identified as a member of *Rhodococcus* sp. based on 16S rRNA sequence. The degradation assays suggested that the strain NJUST16 could utilize picric acid as the sole source of carbon, nitrogen and energy. The isolate grew optimally at 30 °C and initial pH 7.0–7.5 in the mineral salts medium supplemented with picric acid. It was basically consistent with degradation of picric acid by the isolate. Addition of nitrogen sources such as yeast extract and peptone accelerated the degradation of picric acid. However, the stimulation was concentration dependent. The degradation was accompanied by release of stoichiometric amount of nitrite and acidification. The degradation of picric acid at relatively high concentrations (>3.93 mM) demonstrated that the degradation was both pH and nitrite dependent. Neutral and slightly basic pH was crucial to achieve high concentrations of picric acid degradation by the NJUST16 strain.

1. Introduction

Nitroaromatic compounds (NACs) are widespread in the environment due to the manufacturing and processing of a variety of industrial products, such as pharmaceuticals, pigments, dyes, plastics, pesticides and fungicidal agents, explosives and industrial solvents [1–4]. NACs have been classified under hazardous group of chemicals because of their proven toxic effects [5–9]. For this reason, their presence in wastewater is severely regulated. There is interest in removing it from contaminated sites. To treat NACs, the biological treatment is superior to the physicochemical methods, because the latter ones have higher treatment costs and possibilities of causing a secondary pollution [9]. But the poor biodegradability of NACs [10,11] limits the application of conventional biological techniques. However, bioremediation of these compounds is an attractive means for decontaminating the environment and industrial wastewaters.

NACs are rare amongst natural compounds. Due to the pronounced electron-withdrawing character of the nitro groups, NACs harbor a highly electron deficient π-electron system, generating a highly xenobiotic character. As a result, the electrophilic attack which is usually the first step in aromatic biodegradation becomes more difficult. Thus polynitroaromatic compounds such as 2,4,6-trinitrophenol (TNP, also known as picric acid) are subject to initial reductive transformation [10,12]. However, NACs with few nitro groups such as mononitrophenol, and mononitrotoluene, are relatively easily degraded through an oxidative attack [4,13,14]. Dinitroaromatic compounds and particular trinitroaromatic compounds are more resistant to mineralization [11,15].

TNP is truly xenobiotic. Numerous military and industrial sites are highly contaminated with the substance. As a trinitroaromatic compound, this substance is resistant to microbial degradation due to the presence of substituted nitro groups on phenol, especially at high concentrations. Therefore, the screening of microbes capable of degrading TNP is a critical step for formulating an effective strategy for bioremediation of TNP. For the purpose of TNP bioremediation, several microbes having ability to use TNP as the sole nitrogen source under aerobic conditions were isolated [15,16]. However, reports indicated that the use of TNP by microbes as the sole source of carbon, nitrogen and energy was rare. All of the TNP-degrading isolates described previously belonged to Actinomycetales family, such as the genera *Rhodococcus* [17] and *Nocardioides* [15,18]. Due to the small number of collections of such bacteria, knowledge about TNP degradation is yet limited. Consequently, in solving the serious problems of pollution caused by TNP, it is important to screen bacterial strains indigenous to sites contaminated with TNP for TNP degradation.

In addition to the isolation of microbes capable of degrading TNP, an understanding of the interplay between the biotic and abiotic factors is also important. However, up to now, most of the reports have focused on the pathways catalyzed by the enzymes for TNP degradation [15,19–22]. Optimized process parameters aiming at a

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successful application for remediation of contaminated soils and waters are still lacking.

The purposes of the present paper are (a) to isolate an indigenous bacterial strain able to use TNP as the sole carbon, nitrogen and energy source, and (b) to examine the effects of various abiotic factors on the degradation of TNP and growth of NJUST16 strain in liquid cultures, aiming at an effective bioremediation strategy.

2. Materials and methods

2.1. Chemicals

TNP of analytical reagent grade was obtained from Shantou Xilong Chemical Factory (Guangdong, China). All other chemicals were of the highest purity available and were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Bidistilled water was used throughout this study. The soil sample used to isolate the TNP-degrading bacteria was taken from a site contaminated by TNP for more than 10 years at the Nanjing Taowu Chemical Factory.

2.2. Selection and identification of TNP-degrading bacteria

Enrichment and selection of bacteria capable of degrading TNP was performed in mineral salts medium [15] containing phosphate buffer (14 mM, pH 7.0), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $CaCl_2$ (0.05 g/l), SL-4 (10 ml/l), supplemented with a certain amount of TNP stock solution. The phosphate buffer contained $Na_2HPO_4 \cdot 12H_2O$ (3.057 g/l) and KH_2PO_4 (0.743 g/l). SL-4 contained: EDTA (0.5 g/l), $FeSO_4 \cdot 7H_2O$ (0.2 g/l), trace element solution SL-6 (100 ml/l). Trace element solution SL-6 contained: $ZnSO_4 \cdot 7H_2O$ (0.1 g/l), $MnCl_2 \cdot 4H_2O$ (0.03 g/l), H_3BO_3 (0.3 g/l), $CoCl_2 \cdot 6H_2O$ (0.2 g/l), $CuCl_2 \cdot 2H_2O$ (0.01 g/l), $NiCl_2 \cdot 6H_2O$ (0.02 g/l), $Na_2MoO_4 \cdot 2H_2O$ (0.03 g/l). The TNP stock solution contained 17.47 mM TNP, and was adjusted to pH 7.0 with 1 mol/l of NaOH. The initial pH of the medium was 7.0. 50 ml of the media was transferred to 150 ml of an Erlenmeyer flask and autoclaved at 121 °C for 20–30 min. The experimental cultures were incubated on a rotary shaker at 180 rpm and 25 °C during the enrichment and selection of bacteria. Agar plates contained 15 g of agar per liter of medium.

