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Degradation of *o*-chloronitrobenzene as the sole carbon and nitrogen sources by *Pseudomonas putida* OCNB-1

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Abstract

A bacterial strain that utilized *o*-chloronitrobenzene (*o*-CNB) as the sole carbon, nitrogen and energy sources was isolated from an activated sludge collected from an industrial waste treatment plant. It was identified as *Pseudomonas putida* based on its morphology, physiological, and biochemical characteristics with an automatic biometrical system and the 16S rRNA sequence analysis. Microcosm study showed that the biodegradation of *o*-CNB was optimized at culture medium pH 8.0 and 32°C. At these conditions, the strain degraded 85% of *o*-CNB at a starting concentration of 1.1 mmol/L in 42 h. *o*-Chloroaniline was identified as the major metabolite with high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The study showed that *o*-CNB degradation by *Pseudomonas putida* OCNB-1 was initiated by aniline dioxyenase, nitrobenzene reductase and catechol-1,2-dioxygenase.

Key words: *o*-chloronitrobenzene; *Pseudomonas putida*; characterization; degradation

Introduction

Chloronitrobenzenes (CNBs) are widely used as a basic chemical in the industrial production of dyes, pesticide, herbicide, lumber preservatives, pharmaceuticals, photograph film, antioxidants, gasoline additives, corrosion inhibitors and other industrial chemical raw material. The total production of *p*-chloronitrobenzene (*p*-CNB) and *o*-CNB in China was about 5.25×10^5 tons in 2006, which was about 65% of the total world-wide production (Liang, 2007). CNBs possess a wide range of toxicity for humans and animals including hematotoxicity which can cause methemoglobinemia and/or anemia, splenotoxicity, nephrotoxicity, hepatoxicity, genotoxicity, immunotoxicity and carcinogenis (Nair *et al*., 1986a, 1986b; Travlos *et al*., 1996; Li *et al*., 1998, 1999). In the aquatic environment, CNBs are considered as relatively recalcitrant due to the electron-withdrawing property of the nitro and chlorine groups on the aromatic ring. The sources of these chemicals are mainly from discharge of wastewater and spillage of their pure forms due to inappropriate handling or transportation accidents. They were detected in high concentration range as 0.3–0.7 mmol/L in wastewater of chemical plants synthesizing CNBs (Xiang *et al*., 2003). The Chinese government set an effluent standard of 0.003 mmol/L for CNB_S (GB 8978-1996), which is less stringent than the USA standard. To meet the discharge standards, available physicochemical process-based technologies such as ozonation (Chen *et al*., 2006), photolysis (Priya and Madras, 2006; Zhang *et al*., 2006), electrocatalysis (Xu *et al*., 2006) and adsorption (Guo *et al*., 2005) have been widely used for removal of CNBs from wastewater.

Biological process-based technologies are presumably more cost-effective than physicochemical processes. However, information on microbial degradation of chloronitrobenzene is very limited. Prior studies have reported that CNBs can be degraded by microorganisms. Katsivela *et al*. (1999) found that a bacterial strain LW1 in the family Comamonadaceae could transform 1.3 mmol/L *p*-CNB in 35 h. Park *et al*. (1999) reported that *m*- and *p*-CNB were degraded by a co-culture of *Pseudomonas putida* and *Rhodococcus* sp. Kuhlmann and Hegemann (1997) examined degradation of *o*-, *m*- or *p*-CNB in an aerobic batch culture *Pseudomonas acidovorans* strain CA50, which occurred only when alternative carbon and nitrogen sources were added. In the study by Kuhlmann and Hegemann (1997) the degradation rate of *o*-CNB was slow significantly. More recently, *Comamonas* sp. strain CNB-1 was isolated for degradation of *p*-CNB (Wu *et al*., 2004). A strain *P. putida* ZWL73 can grows on *p*-CNB as sole source of carbon, nitrogen, and energy (Zhen *et al*., 2006). Liu *et al*. (2005) declaimed that the strain *Pseudomonas stutzeri* could degrade 0.5 mmol/L *o*-CNB as the sole source of carbon, nitrogen, and energy over a time period of 84 h. In current study, we detected that *P. putida* could also utilize *o*-CNB as the sole carbon,

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nitrogen, and energy source. The kinetics and pathway of *o*-CNB biodegradation were systematically examined with identification and quantification of the metabolites and enzyme assay.

