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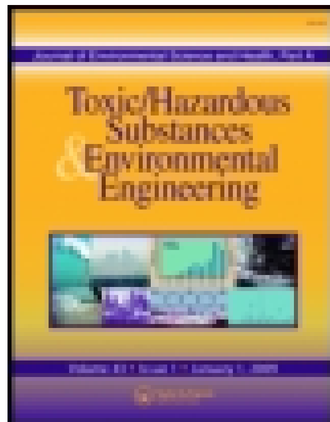

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Degradation of aromatic compounds and degradative pathway of 4-nitrocatechol by Ochrobactrum sp. B2

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Degradation of aromatic compounds and degradative pathway of 4-nitrocatechol by *Ochrobactrum* sp. B2

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The potential capacity of a soil methyl parathion-degrading bacterium strain, *Ochrobactrum* sp. B2, for degrading various aromatic compounds were investigated. The results showed B2 was capable of degrading diverse aromatic compounds, but amino-substituted benzene compounds, at a concentration up to 100 mg L^{-1} in 4 days. B2 could use 4-nitrocatechol (4-NC) as a sole carbon and energy source with release of nitrite ion. The pathway for 4-NC degradation via 1,2,4-benzenetriol (BT) and hydroquinone (HQ) formation in B2 was proposed based on the identification and quantification of intermediates by gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatography (HPLC). Degradation studies carried out on a plasmid-cured derivative showed that the genes for 4-NC degradative pathway was plasmid-borne in B2, suggesting that B2 degrades both *p*-nitrophenol and 4-NC by enzymes encoded by genes on the same plasmid.

Keywords: Biodegradation; degradative pathway; 4-nitrocatechol; *Ochrobactrum* sp. B2; plasmid.

Introduction

Nitroaromatic compounds are among the most important and versatile industrial organic compounds, and are widely distributed in the environment because of their extensive use as pesticides, pharmaceuticals, dyes, plastics, explosives and solvents.[1] These compounds can also be found in the soil as a result of the hydrolysis of several organophosphorus insecticides such as methyl parathion.[2] Nitroaromatics and products of their incomplete degradation have relatively high acute toxicity, and some may be potential carcinogens.[3] Therefore, these compounds occur as contaminants of industrial effluents, soil and groundwater where they have deleterious consequences.[1]

p-Nitrophenol (PNP) is probably the most important among the mononitrophenols in terms of the quantities used and the potential environmental contamination.[4] It have been documented that PNP can be degraded via three monooxidative pathways through different intermediates by different species.[5] 4-nitrocatechol (4-NC) has been known for a relatively long time as an intermediate in the degradation of PNP by some isolates such as *Arthrobacter* sp., *Bacillus* sp., and *Nocardia* sp. TW2,[6–8] but studies on 4-NC degradation are relatively sparse. Organisms capable of degrading both PNP and 4-NC have previously been documented.[9,10] However, Navratilova et al.[11] recently reported that *Rhodococcus wratislaviensis* strain J3 was able to degrade 4-NC, rather than PNP. The complete degradation pathway of 4-NC was described in *Burkholderia cepacia* RKJ200 by Chauhan et al.[9] and in *Arthrobacter protophormiae* by Chauhan et al.[10] These findings showed that the first step in 4-NC degradation by both strains involved the conversion of 4-NC into 1,2,4-benzenetriol (BT), followed by a similar reductive aerobic dehydroxylation of 2-hydroxy-1,4-benzoquinone (HBQ) with the formation of *p*-benzoquinone (BQ) and hydroquinone (HQ).[9,10] With relatively limited information available, it is not known if this is the only pathway in 4-NC degradation.

We have isolated a bacterium, *Ochrobactrum* sp. B2, which hydrolyzes methyl parathion to PNP and then converts this to nitrite and carbon dioxide.[12] In this study, we are to determine whether this bacterium is able to utilize other aromatic compounds. In addition, although we have

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found in our previous work that 4-NC is one of the metabolites of MP degradation pathway,[12] it remains to be investigated whether 4-NC shares the same degradation pathway and uses the same genes as PNP degradation in B2. To seek answers to these questions, we conducted biodegradation studies with 4-NC as the starting substrate.