Five grams of soil sample described above were inoculated into mineral salts medium supplemented with 0.218 mM TNP until the yellow color associated with TNP faded out (approximately 10 days). Five milliliters of the decolorized cultures were transferred to fresh mineral salts medium to initiate the enrichment. From then on, the cultures were transferred successively to fresh mineral salts medium using identical growth conditions at each transfer, except that the TNP concentration was increased stepwise varying from 0.218 mM to final 2.18 mM at 0.218 mM interval. Each fresh medium was incubated for about 48 h. 20 days later, the initial TNP concentration in the final enrichment cultures was brought up to 2.18 mM. The final enrichment cultures were serially diluted and streaked onto mineral salts solid media containing 2.18 mM TNP as the only source of carbon and nitrogen. About 2 weeks later, single colonies were picked and re-streaked for purification three times. All the colonies exhibited the same morphology on agar plates. They were then tested for their biodegradation ability on TNP. Finally, a bacterial isolate designated NJUST16 strain, which exhibited the best TNP biodegradation ability, was obtained and kept on mineral salts agar plates.

The NJUST16 strain was further characterized by Gram staining, biochemical tests, and 16S rDNA sequence determination. The deoxyribonucleic acid (DNA) was extracted from cells grown in the mineral salts medium according to Yang et al. [23]. PCR amplification of the DNA using the primers: 5′-GAATTTGATCCTGGCTCAGAACGAACGCT-3′ (forward) and 5′-TACGGCTACCTTGTTACGACTTCAC-3′ (reverse) was performed with a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler. Reaction mixture contained each primer (0.5 μl), deoxy-nucleotide triphosphates (dNTPs, 0.5 μl), 10× reaction buffer (2 μl), Taq polymerase (0.5 μl), DNA template (1 μl), Mg^{2+} solution (2 μl) and sterilized water (13 μl) to achieve a final volume of 20 μl. PCR was performed under the following conditions: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 58 °C for 30 s, and 72 °C for 2 min; and a final extension step at 72 °C for 8 min. After thermocycling, the samples were loaded on an 3700 DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions. Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information Databases).

2.3. Biodegradation test

The experiments for TNP degradation were conducted with pure cultures of the NJUST16 strain. The inocula for the experiments of TNP degradation study were prepared by growing bacteria in mineral salts medium supplemented with 2.18 mM TNP, on a shaker at 180 rpm and 30 °C. 2 ml of this cell culture was added to fresh medium (2.18 mM TNP) as inoculum. 32 h later, a third fresh medium was also inoculated with 2 ml of the last culture, to ensure that bacteria were already adapted to TNP. The induced cells at late exponential growth phase (cell concentration of about 2×10^7 cells/ml, OD_{600} around 0.3 absorbance units) were then transferred to each experimental flask containing the prepared medium as the inocula.

The degradation experiments were conducted with a series of 150 ml Erlenmeyer flasks with 50 ml culture media. In order to find out the optimum temperature for the growth of NJUST16 strain, mineral salts media with TNP concentration of 2.18 mM and pH 7.0 were prepared. All the reactors were inoculated with 2 ml inocula described above, and incubated at 20, 25, 30, 35, 40 °C, respectively, at 180 rpm. Various pHs (6.0–8.0) of inorganic culture media were prepared to determine the optimum pH for the removal of TNP by the bacterial isolate. To maintain pHs constant, the phosphate buffer systems (14 mM) were used. The pHs were changed through changing the proportion of $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 in the buffer systems. The initial TNP concentration in the mineral salts media was set as 2.18 mM. The reactors were shaken at 180 rpm and 30 °C.

To study the effect of TNP concentration on the rate of degradation by NJUST16 strain, inocula of the NJUST16 strain described above were inoculated into mineral salts media containing 0.44, 1.31, 2.18, 3.06, 3.93 mM TNP, respectively. Mineral salts media containing 2.18 mM TNP, supplemented with different nitrogen sources were used to study the effect of additional nitrogen sources on TNP degradation. Five nitrogen sources were used, namely, sodium nitrate, ammonium sulphate, urea, yeast extract and peptone. For sodium nitrate, ammonium sulphate and urea, several concentrations (1.09, 2.18, and 10.9 mM) were tested. For yeast extract and peptone, concentrations such as 0.1, 0.2, and 1.0 g/l were tested. Controls without any additional nitrogen source were included in the study. The pH of the culture media was 7.0. The batch reactors were shaken at 180 rpm and 30 °C to monitor the variation of TNP concentration, the OD of growth at 600 nm.

The results were reported as an average of three independent experiments. Maximum deviations from the average (error bars) were indicated.

2.4. Analytical methods

To determine the amount of remaining TNP and the nitrite released, samples were passed through a 0.22 μm filter and submitted to analysis. TNP in the supernatant was identified and quantified by HPLC (Waters 2996, Waters Incorporation, USA) conducted at room temperature using a Waters RP18 column (5 μm, 3.9 mm × 150 mm) and a diode array detector at a flow rate of 1.00 ml/min. The mobile phase consisted of 30% acetonitrile, 70% water and 0.26% H_3PO_4 (v/v/v). The analysis was performed at 254 nm, with column temperature at 35 °C. Nitrite ion and COD (Chemical Oxygen Demand) analysis were quantitatively determined by the method of Behrend and Heesche-Wanger [15]. COD values were obtained using mineral salts medium supplemented with the corresponding concentration of nitrite in the sample as the blank.

The optical density (OD) of cell growth at regular intervals was determined at 600 nm using a UV–vis Spectrophotometer (TU-1901, Purkinje General Instrument Co. Ltd., China) throughout the studies. As the culture supernatant turned red during fermentation, a cell free sample of the corresponding supernatant was used as the blank. The OD value minus the initial OD value made the OD increase. The natural logarithm of these measurements was plotted against the incubation time. The growth rates were calculated from slopes of the plots. The number of viable cells was determined as follows. Serial dilutions of the culture were conducted and 0.05 ml of the diluted culture was spread on a LB plate. The plate was then incubated at 30 °C for 96 h and the number of colonies was counted. The pH was measured in a pH meter (PHS-3B, Shanghai Precision & Scientific Instrument Co. Ltd., China).