1 Materials and methods

1.1 Chemicals and mineral salt medium (MSM)

NB (99%), CB (97.5%), *o*-CNB (99%), 2-aminophenol (99%), catechol (99%), 4-chlorocatechol (99%), and 4-chloro-2-aminophenol (99%) were purchased from Aldrich-Sigma Chemical Co., Germany and China Shanghai Reagent Co. The water used was pure water (Pure Lab Plus UV/UF, Pall Co., USA). A mineral salts medium (MSM) used for growth of microorganisms contained (mg/L) Na_2HPO_4 1 000, KH_2PO_4 500, $CaCl_2$ 30, $MgSO_4{\cdot}7H_2O$ 70, $FeSO_4{\cdot}7H_2O$ 30, KI 0.83, H_3BO_3 6.2, $MnSO_4{\cdot}4H_2O$ 22.3, $ZnSO_4{\cdot}7H_2O$ 8.6, $Na_2MoO_4{\cdot}2H_2O$ 0.25, $CuSO_4{\cdot}5H_2O$ 0.025, $CoCl_2$ 0.025, at pH 8.0. This MSM did not contain either nitrogen- or carbon-bearing chemicals. All the chemicals were of analytical grades and were purchased from Guangzhou Chemical Reagent Company of China.

1.2 Isolation and culture conditions

To obtain the CNB-degrading microorganisms, a wastewater sample was taken from Changzheng Chemical Limited Company in Zhejiang, China. Aliquots of the wastewater were transferred to incubation bottles that contained a nitrogen- and carbon-free MSM spiked with 1.1 mmol/L *o*-CNB. The bottles were placed on a shaker preset at 120 r/min and 32°C. After 1 week of incubation, the culture (approximately 5% inoculum) was transferred to a fresh medium of the same chemical compositions and was incubated under the same conditions. This procedure was repeated seven times. The eighth inoculums were plated onto LB agar plates. Developed colonies were incubated individually in liquid mineral salts media containing *o*-CNB for screening *o*-CNB-degrading strains by monitoring its depletion and chlorine released. The pure cultures were used subsequently for Gram staining. The cell was observed under fluorescence microscope (DP70, Olypus, Japan), identified using both BioMerieux Vitek System (bioMerieux, Marcy 1'Etoile, France) and 16S rRNA technique.

1.3 16S rRNA characterization

After the pure cultures had been inoculated in MSM containing *o*-CNB (1.1 mmol/L) for 48 h at 32°C at 120 r/min, the cell-containing solutions were withdrawn and centrifuged at 15000 r/min for 3 min at 4°C. The harvested cell pellets were washed twice with 0.5 mL sterile water to remove background chemicals and *o*-CNB. The washed cells were then resuspended in 567 μL of TE buffer (10 mmol/L Tris/HCl, 10 mmol/L EDTA, pH 8.0). A 30-μL of 10% SDS and 15 μL of 20 mg/mL proteinase K (Takara, Dalian, China) were added, and were incubated at 37°C for 15 min. Then 100 μL of 5 mol/L NaCl and 80 μL CTAB/NaCl (5 g CTAB was dissolved in 100 mL of 0.5 mol/L NaCl) were added and the mixture was incubated again at 50°C for 30 min. Then 780 μL of phenol/chloroform/isoamyl alcohol (25/24/1) was added and mixed. The mixture was centrifuged at 5000 r/min for 5 min at 4°C. The supernatant was withdrawn in a new tube and 500 μL isopropyl alcohol was added and mixed lightly. DNA was precipitated and the supernatant was removed. Precipitated DNA was washed twice with 70% (*V/V*) ethanol and finally resuspensed in 0.2 mL of sterile water. Universal primers were used to amplify 16S rDNA.