Materials and methods

Chemicals

4-Nitrocatechol (98%) was purchased from Fluka (Buchs, Switzerland); 1,2,4-benzenetriol (99%) from Aldrich (Milwaukee, WI, USA); hydroquinone (>98%) from Tianjin Chemical Plant (Tianjin China); 2,2′-dipyridyl (>99.9%) from Beijing Siying Chemical Plant (Beijing, China). All other chemicals used were of the highest purity available.

Bacterial strains

The wild-type *Ochrobactrum* sp. B2 and its acridine orange cured derivative (PNP^-)[12] were used in this study. The *Ochrobactrum* sp. B2 was isolated from a soil sample obtained from the yard of a pesticide factory (located in Gaomi, Shandong Province, China) which manufactures organophosphorus insecticides. *Ochrobactrum* sp. B2 is a rod-shaped, Gram-negative soil bacterium, which has been described by Qiu et al.[12]

Biodegradation studies

The inocula for all the experiments of the degradation study were prepared by growing bacteria in 50 mL of MSM[13] supplemented with 0.1% glucose and incubated for 36 hours at 30°C on a shaker at 150 rpm. The culture was aseptically harvested and the cells washed thoroughly with MSM (8000 × g, 10 min, 4°C). The cells were suspended in sterile MSM and added as the inoculum to MSM containing chemicals (at 100 mg L^{-1}, except 4-NC) and incubated on a rotary shaker (150 rpm) at 30°C. After incubating for 4 days, portions of the supernatants of cultures (10000 × g, 30 min, 4°C) were filtered (0.22 μm) and used for residual analysis by HPLC. All the experiments were performed independently in triplicate. Un-inoculated control tubes were maintained in all experiments.

For the 4-NC degradation pathway study, 0.063 mmol L^{-1} 4-NC was added in the culture as mentioned here. After regular intervals, samples were taken for quantitation of cell growth, 4-NC, and nitrite ion after centrifugation and filtration. The pelleted cells were washed thoroughly with MSM (8000 × g, 10 min, 4°C) and suspended in MSM. The growth of cells was determined by monitoring the OD_{600} of suspended cells. The filtrates were used to determine the concentrations of 4-NC and nitrite ion, and to identify the metabolites. Nitrite ion was quantitatively determined based on the standard curves prepared using sodium nitrite (DU-800 spectrophotometer, Beckman Coulter Inc., Fullerton, California, USA) by the method of Montgomery and Dymock.[14]

The residuals of 4-NC and the other tested chemicals were quantified by Agilent 1100 HPLC. (Agilent Technologies, Palo Alto, CA, USA). HPLC studies were conducted at room temperature using an Agilent Zorbax 300SB-C18 column and acetonitrile: water = 20:80 (water contains acetic acid, 650:1,v: v,pH = 3.0) as the mobile phase at a flow rate of 1 mL min^{-1}. The analysis was performed at 354 nm (DAD detector), column pressure of 126 bar, column temperature at 40°C. Injection volume was 5 μL.

Identification of metabolites

Metabolites were identified by GC-MS and HPLC using culture extracts of B2 that had been incubated with 4-NC as the degradation study. Portions of the cultures were extracted twice with equal volumes of ethyl acetate, and extracts were dehydrated with sodium sulfate. These extracts were filtered (0.45 μm) and used to identify metabolites by GC-MS. GC-MS was obtained on an Agilent Technologies 6890N GC-5973N MSD (Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with a DB-5MS (95% polydimethyl siloxae 5% poly-1,4-bis-dimethylsiloxae phenylene siloxae, 60 m × 0.25 mm ID, film thickness 0.25 μm) capillary column and operated in splitless mode. Helium (>99.999%) was used as carrier gas with a constant flow rate 1 mL min^{-1}. The injection temperature was 250°C and the GC-MS transfer line temperature was 280°C, ion source 230°C, quadrupole 150°C. All samples were analyzed with 70 eV nominal electron energy and scan range 30–300 amu.

Quantitative analysis of intermediates in 4-NC degradation by B2

Quantitative analysis of intermediates in 4-NC degradation was performed by HPLC studies. Incubations were set up as in the biodegradation studies described above. 2,2′-dipyridyl (1 mmol L^{-1}) (an inhibitor of the ring cleavage of some aromatic compounds[10]) was also added in order to detect accumulating intermediates from 4-NC. Metabolites were identified and by comparison of retention times with those of authentic compounds. BT and HQ concentrations were respectively quantified from their concentration curves.