For FTIR analysis, the reaction solution was filtered by a 0.22 μm filter and concentrated with a Buchi R-200 rotary vacuum evaporator (Switzerland), and was then desiccated to get solid samples. IR spectrum was obtained with KBr disc using a Bomen MB154S FTIR spectrometer (Canada). The mechanism of TNP degradation was further analyzed by MS or MS/MS. The mass spectrometer (TSQ Quantum Ultra AM, Thermo Finnigan, USA) was equipped with an electrospray ionization (ESI) source and operated in negative ESI mode. The capillary voltage was 3.0 kV and the cone voltage was 35 V. The collision energy was set to 30 eV for MS measurements, 25 eV for the MS/MS measurement.

3. Results

3.1. Selection and characterization of TNP-degrading bacteria

An indigenous bacterial strain able to grow on TNP was isolated from a soil contaminated by TNP. During the incubation of the soil sample, a transient intense orange-red color of the culture medium appeared. Finally both the orange-red color associated with metabolites and the yellow color associated with TNP faded out. Single colonies were picked.

The strain which exhibited the best TNP biodegradation ability was named after NJUST16 strain. During the incubation of the NJUST16 strain, the TNP concentration in mineral salts medium declined slowly from 2.18 mM after inoculation and then decreased more rapidly after an initial lag phase of 11 h (Fig. 1). When the TNP concentration was undetectable after 32 h, final OD_{600} increase reached maximum value of 0.38. The release of about 2.62 mol of nitrite per mol of TNP was observed in the culture. The measured COD during the batch growth period of the NJUST16 strain was always above the expected COD due to the remaining TNP concentration (0.978 mg COD/mg TNP) (Fig. 1). This difference could be attributed to intermediate metabolite. However, the difference was
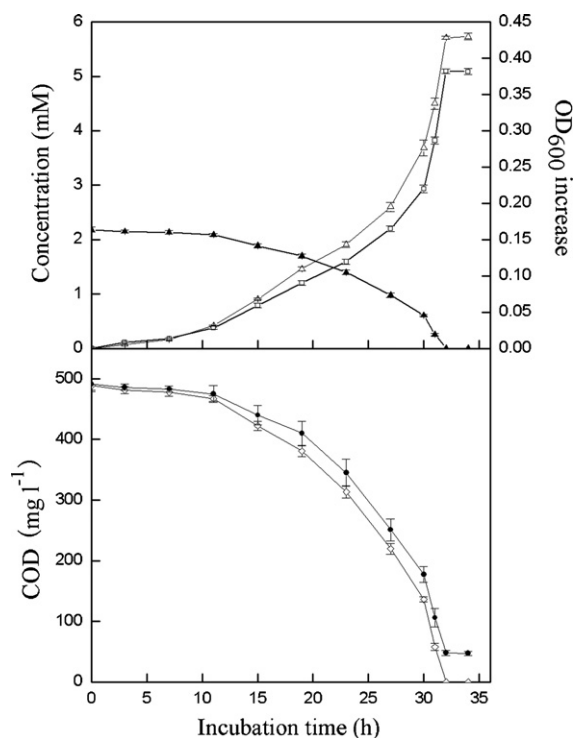
Fig. 1. Growth of NJUST16 strain with TNP as the sole carbon, nitrogen, and energy source. (▲) Concentration of TNP; (□) OD_{600} growth; (△) release of nitrite; (●) measured COD; (◇) expected COD.

rather low (<10% of the initial COD), indicating that intermediate metabolite leakage was not evident.

The NJUST16 strain was gram-positive, catalase positive and strictly aerobic rod. The partial 16S rDNA sequence of NJUST16 strain (comprising 1339 nucleotides) was determined. The phylogenetic analysis was carried out based on 16S rDNA sequence. The sequence was deposited in the GenBank database under accession no. EF635425. The strain was closely related to *Rhodococcus* sp. NCIMB12038 (GenBank accession no. AY426699) and *Rhodococcus koreensis* (GenBank accession no. AF124342), showing >99% sequence identity. Therefore, the isolate was designated *Rhodococcus* sp. NJUST16.

3.2. Optimum cell growth and TNP degradation temperature

The experiments carried out to determine the optimal temperature for the cell growth and the TNP degradation showed that the degradation of TNP and the growth of the NJUST16 strain in the mineral salts media occurred over a wide range of temperatures (Fig. 2). The greatest growth of the NJUST16 strain and TNP depletion was observed at 30 °C by utilizing 2.18 mM of TNP as substrate. The strain grew well from 20 to 35 °C, but temperatures over 35 °C and below 20 °C were proven to be unfavorable for both cell growth and TNP degradation. Complete growth inhibition of the NJUST16 strain occurred at 40 °C. Similar results were obtained in the LB media, which indicated that the NJUST16 strain is a mesophilic bacterium.
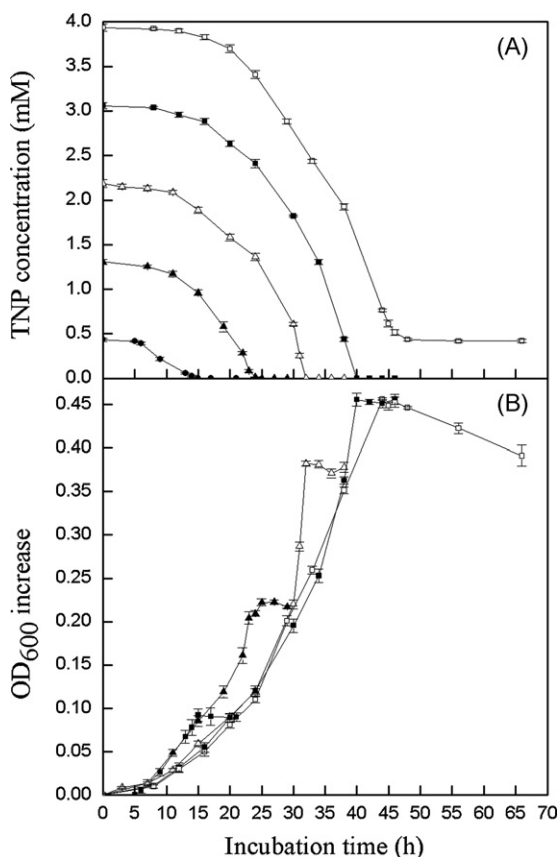
3.3. Effect of TNP concentration on cell growth and TNP degradation