PCR (polymerase chain reaction) conditions were as follows: pre-heating at 95°C for 4 min; 35 cycles of denaturing at 95°C for 1 min; annealing at 55°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were stored at 4°C. The PCR product purification and DNA sequencing was obtained in Shanghai Yingjun Biotechnology Ltd., China. GenBank databases were used to search for 16S rDNA sequence similarities.

1.4 Growth of bacteria and biodegradation of *o*-CNB

Single colonies of the isolated bacteria were withdrawn from agar plates and inoculated in 250 mL Erlenmeye bottles filled with 50 mL of sterile MSM containing 1.1 mol/L *o*-CNB. The flasks were then incubated for 54 h at 32°C, 120 r/min. In the late exponential phase, the culture (approximately 10% inoculum) was transferred to a fresh medium of the same chemical compositions and was incubated under the same conditions. This procedure was repeated until the culture OD_{600} increased to 0.2. Cultures were further inoculated (10%, *V/V*) with the preculture that OD_{600} was 0.2 in 250-mL Erlenmeyer flasks with 100 mL MSM for studying its optimization of growth parameters: temperature, pH and oxygen availability. Then its ability to utilize *o*-CNB as the sole carbon, nitrogen and energy sources was further characterized in the optimization of growth parameters. Growth was monitored spectrophotometrically by measuring culture turbidity at 600 nm. The degradation rate of *o*-CNB was qualified by detecting the release of chloride. The concentrations of *o*-CNB, Cl^-, and TOC in the solution phase were quantified. All tests were conducted in triplicates. Sterile controls were prepared by autoclaving before introduction of *o*-CNB.

The effect of temperature was investigated in the dark at 20, 25, 28, 32, 35, and 40°C. Likewise, the optimal growth was determined in the pH range from 4 to 10. The effect of dissolved oxygen content was studied under different agitation conditions at 40, 90, 120, 140, and 180 r/min.

1.5 Isolation of metabolites

Culture medium (100 mL) was centrifuged for 30 min at 16000 r/min. The obtained supernatant was acidified to pH 2 with HCl, and extracted three times with 10 mL of dichlormethane. After extraction, the supernatant was adjusted to pH 12 with 5 mol/L NaOH, and was again extracted three times with 10 mL dichlormethane. The extracts (60 mL) were combined, dried with anhydrous

sodium sulfate, and condensed with a rotary evaporator. The residue was dissolved in 0.5 mL methanol and was analyzed with GC-MS for identification of possible metabolites.

1.6 Enzyme assays

Cells were grown in 250 mL flasks with 1.1 mmol/L *o*-chloronitrobenzene as sole sources of carbon, nitrogen. Cells were harvested in exponential phase of growth (OD_{600} about 0.2) and were centrifuged for 20 min at 10000 r/min by a Sigma 2-16K centrifuge at 4°C, and washed twice in 50 mmol/L phosphate buffer (pH 7.0). Fresh cell suspension was disrupted by supersonic treatment for 10 min, and then was centrifuged for 10 min at 15000 r/min at 4°C. Solid was removed and the supernatant was used immediately for enzyme assayed.

Catechol-1,2-dioxygenase and catechol-2,3-dioxygenas were identified by detecting two ring cleavage products of muconic acid at 260 nm and muconic semialdehyde at 375 nm (Hayaish *et al*., 1957; Sala-Trepat and Evans, 1971). Nitrobenzene reductase activity was measured by monitoring decrease in absorbance at 340 nm resulting from conversion of NADPH to NADP (Somerville *et al*., 1995). 2-Amino-1,6-dioxygenase activity and aniline dioxygenase activity were determined with previously described methods (Nishino and Spain, 1993; Fukumor and Saint, 1997).