Results and discussion

Degradation study of some aromatic compounds

B2 was capable of degrading diverse aromatic compounds at a concentration up to 100 mg L^{-1} in four days. The degradation rates were in the order of 4-nitrophenol > p-methyl phenol > phenol > 2-nitrophenol > nitrobenzene

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Table 1. Degradation of aromatic compounds by *Ochrobactum* sp. B2

Chemicals	*Retention time (min)*	*Without B2 (mg L^{-1})*	*With B2 (mg L^{-1})*	*Degradation (%)*
4-nitrophenol	5.361	103.9 ± 3.7	0	100
2-nitrophenol	7.516	98.6 ± 1.9	57.4 ± 0.5	41.8
aniline	2.913	100.2 ± 1.3	99.8 ± 4.0	0
4-nitroaniline	4.980	100.4 ± 0.4	100.1 ± 0.2	0
2,6-dinitroaniline	6.183	100.2 ± 0.9	99.9 ± 1.9	0
phenol	4.382	101.2 ± 1.1	41.9 ± 2.5	58.6
p-methyl phenol	5.890	100.3 ± 0.4	31.6 ± 0.4	68.5
nitrobenzene	8.927	99.6 ± 0.5	55.9 ± 1.1	40.9

MSM containing 100 mg L^{-1} of chemicals was inoculated with B2 cells (initial OD_{600} was 0.02) and incubated on a rotary shaker (150 rpm) at 30°C for 4 days. The data were the mean ± standard deviation of 3 replicates.

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(Table 1). No degradation of all the three tested amino-substituted benzene compounds was detected. These results indicated that B2 could be used in the remediation of nitro-, hydroxyl-substituted aromatic compounds.

Degradation study of 4-NC

No degradation of 4-NC was observed in an uninoculated control (data not shown). Strain B2 degraded 0.063 mmol L^{-1} of 4-NC after 36 h (Fig. 1). B2 released stoichiometric amounts of nitrite (0.063 mmol L^{-1}) in the medium, indicating the involvement of an initial oxidative step in the degradation of 4-NC.[8] *Ochrobactrum* sp. B2 cells grew with a significant lag period (about 12 h). Cell growth accompanied significant degradation of 4-NC and simultaneous release of nitrite ions after 12 h of incubation, suggesting that the 4-NC degrading enzymes seemed to be induced or activated by 4-NC.[7]

There are few reports regarding the biodegradation of 4-NC. A relatively detailed study was made by Navratilova et al.[11] They found that *Rhodococcus wratislaviensis* J3 degraded 0.043 mmol L^{-1} of 4-NC with a stoichiometric nitrite release after 10 h. Then, 0.1 mmol L^{-1} of 4-NC was degraded after 24 h and a higher concentration of 4-NC (0.7 mmol L^{-1}) after 62 h. *Ochrobactrum* sp. B2 could totally degrade 0.063 mmol L^{-1} of 4-NC within 36 h (Fig. 1), suggesting that B2 could be used for bioremediation.

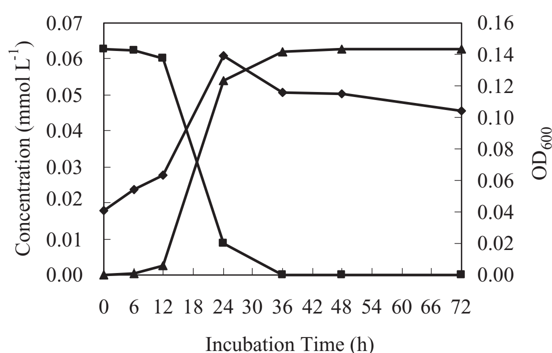

Fig. 1. Growth of *Ochrobactrum* sp. B2 in MSM media containing 4-NC as the sole carbon source and changes in the concentrations of 4-NC and nitrite ions. (■) 4-NC, (▲) Nitrite ion, and (♦) OD_{600}. MSM containing 0.063 mmol L^{-1} 4-NC was inoculated with B2 cells (initial OD600 was 0.04) and incubated on a rotary shaker (150 rpm) at 30°C. All the data points are means ±SD of values from 3 replicates.

Catabolic pathway of 4-NC degradation by B2

HPLC studies revealed the appearance of additional peaks in the samples drawn from 22–30 hour intervals not seen in the control samples, of which the retention times correlated well with those of authentic 1,2,4-benzenetriol (BT) (6.015 min), and hydroquinone (HQ) (6.793 min) (Fig. 2). GC-MS analysis indicated that the presence of two metabolites, identified as BT, and HQ based on retention time (Table 2) and mass spectra (Fig. 3). Taken together, the above results indicated that the BT, and HQ were probable metabolites.