In order to evaluate the effect of TNP concentration on microbial growth and TNP degradation, *Rhodococcus* sp. NJUST16 was cultivated in mineral salts media at TNP concentrations between
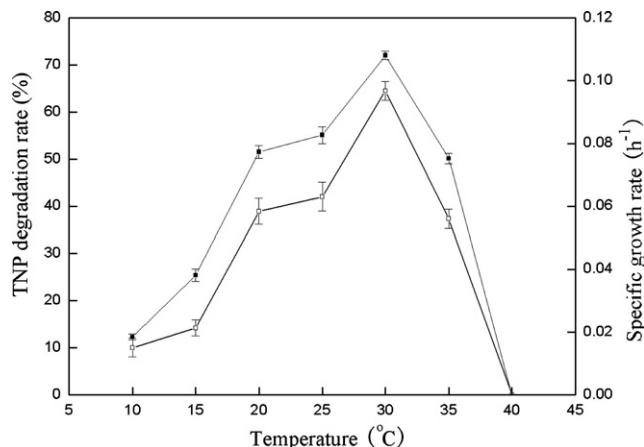
Fig. 2. The effect of temperature on the NJUST16 strain growth and TNP degradation. (■) Degradation rate; (□) specific growth rate. The data of degradation rate were the results after incubation for 30 h.

0.44 and 3.93 mM. As Fig. 3 shows, in the mineral salts media with an initial pH of 7.0, the NJUST16 strain was capable of completely degrading TNP at a concentration lower than 3.06 mM. At initial TNP concentrations of 0.44, 1.31, 2.18 and 3.06 mM, complete degradation was achieved within 15, 25, 32 and 40 h, respectively. Correspondingly, the OD_{600} increases of the cultures reached maximum of 0.09, 0.22, 0.38 and 0.46, respectively, at the time of complete exhaustion of TNP. Due to the large inoculum size, the lag phase was not remarkable. When the inoculum size was small,

Fig. 3. Biodegradation of TNP (A) and growth of the NJUST16 strain (B) at different TNP concentrations. (●) 0.44 mM; (▲) 1.31 mM; (△) 2.18 mM; (■) 3.06 mM; (□) 3.93 mM.
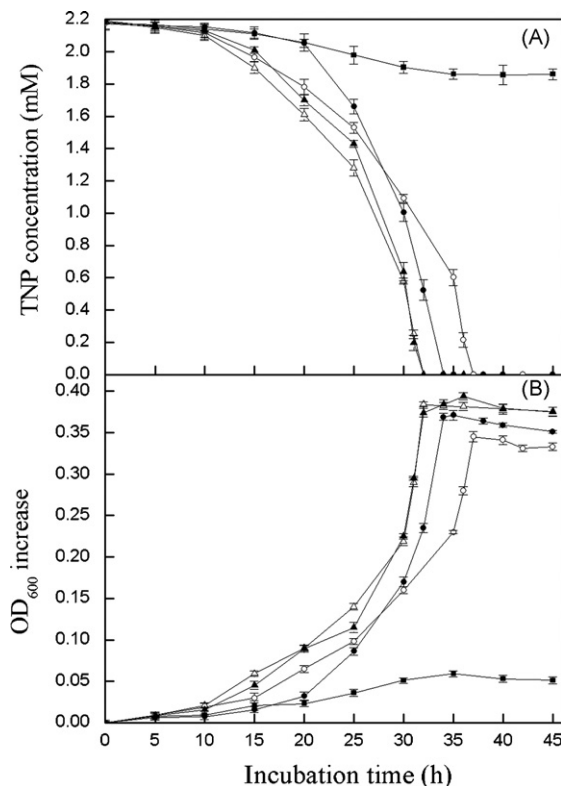

Fig. 4. The effect of pH on the biodegradation of TNP (A) and growth of the NJUST16 strain (B). (■) pH 6.0; (○) pH 6.5; (△) pH 7.0; (▲) pH 7.5; (●) pH 8.0.

there was an obvious lag phase. The lag phase increased with increasing initial TNP concentration, though the well-acclimatized inocula were used in these experiments. Similar phenomenon was observed by others in the *p*-nitrophenol biodegradation system [4]. Wan et al. [4] concluded that NACs were toxic to microorganisms. It appeared that the $-NO_2$ group inhibited the microbial metabolism even at relatively low concentration. We had experimentally verified that high TNP concentrations resulted in increased viability loss. For example, at initial TNP concentration up to 3.49 mM, the viable cell counts decreased from 8×10^5 to 3×10^5 cells/ml before the cells got rid of the lag phase. While at TNP concentration of 3.93 mM, degradation was incomplete with about 89% of TNP degraded after 48 h, but no further degradation was observed for a longer time. Although phosphate buffer was present in the media, pH decreased from 7.0 to 6.23 at the end of study with the initial TNP concentration of 3.93 mM.