One unit (U) of enzyme activity represents the conversion of 1 μmol of substrate per min at 25°C. Specific activity is defined as 1 μmol of substrate converted per min per gram of protein at 25°C and is expressed as U/g protein.

The protein contents of cell extracts were determined by the method of Bradford (1976) with bovine serum albumin (Dingguo, Beijing, China) as standard.

1.7 Analytical methods

The aqueous concentrations of chloride, ammonium and nitrite were quantified with an ion chromatography (model 792Basic IC, Metrohm Corporation, Switzerland). The total organic carbon (TOC) contents of aqueous samples were determined with a TOC analyzer (model TOC-5050, Shimadzu Corporation, Japan). Aqueous phase CNB concentrations were quantified with a high performace liquid chromatography (HPLC) (Agilent 1100 series, Agilent Technologies Ltd., Germany) equipped with a VWD detector and Agilent Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, particle size 5 μm). The mobile phase was a mixture of methanol and water at the ratio of 85:15 (*V/V*), and the flow rate was 1 mL/min. UV-detection was set at 210 nm. Under these chromatographic conditions, baseline separation *o*-CNB could be achieved within 8 min. Culture media was centrifuged for 30 min at 16000 r/min. The supernatant was filtered by 0.2 μm filter membrane and was analyzed by chromatography of ions, TOC, or HPLC, which was run at ambient temperature. *o*-CNB degradation metabolites were identified using a gas chromatography-mass spectrometry (GC-MS) (GCMS-QP2010, Shimadzu, Japan). The GC-MS analysis was carried out as follows: a model GCMS-QP2010 equipped with a type DB-5MS column (30 m × 0.25 mm, 0.25 μm thickness) was used. The oven temperature maintained at 60°C for 2 min, and then was increased to 150°C at a rate of 20°C/min and finally to 280°C at a rate of 6°C/min, and held at 280°C for 5 min. The injection volume was 1 μL and the carrier gas was helium (1.0 mL/min). The mass spectrometer was operated at electron ionization energy of 70 eV. Instrumental library searches, comparison with available authentic compounds, and mass fragmentation pattern were used to identify the suspected metabolites.

2 Results and discussion

2.1 Isolation and characterization

One bacterial species OCNB-1 capable of utilizing *o*-chlorobenzene as the sole source of carbon, nitrogen and energy was isolated from the culture medium. The colony was colorless and transparent, the cell was irregular rods ((0.8–1.0) μm × (2.5–8.0) μm) with green fluorescence (Fig. 1), Gram-negative, oxidase-positive, catalase-negative, and was able to reduce nitrate and use glucose as carbon source. The strain was identified by WSVTK-R07.02 system. The results showed that it was similar to *Pseudomonas mendocina* 52% and similar to *Pseudomonas fluorescens* or *P. putida* 40%. Sequences of its 16S rDNA gene were amplified and analysis in the GenBank database. The result showed that the strain was closely related to *P. putida* (ATCC12633T-Genbank) with similarity value (*S*) of 0.98.

Isolates strain OCNB-1 was identified as *P. putida* by the 16S rDNA sequence analyses, similarity value (*S*) of 0.98, but the results obtained from the biochemical analysis and the BioMerieux Vitek System, the isolated strain was similar to *P. putida* only 52%. Biochemical characteristics can be effected by the change of environment and the induction of drug, but the 16S rDNA sequence is highly conserved sequence and is the most important standard for the classification of bacteria.

2.2 Effect of pH

The hydrogen ion concentration in the culture medium

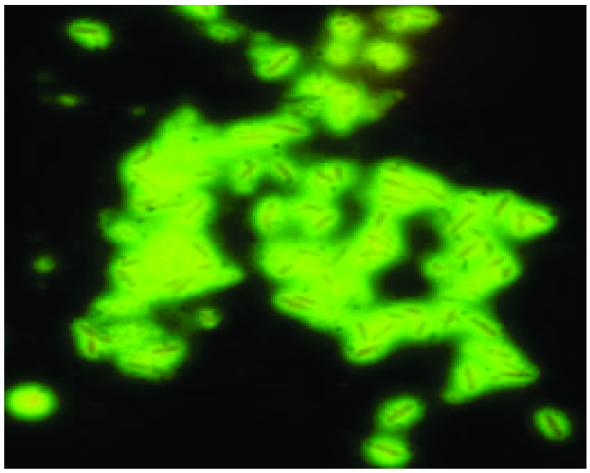

Fig. 1 Micrographs of strain OCNB-1 (1000×).