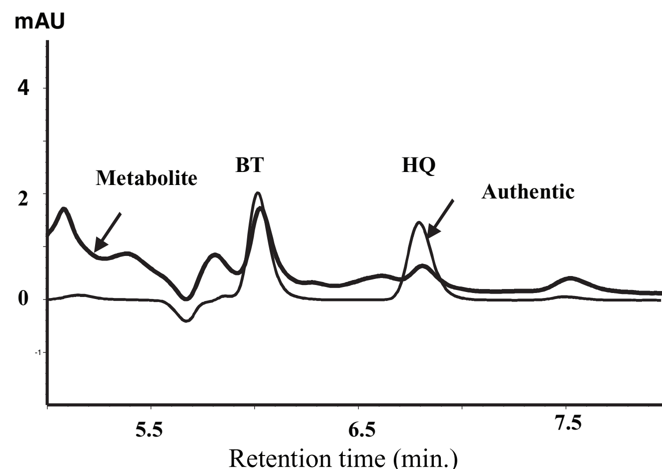

Fig. 2. HPLC chromatogram of the metabolites in 4-NC degradation by B2. Samples were drawn at the 22 h growth interval. Culture conditions were as in Figure 1.

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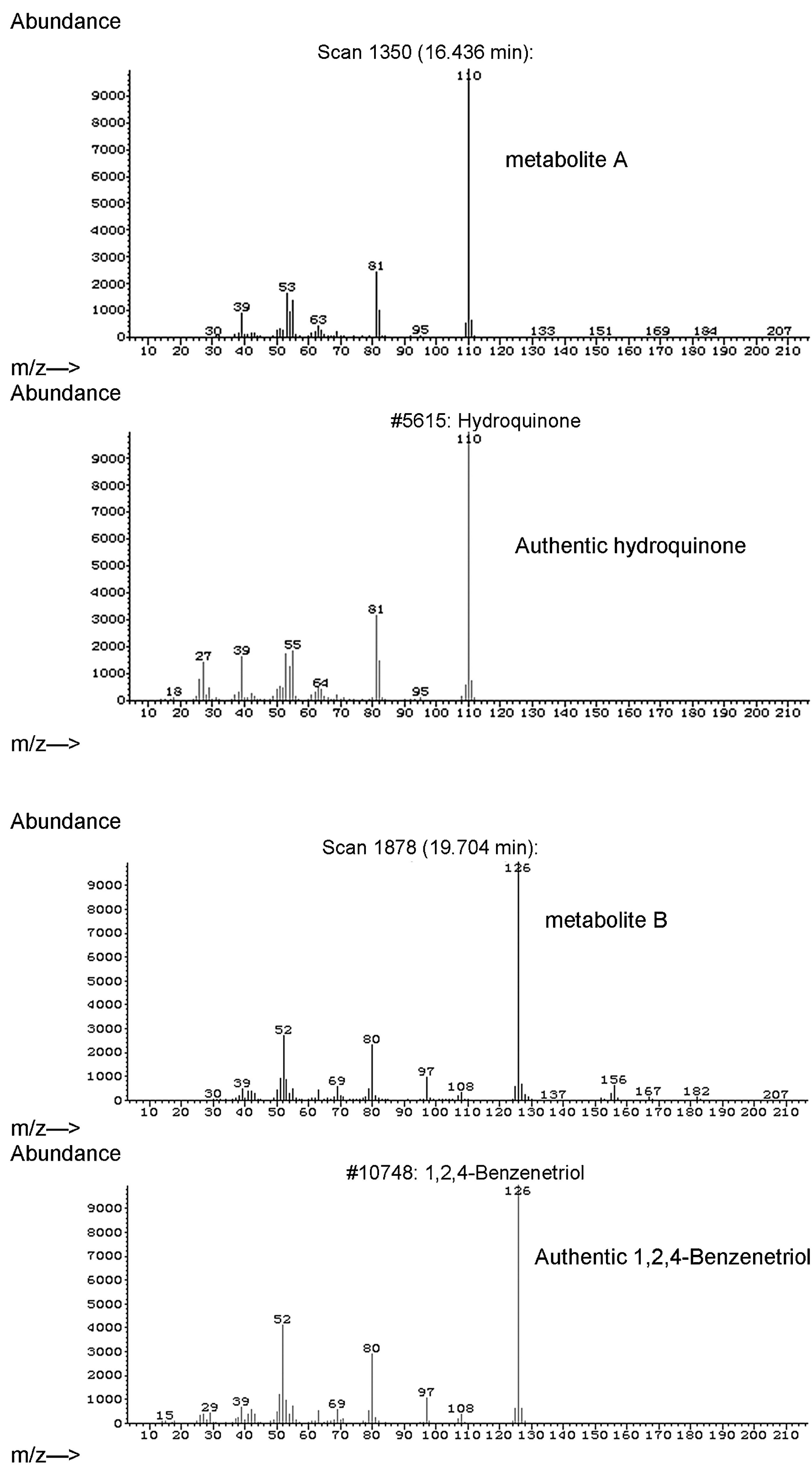