3.4. The pH dependence of metabolism

Mineral salts media with various pHs were prepared to explore the effect of pHs. The growth of the strain NJUST16 and TNP degradation were optimal at an initial pH of 7.0–7.5 (Fig. 4), showing that a neutral to slightly alkaline pH might be required for the degradation of TNP. At an initial pH of 6.0, only about 10% of the added 2.18 mM TNP was degraded within 25 h, but no further degradation was observed within 45 h. At an initial pH of 8.0, the lag phase prolonged, but the growth of the NJUST16 strain and TNP degradation were accelerated after the lag phase. No TNP degradation was observed in the incubations without the inocula at any tested pH condition.

In order to clarify whether the cell death that occurred at higher initial TNP concentration (>3.93 mM) was due to pH drop during the incubation, the pH dependence of TNP degradation was
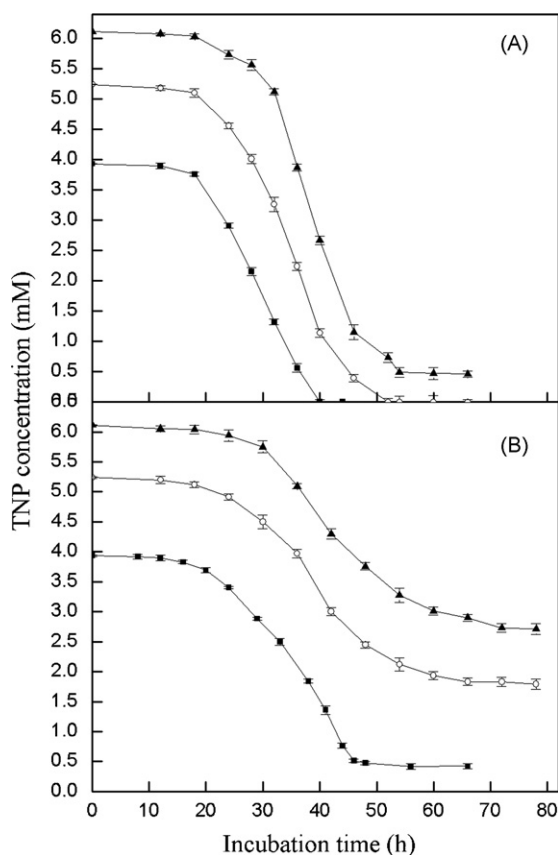
Fig. 5. The degradation of TNP at high concentrations in the mineral salts media with different buffer capacity. (A) Concentrated phosphate buffer (28 mM); (B) less concentrated phosphate buffer (14 mM). (■) 3.93 mM of TNP; (○) 5.24 mM of TNP; (▲) 6.11 mM of TNP.

examined (Fig. 5). More concentrated phosphate buffer (28 mM, pH 7.0) was used, and the mineral salts media with a larger buffer capacity was obtained. In the new phosphate buffer system, at initial TNP concentrations of 3.93 and 5.24 mM, complete degradation was achieved within 40 and 52 h, respectively (Fig. 5(A)). pHs dropped gradually from initial 7.0 to final 6.55 and 6.41, respectively. While in the less concentrated phosphate buffer system (14 mM, pH 7.0), at initial TNP concentrations of 3.93 and 5.24 mM, pHs dropped from initial 7.0 to final 6.23 and 6.13, respectively, with

Table 1
Effect of pH on number of viable cells

Initial TNP concentration (mM)	Incubation time (h)	Concentration of phosphate buffer (mM)	Number of viable cells/ml
3.93	Time[a]	14	1.6×10^7
3.93	Time[b]	14	4.8×10^5
3.93	Time[a]	28	9.2×10^{10}
3.93	Time[b]	28	6.4×10^{10}
5.24	Time[a]	14	4.6×10^5
5.24	Time[b]	14	7.8×10^4
5.24	Time[a]	28	1.2×10^{11}
5.24	Time[b]	28	8.2×10^{10}
6.11	Time[a]	14	6.8×10^4
6.11	Time[b]	14	5.6×10^3
6.11	Time[a]	28	4.2×10^6
6.11	Time[b]	28	9.4×10^4

[a] Number of viable cells was recorded at the time when the degradation finished or ceased.

[b] Number of viable cells was recorded 10 h after the degradation finished or ceased.

Table 2
Effect of various nitrogen sources on degradation of TNP by NJUST16 strain at initial concentration of 2.18 mM

Nitrogen source	Concentration	OD[a]	Time required for complete TNP degradation (h)
Sodium nitrate	1.09 mM	0.379 ± 0.0017	32.33 ± 0.58
Sodium nitrate	2.18 mM	0.369 ± 0.0026	32.67 ± 0.58
Sodium nitrate	10.9 mM	0.354 ± 0.0019	33 ± 1
Ammonium sulphate	1.09 mM	0.385 ± 0.0021	31.33 ± 0.58
Ammonium sulphate	2.18 mM	0.392 ± 0.0046	29 ± 1
Ammonium sulphate	10.9 mM	0.374 ± 0.0035	33.33 ± 0.58
Urea	1.09 mM	0.378 ± 0.0025	31 ± 1
Urea	2.18 mM	0.375 ± 0.0046	29.33 ± 0.58
Urea	10.9 mM	0.383 ± 0.0034	32 ± 1
Yeast extract	0.1 g/l	0.435 ± 0.0036	28.67 ± 0.58
Yeast extract	0.2 g/l	0.474 ± 0.0038	25 ± 1
Yeast extract	1.0 g/l	1.466 ± 0.0142	40 ± 1
Peptone	0.1 g/l	0.464 ± 0.0031	27 ± 1
Peptone	0.2 g/l	0.483 ± 0.0067	24.33 ± 0.58
Peptone	1.0 g/l	1.453 ± 0.0183	39.33 ± 0.58
Without additional nitrogen sources	–	0.382 ± 0.0032	31.67 ± 0.58

±Standard deviation.