greatly influences the bacterial growth since pH limits the activity of enzymes. The relationship between the chloride released and pH for *Pseudomonas putida* OCNB-1 is shown in Fig. 2. The amount of chloride released indicates that stoichiometric elimination occurred. When the pH was lower than 5.0 or higher than 9.0, the growth of the strain and the degradation of *o*-CNB decreased sharply. The degradation rate of *o*-CNB increased quickly when pH value of the culture was increased from 5.5 to 8.0. The highest degradation rate was achieved for *P. putida* OCNB-1 at pH 8.0. The optimum pH value for *o*-CNB degradation by *P. putida* OCNB-1 was 8.0 in this study.

2.3 Effect of temperature

When the effect of temperature on *o*-CNB degradation by *P. putida* OCNB-1 was assessed (Fig. 3), the degradation rate increased with the increase of temperature from 20 to 32°C. Higher temperature resulted in a lower degradation rate. The optimum temprature was 32°C at which the degradative enzyme reached the highest activity.

2.4 Effect of oxygen availability

The effect of dissolved oxygen content on *o*-CNB degradation by *P. putida* OCNB-1 was determined by comparing the chloride released under different agitation conditions (Fig. 4). The increase in agitation rate up to 120 r/min resulted in an increase in the strain optical density and the rate of chloride release. However, the strain could grow and the chloride released in standing condition. Because *P. putida* OCNB-1 is an aerobic microorganism, dissolved oxygen serves as an essential electron acceptor and plays an important role in the physiology growth of the microorganism. Better mass transfer efficiency, resulting from a high mixing rate, may lead to high dissolved oxygen content in the culture medium. However, further increasing the agitation rate beyond 120 r/min did not improve the rate constant. Therefore, a rate of 120 r/min was selected as optimum.

2.5 Biodegradation of *o*-CNB

A growth curve obtained with *o*-CNB as the only source of carbon, nitrogen and energy is shown in Fig. 5. The initial concentration of the dissolved carbon source in the culture fluid was 1.1 mmol/L. The exponential growth phase lasted 18–42 h. In the equilibrium phase the strain $OD_{600\ nm}$ was about 0.18. Chloride anion was released and total organic carbon (TOC) was reduce simultaneously to *o*-CNB degradation. Accumulation of ammonium or nitrite was not observed, as determined by the chromatography of ions. In a negative control without cells, *o*-CNB concentration declined as a result of its volatility but was still present at 0.95 mmol/L after 42 h.

Pseudomonas putida has been shown to degrade a wide range of organic pollutants such as benzene, toluene, phenol (Reardon *et al*., 2000; Juang and Tsai,

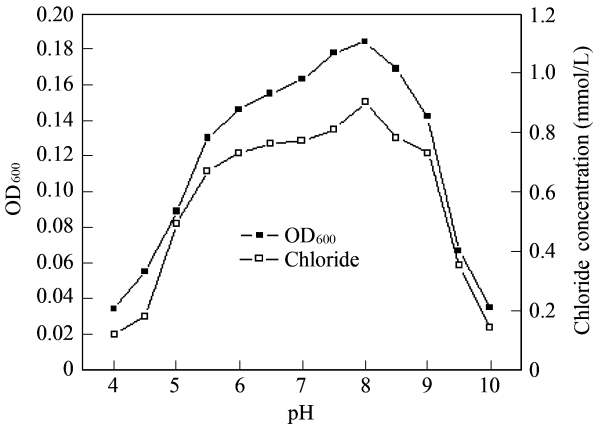

Fig. 2 Effect of pH on *o*-CNB degradation by *Pseudomonas putida* OCNB-1.

