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Isolation and Characterization of a Nitrobenzene Degrading *Streptomyces* Strain from Activated Sludge

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Nitrobenzene is widely used in the manufacture of organic products, such as aniline, lubricating oils, dyes, drugs, pesticides and synthetic rubber (ATSDR, 1990). Its wide usage in industry has been contaminating the environment. The Songhua River, which supplies drinking water to Harbin, a northeastern Chinese city, was contaminated with nitrobenzene on November 25, 2005, when a chemical factory exploded. Chronic exposure to nitrobenzene in humans can result in methemoglobinemia and liver damage (http://www.epa.gov/ttn/atw/hlthef/nitroben.html). Nitrobenzene is listed as a priority pollutant by the US Environmental Protection Agency (He and Spain, 1997).

Wastewater loaded with nitrobenzene can be treated with many methods, such as physical-chemical and biological methods. Physical-chemical methods such as adsorption, solvent extraction, ozonisation, H_2O_2 oxidation, etc., were reported (Tang et al., 2003). Compared to physical-chemical methods, biological methods can mineralize nitrobenzene at a lower cost (Li et al., 2006). Aerobic degradation of nitrobenzene has been studied by many researchers. Bacterial strains and white rot fungi capable of degrading nitrobenzene were described. However, reports on the degradation of nitrobenzene by *Streptomyces* strains are very few. Moreover, although much research has been done on the nitrobenzene-degrading microorganisms, most of them focused on the catabolism pathways and growth conditions. As the nitrobenzene industrial wastewater usually contains high inorganic salts (specifically NaCl) and multi-organic compounds (Yao et al., 2003), the nitrobenzene degradation by strains in high salinity or in the presence of other toxicants should be tested to demonstrate their degradation effectiveness and application potentials. This article reports on the isolation and characterization of a nitrobenzene-degrading *Streptomyces* strain, with a stress on the capacity of the strain to degrade nitrobenzene.

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Materials and Methods

The activated sludge used in this study was collected from a nitrobenzene-contaminated site, located in Dalian (Liaoning Province, People's Republic of China). The mineral salts medium (MS medium) contained (per liter): $Na_2HPO_4{\cdot}12\ H_2O$, 7 g; KH_2PO_4, 1 g; $CaCl_2{\cdot}2\ H_2O$, 10 mg; $FeCl_3$, 2 mg; and $MgSO_4{\cdot}7\ H_2O$, 20 mg. The pH of the MS medium was adjusted to 7.0. Ten milliliters of the activated sludge was aseptically added to 90 mL of the sterilized MS medium in a 250 mL flask. The 250 mL flask was supplemented with nitrobenzene (analytical reagent) to a final concentration of 200 $mg{\cdot}L^{-1}$ and incubated on a rotary shaker (180 rpm) at 30°C. When the culture became obviously turbid, 10 mL of the culture was transferred to 90 mL fresh MS medium in a new 250 mL flask with 200 $mg{\cdot}L^{-1}$ nitrobenzene. This operation was repeated until the degradation of nitrobenzene came to a stable level. And then the culture was diluted and spread onto agar (1.5%)

plates with MS medium and nitrobenzene. The agar plates were incubated at 30°C for 4–5days. Colonies appearing on the agar plates were subcultured. Purity of the cultures was confirmed by microscopic examination. One of the most efficient isolates, strain Z2, was selected.

One loop of the stock culture on the agar plates was transferred aseptically to 100 mL sterilized MS medium in a 250 mL flask with 200 $mg \cdot L^{-1}$ nitrobenzene. Ten milliliters of the culture in the late exponential phase was aseptically inoculated into 90 mL sterilized MS medium in a 250 mL flask. The 250 mL flask was supplemented with nitrobenzene at a starting concentration ranging from 200–400 $mg \cdot L^{-1}$. The cultures were incubated on a rotary shaker (180 rpm) at 30°C. All cultivations were repeated five times. A flask with the same amount of the autoclaved cells (10 min, 60°C) was used as a control. Nitrobenzene-degrading cells in the late exponential phase were immobilized by FA solution (Formaldehyde: Acetic acid = 4:10) for 1–2 hr. The suspension was dehydrated gradually by ethanol and photographed on a GEM-1200EX scanning electron microscope (Japan). The dry cell mass was determined as described by Du et al. (2001). Nitrobenzene concentrations were measured with a V-560 UV-vis spectrophotometer (JASCO, Japan) using N-(1-Naphthyl) ethylenediamine dihydrochloride as per standard procedure (SEPA, 2002). Prior to a total organic carbon (TOC) content analysis, samples were centrifuged (150°C) at 20,000 × g for 20 min to remove the biomass. The TOC contents were determined using a TOC-5000 analyzer (Shimadzu, Japan) (Wu and Wang, 1995).