[a] Cell density (OD) was recorded at the time of complete exhaustion of TNP.

the incomplete degradation of TNP (Fig. 5(B)). At even higher initial TNP concentrations of 6.11 mM, the chemical in the both phosphate buffer systems was incompletely degraded, however, in the concentrated phosphate buffer system, more TNP was degraded. While in the previous mineral salts media containing less concentrated phosphate buffer, at higher TNP concentrations (>3.93 mM), independent of the initial TNP concentration, only about 3.46 mM TNP was degraded (Fig. 5(B)). Subsequently cell death occurred (Table 1). Numbers of viable cells at the time when the degradation finished or ceased in the different buffer system were determined using the spread plate method. In the less concentrated phosphate buffer system, numbers of viable cells were much smaller than that in the buffer system with a larger buffer capacity. Ten hours after the degradation finished or ceased, viability loss was observed under all the test conditions, however, in the less concentrated phosphate buffer system, the number of viable cells decreased dramatically.

3.5. Effect of additional nitrogen source

Various nitrogen sources (sodium nitrate, ammonium sulphate, urea, yeast extract and peptone) were used to find their effect on the degradation of 2.18 mM TNP (Table 2). Addition of sodium nitrate at 1.09 and 2.18 mM did not exert significant effect on TNP degradation. However, addition of ammonium sulphate and urea at 1.09 and 2.18 mM stimulated TNP degradation slightly. High concentration of sodium nitrate, ammonium sulphate and urea (10.9 mM) contributed to the decrease of TNP degradative activity of NJUST16 strain. Addition of yeast extract and peptone at 0.1 and 0.2 g/l stimulated both biomass formation and TNP degradation, while at 1.0 g/l, biomass formation was enhanced at the cost of delayed TNP degradation.

3.6. Metabolites analysis and identification

Fig. 6 illustrates the FTIR spectra of TNP and TNP biodegradation products with an initial TNP concentration of 2.18 mM. Most of bands of TNP, e.g., the characteristic band of γ(C–H) of benzene ring at wavenumber of about 3070 cm^{-1}, the bands of γ(C=C) of benzene ring at wavenumber of about 1480 cm^{-1}, the bands of γas(NO$_2$) of benzene ring at wavenumber of about 1550 cm^{-1}, the bands of γs(NO$_2$) of benzene ring at wavenumber of about
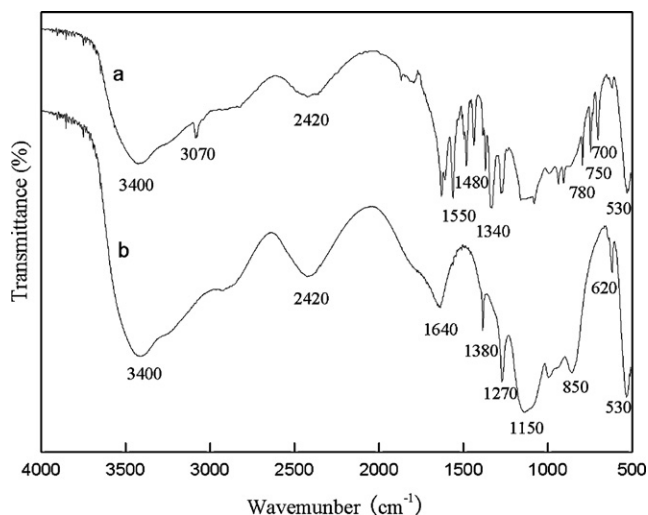
Fig. 6. FTIR spectra of TNP. (a) Authentic TNP; (b) products under NJUST16 strain treatment for 40 h.

$1340\,cm^{-1}$, and peaks at $675\text{–}910\,cm^{-1}$ attributed to δ(C–H) of benzene ring, significantly decreased in the spectrum of the TNP degradation products under the treatment of NJUST16 strain. The cleavage of benzene ring and elimination of nitro groups was unambiguously identified.