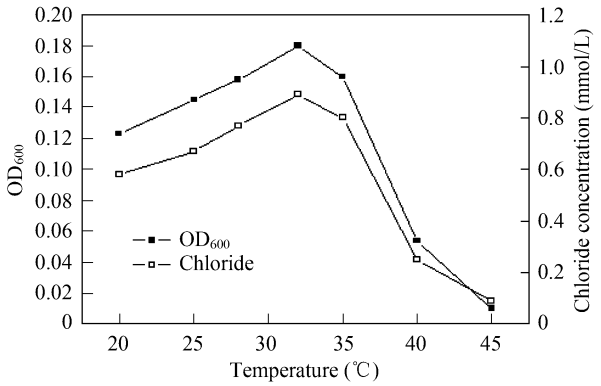

Fig. 3 Effect of temperature on *o*-CNB degradation by *Pseudomonas putida* OCNB-1.

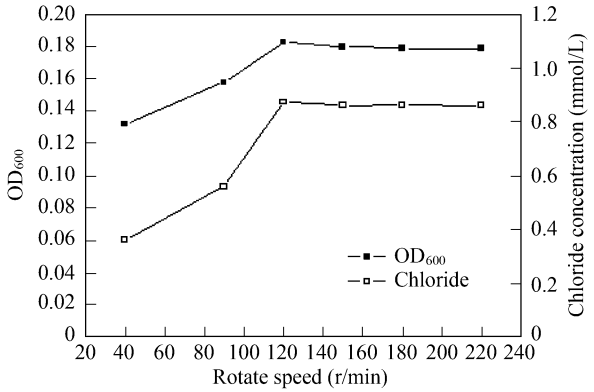

Fig. 4 Effect of rotate speed on *o*-CNB degradation by *Pseudomonas putida* OCNB – 1.

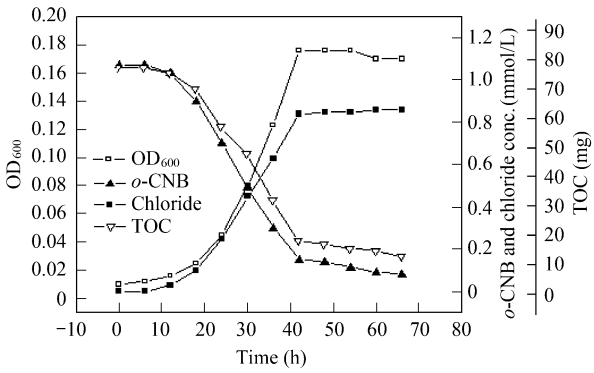

Fig. 5 Degradation of *o*-CNB and growth of bacterial strain OCNB-1 with *o*-CNB as the sole carbon, nitrogen, and energy source.

2006), chlorophenol (Farrell and Quilty, 2002), nitrobenzene (Jung *et al*., 1995), naphthalene and phenanthrene (Balashova *et al*., 1997; Purwaningsiha *et al*., 2004), dibenzofuran (Hong *et al*., 2000), carbazole (Loh and Yu, 2000), *p*-chloronitrobenzene (Xiao *et al*., 2006). However, the *o*-CNB degradation by *P. putida* was not reported. In this study, a strain *P. putida* OCNB-1 was found to be able to utilize *o*-CNB as the sole source of carbon, nitrogen, and energy. At the initial *o*-CNB concentration of 1.1 mmol/L, the chloride released was 0.84 mmol/L and the initial amount of TOC was reduced by 80% within 42 h. This was more efficient than *Pseudomonas stutzeri* that degraded 0.5 mmol/L *o*-CNB in 84 h (Liu *et al*., 2005).