Fig. 3. GC-MS of metabolites in the degradation of 4-NC by B2. Samples were drawn at the 22 h growth interval. Culture conditions were as in Figure 1.

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Table 2. GC-MS characterization of the metabolites formed during degradation of 4-NC by *Ochrobactrum* sp.B2

Retention time (min)	*Mass spectral properties {m/z(% relative intensity) [molecular ion]}*	*Metabolite identified as*[a]
16.44	110*(100)[M+], 82(12), 81(26), 55(14), 54(12), 53(18)	Hydroquinone (HQ)
19.70	126*(100)[M+], 108(6), 97(12), 80(26), 69(8), 53(10), 52(28), 51(11)	1,2,4-Benzenetriol (BT)
25.90	155*(100)[M+], 139(7), 125(27), 109(25), 107(20), 97(8), 81(32), 79(15), 63(15), 55(22), 53(28), 51(20)	4-Nitrocatechol (NC)

[a]The metabolites were identified after comparison with standard compounds.

BT and HQ present in the medium were quantified during the growth period by HPLC analysis. In the presence of 2,2′-dipyridyl, 1 main compound was detected after 22 h of incubation: 0.35 mg L^{-1} BT; after 24 h, 2 compounds were detected: 0.34 mg L^{-1} BT, and 0.11 mg L^{-1} HQ; after 26h, no BT was detected, and the concentration of HQ reached its peak (1.27 mg L^{-1}). When the same studies were carried out without 2,2′-dipyridyl, 0.18 mg L^{-1} BT and 1.12 mg L^{-1} HQ were detected after 22 h; after 24 h, 1.11 mg L^{-1} of HQ was detected only in the medium; after 26 h, both HQ and BT were undetectable. Neither of these compounds could be detected in samples drawn after 30 h of incubation, suggesting that B2 could mineralize the 4-NC under the conditions of our experiments. Comparing the concentrations of HQ at different sampling intervals, we found that there was significant accumulation of HQ after 26 h of incubation in the presence of 2,2′-dipyridyl, suggesting the inhibition of the ring cleavage of HQ. The kinetics of BT and HQ demonstrated that HQ formed after BT in 4-NC degradation (Fig. 4).

Based on our results described here, and the findings made by Chauhan et al.,[9,10] we proposed the pathway for degradation of 4-NC in B2 as in Figure 5. This pathway is similar to those reported in the cases of *Arthrobacter protophormiae* and *B. cepacia* RJ200,[9,10] suggesting a common pathway occurs among different bacteria. It would be of importance to compare 4-NC degrading genes of these strains to see if they share a common origin. Interestingly, although *Arthrobacter portophormiae* and *Ochrobactrum* sp. B2 have similar 4-NC degradation pathways, they degrade PNP via different routes: the first step of PNP degradation by *Arthrobacter protophormiae* is the formation of BQ,[9,10] while *Ochrobactrum* sp. B2 degrades PNP via the formation of 4-NC.[12] Based on the fact that B2 degrades PNP via the formation of 4-NC[12] and identical metabolites in PNP and 4-NC degradation were detected,[12] it is likely that the same set of genes encode the further degradation of 4-NC in PNP and 4-NC degradation. Cloning and characterization of genes encoding activities in PNP degradation is underway in our laboratory, which will benefit the understanding of the genetics and evolution of nitrophenol degradation.