The degradation products were further analyzed by using MS (Fig. 7). Based on exact mass measurements, the elemental composition of the ion was determined to be TNP at m/z 228. In the product ion spectrum, the most abundant fragment ions were detected at m/z 232, 183, 189 and 204. Using exact mass measurements, the elemental compositions of these product ions were determined to be the dihydride Meisenheimer complex of TNP ($2H^{-}$–TNP), the hydride Meisenheimer complex of DNP (H^{-}–DNP), 2,4-dinitrocyclohexanone (2,4-DNCH) and 4,6-dinitrohexanoate (4,6-DNH), respectively. The pathway has been described for TNP degradation in the literature, in which the genes and enzymes involved in TNP degradation have been characterized [11,21]. The degradation products observed in this study coincided with those observed in the literature [11,21]. Thus the TNP catabolic pathway of NJUST16 strain could be described as below (Fig. 8): TNP is firstly hydrogenated at the aromatic nucleus. The hydride Meisenheimer complex of TNP (H^{-}–TNP) thereby formed is further hydrogenated, producing the dihydride Meisenheimer complex of
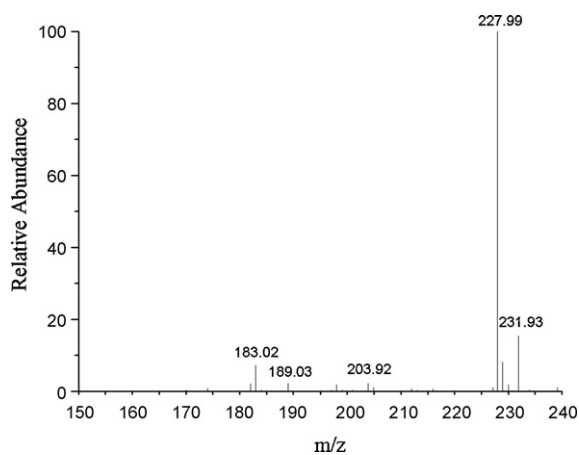
TNP ($2H^{-}$–TNP). Then the nitrite is eliminated from $2H^{-}$–TNP to produce the hydride Meisenheimer complex of DNP (H^{-}–DNP). H^{-}–DNP was further hydrogenated, forming $2H^{-}$–DNP, which is protonated to form 2,4-DNCH. 2,4-DNCH was finally hydrolyzed and converted to 4,6-DNH. 4,6-DNH can disappear rapidly with the release of nitrite [21], although a product could not be identified.
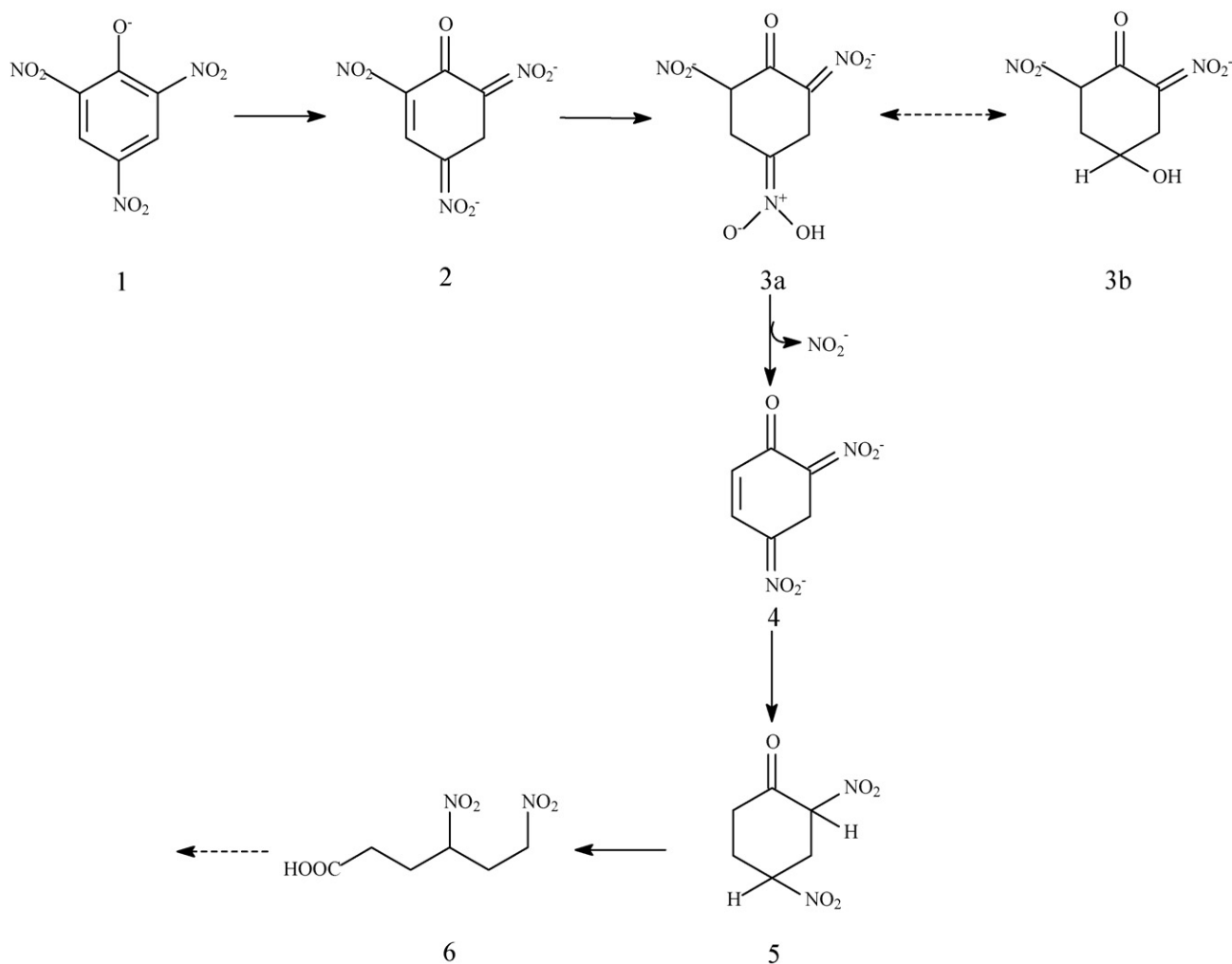
4. Discussion

TNP has been reported to be highly toxic to some microorganisms [24,25]. The toxicity of higher concentrations of nitroaromatics limits its degradation by microbes. However, *Rhodococcus* sp. NJUST16, which was isolated from a soil contaminated by TNP has been found to be TNP-tolerant, and can grow well in the mineral salts media, in which picric acid could be degraded almost completely. No depletion of TNP concentration or increase in the values of OD_{600} was noticed in the abiotic controls, and no growth was detected in the controls without TNP. In addition, no other carbon or nitrogen sources were present in mineral salts media used during the selection of the strain. It indicates that TNP is utilized as the sole source of carbon, energy, and nitrogen.

What's more, the stoichiometric release of nitrite and the color change during the degradation of TNP were in agreement with previous observations [15,17]. As shown in the references, the orange-red color compound which was transiently observed by us may be the well known hydride Meisenheimer complex. The degradation pathway of TNP has been proposed. Firstly, two hydrogenations take place in the initial attack on TNP. Then nitrite is eliminated from $2H^{-}$–TNP to produce the hydride Meisenheimer complex of DNP (H^{-}–DNP). H^{-}–DNP is further hydrogenated and cleavage of ring fission takes place, forming 4,6-DNH. The cleavage of benzene ring and elimination of nitro groups is also unambiguously identified by FTIR analysis.