2.6 Identification of enzyme activities

The activities of various possible pathway enzymes in cell extracts are shown in Table 1. For LB-grown cells, enzyme activities were significantly low in contrast to the *o*-CNB-grown cells which was high nitrobenzene reductase, catechol 1,2-dioxygenase and aniline dioxygenas. In both media no 2-aminophenol 1,6-dioxygenase was observed and catechol 2,3-dioxygenase was dramaticaly low. According to the identification of enzyme activity, catabolic enzyme activity was very low in LB-grown cells. It was showed that catabolic enzymes were likely induced by *o*-chloronitrobenene.

The observed nitrobenzene nitroreductase activity in OCNB-1 cell extracts suggested that the initial attack on the nitro group was reductive. Nitrobenzene nitroreductase is known to have a rather relaxed substrate specificity, and this enzyme seems to attack *o*-CNB, resulting in the formation of *o*-chloroaniline, similar to that of *Pseudomonas acidovorans* CA50 (Kuhlmann and Hegemann, 1997) for the degradation of *o*, *m* or *p*-CNB and that of *Rhodosporidium* sp. (Corbett and Corbett, 1981) for the degradation of *p*-CNB. The reductive transformation of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene was reported to result in the formation of amino-substituted toluenes (Gilcrease and Murphy, 1995; Vanderberg *et al*., 1995; Noguera and Freedman, 1996; Fiorella and Spain, 1997). It was also reported that *Pseudomonas* sp. strain CBS3 converted various nitroaromatic compounds to the corresponding amino aromatic compounds under aerobic resting conditions, mononitro-compounds to aniline, and 1-chloro-2,4 dinitrobenzene to the two chloronitroanilines (Schackmann and Muller, 1991). Nitrobenzene nitroreductase in some strains (Katsivela *et al*., 1999; Park *et al*., 1999; Wu *et al*., 2006; Xiao *et al*., 2006) was reported to transform nitro groups of 3- or 4-chloronitrobenzene to the corresponding 4- and 5-chloro-2-aminophenol rather than reduction to the corresponding 3- and 4-chloroanilines. However, in this study, we cannot detect 2-aminophenol 1,6-dioxygenase activity and the corresponding intermediate.

Table 1 Specific activities of catabolic enzyme activities in cell extracts of strain OCNB-1

Enzyme	Specific activities (U/g protein)	
	MSM	LB
Nitrobenzene reductase	2200	150
2-Aminophenol 1,6-dioxygenase	< 0.01	< 0.01
Aniline dioxygenase	1567	< 10
Catechol 1,2-dioxygenase	2356	< 20
Catechol 2,3-dioxygenase	85	< 20

2.7 Identification of *o*-CNB degradation metabolites

o-CNB was rapidly transformed in the culture medium inoculated with *P. putida* OCNB-1 when *o*-CNB served as the sole source of carbon and energy. The culture filtrate from *o*-CNB degradation experiment was detected by HPLC. The parent compound peak of *o*-CNB, with HPLC retention time of about 3.42 min, a major metabolite was observed at HPLC retention time of about 3.17 min. Other metabolites with small peaks were also detected in the samples, but could not be isolated for further characterization. The peak at 3.17 min was identified as *o*-chloroaniline by comparing the retention time. To confirm positively the identity of this intermediate, GC-MS was also used. Two main compounds were identified as *o*-CNB and *o*-chloroaniline. The peak at about 3.17 min on HPLC was identified as *o*-chloroaniline by comparing the mass chromatogram of authentic *o*-chloroaniline (Fig. 6). The result obtained by GC-MS was consistent with that by HPLC, indicating the identified intermediate was *o*-chloroaniline.