Location of the catabolic genes for 4-NC degradation

To determine whether 4-NC degrading activity was controlled by a plasmid, we performed a degradation study using the plasmid-cured mutant of *Ochrobactrum* sp. B2

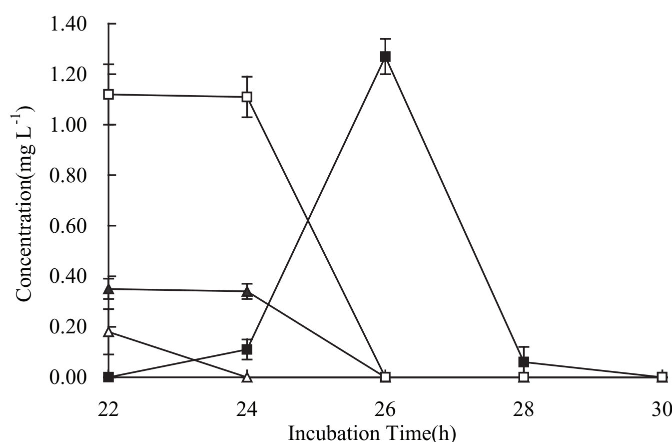

Fig. 4. Quantification of formation of intermediates (BT, HQ) by *Ochrobactrum* sp. B2 by HPLC analysis. (▲) BT and (■) HQ when the ring cleavage was blocked using 2, 2′- dipyridyl (1 mmol L^{-1}). (△) BT, (□) HQ in the absence of 2,2′-dipyridyl. Culture conditions were as in Figure 1. All the data points are means ±SD of values from three replicates.

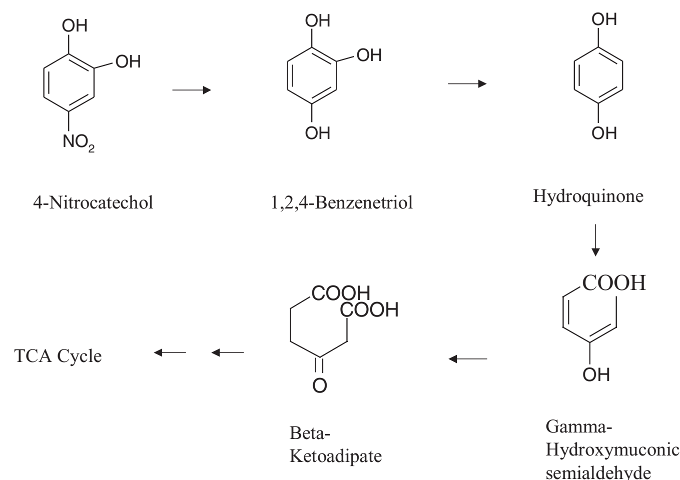

Fig. 5. Proposed pathway for the degradation of 4-NC by *Ochrobactrum* sp. B2.

Table 3. 4-NC degradation by *Ochrobactrum* sp. (B2) and its plasmid-curing derivative (At)

	Residual 4-NC	
	After 0 h	*After 36 h*
Un-inoculated	9.83 ± 0.30	9.68 ± 0.30
B2	9.72 ± 0.21	Not detected
At	9.95 ± 0.58	9.67 ± 0.43

Data are the concentrations of 4-NC (mg L^{-1}) detected in the medium by HPLC. Results are means $\pm$ standard deviation of 3 replicates.

(designated At). The result showed that strain At lost the 4-NC degrading ability (Table 3), indicating loss of the plasmid as the basis for loss of PNP degrading activity. This finding made us suggest the plasmid responsible for harboring genes for 4-NC degradation in B2. A plasmid transformation study could be required in order to confirm this suggestion, and this becomes one subject of future investigation. In our previous report, we demonstrated that the same plasmid has an involvement in PNP degradation via 4-NC formation.[12] The phenomena that genes encoding both PNP and 4-NC degradation reside on the same plasmid were also found in the case of *Arthrobacter protophormiae* strain RKJ100 and *Burkholderia cepacia* RKJ200.[9,10] The findings that B2 degrades PNP and 4-NC by the same pathway and responsible genes reside on the same plasmid make us propose that *Ochrobactrum* B2 share a set of enzymes encoding PNP and 4-NC degradation. We believe that these findings would benefit our cloning and characterization of genes responsible for PNP and 4-NC degradation.

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Conclusion

Ochrobactrum sp. B2 has the ability to use 4-NC as an energy and carbon source. B2 degrades 4-NC via the same catabolic pathway as PNP and the genes encoding PNP and 4-NC degradation are located on the same plasmid in B2.

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