As the stoichiometric release of nitrite occurs during the degradation of TNP by the NJUST16 strain, we suspect that all of the remaining two nitro groups are cleaved from 4,6-DNH, forming carboxylic acids which are readily susceptible to biodegradation. Moreover, at the initial TNP concentration of 2.18 mM, the residual COD was rather low, verifying the lack of dead-end product formation. These results indicate that significant mineralization of TNP occurred during the incubation.

Our results show that the ability of the NJUST16 strain to degrade TNP is influenced by TNP concentration. At low concentrations, the NJUST16 strain degraded TNP completely and rapidly, while incomplete degradation of 3.93 mM TNP by the NJUST16 strain at initial pH of 7.0 was observed, suggesting that cell toxicity of TNP and its transformation products towards the NJUST16 strain exists. Improvement of the rate of TNP degradation by *Rhodococcus* sp. NJUST16 at higher concentrations by optimizing processing parameters is a subject for further investigation.

In order to explore the possible reason for the observed incomplete degradation of TNP by the NJUST16 strain at relatively high concentrations, the pH dependence was investigated. In the phosphate buffer system (28 mM) with a greater buffer capacity, TNP was metabolized at starting concentrations up to 5.24 mM at initial pH of 7.0, and the pH was maintained well above 6.3. While in the phosphate buffer system (14 mM) with a small buffer capacity, only about 3.46 mM TNP could be completely removed with an obvious pH drop. However, the drop in pH during TNP degradation was not described by Behrend and Heesche-Wanger [15]. The authors attributed the cell death that occurred during the consumption of high concentration TNP to nitrite sensitivity [15]. Indeed, the addition of nitrite leads to inhibitory effect on TNP degradation. However, the nitrite sensitivity is not the only reason. For example, although the incubation time doubled, total transformation

Fig. 7. Mass spectrum of the intermediates of TNP biodegradation. The mass spectrometer was operated in negative ESI mode.

Fig. 8. The proposed biodegradation pathway of TNP by NJUST16 strain. (Panel 1) TNP; (Panel 2) H−−TNP; (Panel 3a) aci-nitro form of 2H−−TNP; (Panel 3b) nitro form of 2H−−TNP; (Panel 4) H−−DNP; (Panel 5) 2,4-dinitrocyclohexanone (2,4-DNCH); (Panel 6) 4,6-dinitrohexanoate (4,6-DNH).

of 2.18 mM TNP by the NJUST16 strain was observed at additional nitrite concentration up to 6.54 mM. In addition, in the phosphate buffer system with the small buffer capacity, at an initial pH of 7.5, TNP was completely degraded at starting concentration up to 5.24 mM. From these, we can infer that, besides nitrite sensitivity, pH is also crucial to achieving high concentration of TNP degradation by the NJUST16 strain. Low pH did not favor TNP degradation. An increase in the initial pH enhanced TNP degradation at high concentration. The pH dependence of TNP degradation indicates that the cell death that occurred at higher initial TNP concentration (>3.93 mM) is due to pH drop during the incubation. As acidification during degradation reduces the degradation efficiency and causes cell death, TNP degradation is achieved at neutral and slightly basic pHs.

Researchers have noted the toxicity of nitrous acid (HNO$_2$) and the deleterious effect of low pH on denitrification systems, suggesting that the concentration of nitrous acid, not nitrite, controls the inhibition of denitrification [26,27]. Hence, it is assumed that the toxicity of nitrous acid (HNO$_2$) is consistent with the deleterious effect of low pH on TNP degradation. As described previously, at an initial pH of 8.0, the growth of the NJUST16 strain and TNP degradation were accelerated after the prolonged lag phase. Perhaps it is due to the low concentration of nitrous acid generated. While at an initial pH of 6.0, unsuccessful degradation was observed. An inter-

esting observation concerning a biological aerobic filter system in our laboratory, in which the NJUST16 strain and nitrifying bacteria coexist, was that the degradation of TNP was successful at initial TNP concentration up to 6.55, even at a low effluent pH of 6.5. It indicates that the growth of the NJUST16 strain and TNP degradation are susceptible to changes in both pH of the media and nitrite released during TNP degradation. These results suggest that the pH change during the degradation is an important factor that needs to be considered when making a remediation strategy.

Besides, as previously described, the NJUST16 strain could grow on TNP in the absence of additional nitrogen, which suggested that the additional nitrogen was not a necessary nutrient for its growth. Addition of sodium nitrate, ammonium sulphate and urea did not exert significant effect on TNP degradation. However, organic nitrogen sources such as yeast extract and peptone, which contained a considerable amount of vitamins and other cofactors, could have an important effect in both to stimulate the growth and TNP degradation. On the other hand, yeast extract and peptone also supplied the carbon for biomass growth. Results proved that though addition of yeast extract and peptone enhanced the biomass formation greatly, the stimulation on TNP degradation was concentration dependent. Low concentration of yeast extract and peptone accelerated the degradation of TNP, while high concentration did not. The organism ignores TNP in presence of high concentration of yeast extract

and peptone, thus TNP degradation period was delayed. The concentration of the stimulant such as yeast extract and peptone and that of the compound to be degraded would, therefore, be of prime importance for removal of TNP from contaminated sites.

5. Conclusion

The isolated stain *Rhodococcus* sp. NJUST16 could withstand and mineralize relatively high concentration of TNP, which constituted the basic need in effluent treatment strategies.

The strain was able to actively metabolize TNP at temperature from 20 to 35 °C and initial pH between 7.0 and 7.5. Addition of nitrogen sources such as yeast extract and peptone accelerated the degradation of picric acid, but the stimulation was concentration dependent.

The toxicity of nitrous acid (HNO_2) was consistent with the deleterious effect of low pH on TNP degradation. The drop of pH and the nitrite released weakened the degradation of TNP by the NJUST16 strain. Both of them would be of prime importance while designing an effective remediation strategy.

Acknowledgments

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