The degradation metabolite 2-chloroaniline observed and enzymatic analysis showed that *o*-CNB was mineralized via chloroaniline which was mineralized via chlorocatechol by aniline dioxygenase and subsequent ring cleavage by catechol 1,2-dioxygenase. It was reported that aniline and monochloroanilines were degraded via

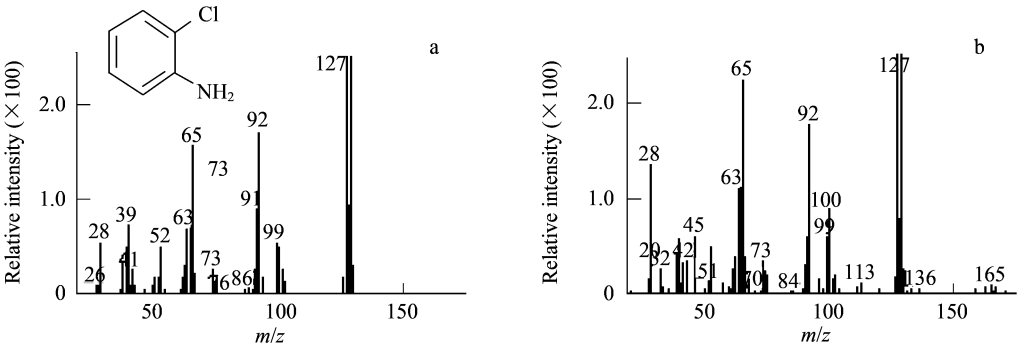

Fig. 6 Mass spectra of authentic *o*-chloroaniline (a) and the intermediate (b).

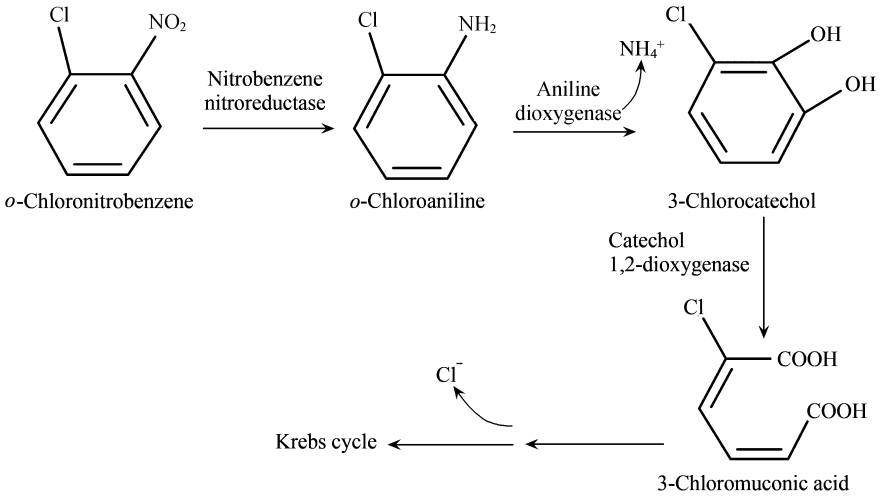

Fig. 7 Proposed pathway for catabolism of *o*-CNB by bacterial strain OCNB-1.

ortho-cleavage pathway which was catalyzed by catechol 1,2-dioxygenase (Zeyer *et al*., 1985; Loidl *et al*., 1990; Hinteregger *et al*., 1992; Boon *et al*., 2001; Helia *et al*., 2003). Based on the identification of metabolic intermediates and enzyme activity formed by *P. putida* OCNB-1, degradation of *o*-CNB by *P. putida* OCNB-1 proceeded through 2-chloroaniline and 3-chlorocatechol before cleavage of aromatic ring, we propose here the metabolic pathway for *o*-CNB as shown in Fig. 7.

3 Conclusions

The results obtained in the present study showed that *o*-CNB could be degraded effectively by a bacterial strain *P. putida* OCNB-1 isolated from the activated sludge in the wastewater treatment plant of a chemical factory. The optimum pH and temperature for the degradation were 8.0 and 32°C, respectively. *o*-Chloroaniline identified as the major metabolite and enzymatic analysis showed that *o*-CNB was mineralized via chloroaniline and degradation was initiated by aniline dioxyenase, nitrobenzene reductase and catechol-1,2-dioxygenase. It may provide theoretical and application basis for the treatment of *o*-CNB in wastewater.

Acknowledgments

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