The capacity of strain Z2 to degrade nitrobenzene in high salinity was tested. Batch experiments were performed five times. Ten milliliters of the culture in the late exponential phase was aseptically inoculated into each 250 mL flask with 90 mL sterilized MS medium. Each 250 mL flask was supplemented with 200 $mg \cdot L^{-1}$ nitrobenzene and 2%–7% (quality concentration) NaCl. The cultures were incubated on a rotary shaker (180 rpm) at 30°C. Samples were withdrawn periodically for nitrobenzene concentration analysis. The same setup was also used for testing the capacity of strain Z2 to degrade nitrobenzene in the presence of aniline or phenol. Phenol concentrations ranged from 50 to 200 $mg \cdot L^{-1}$ and aniline ranged from 25 to 100 $mg \cdot L^{-1}$.

The 16S rDNA sequence was amplified by PCR with primers 27f (5′-GAGTTTGATC (AC)TGGCTCAG-3′) and 1492r (5′-TACGG(CT)TACCTTGTTACGACTT-3′). PCR mixtures contained LA *Taq* (0.5μL), 2.5 mM dNTP (8μL), 10 × LA PCR buffer (5μL), 27f (1μL), 1492r (1μL), d H_20 (up to 50μL), and genomic DNA (50ng). The PCR instrument used was a TaKaRa Thermal Cycler Dice TP600 (TaKaRa Bio Inc.), the electrophoresis instrument used was a Mupid (ADVANCE-BIO Co., Ltd.), and the electrophoresis image-forming instrument used was an ImageMaster® VDS (Pharmacia Biotech). The amplification settings were a starting temperature of 94° for 5 min; 55° for 1 min; 72° for 1 min, 30 cycles; and finally 72° for 5 min. A partial sequence of the PCR products was determined for the isolate using an ABI PRISM™ 3730XL DNA Sequencer (Applied Biosystem). The 16S rDNA sequence of strain Z2 was deposited at the GenBank under accession number DQ855477.

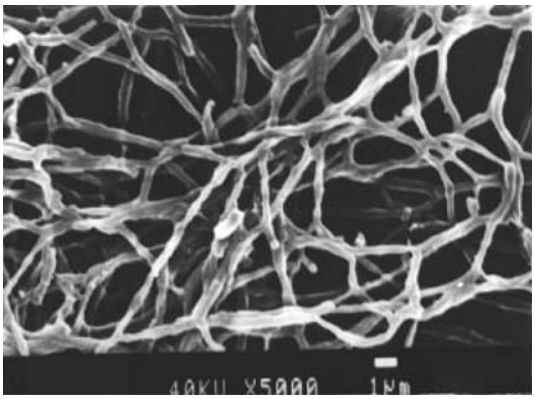

Fig. 1 Scanning electron microscopic photograph of strain Z2

Results and Discussion

Strain Z2 was isolated after six months of enrichment. Strain Z2 was able to utilize nitrobenzene as a sole source of carbon, nitrogen and energy under aerobic conditions. Strain Z2 grew well in the temperature from 25–30°C and pH from 6.0 to 9.0. Figure 1 shows the morphology of strain Z2 in the late exponential phase. The spore-bearing filaments were spiral and the spores were oval. Table 1 shows the cultural characteristics of strain Z2 in seven different media.

The 16S rDNA sequence of strain Z2 was 1433 bp. A maximum phylogenetic likelihood tree was generated by neighbor-joining methods from PHYLIP program (Fig. 2). Based on the sequencing of 16S rDNA with 1412-bp, the homology between strain Z2 and a *Streptomyces sp.* (Genbank acession no. DQ409080) was 100%. It can be concluded that strain Z2 belongs to the genus *Streptomyces*.

Strain Z2 grew on nitrobenzene up to a concentration of 400 $mg \cdot L^{-1}$. Figure 3 shows the results of nitrobenzene consumption and cell growth profiles at initial nitrobenzene concentrations of 200 $mg \cdot L^{-1}$ and 400 $mg \cdot L^{-1}$. Strain Z2 degraded 200 $mg \cdot L^{-1}$ nitrobenzene within 72 h and 400 $mg \cdot L^{-1}$ nitrobenzene within 144 h. In the biodegradation period, mentioned above, over 98% of TOC was removed. These results indicated that most of the organic

Table 1 The cultural characteristics of strain Z2 in seven different media

Medium	Aerial mycelium	Substrate mycelium	Soluble pigment
Dextrose asparagines agar	Lotus seed white	Mustard yellow	None
Glycerol asparagines agar	Hawksbill yellow	Yellow	None
Ammonium salt starch agar	Mustard yellow	Light brown-gray	None
Yeast and malt extract agar	Ash gray	Light brown	Clove-brown
Oat powder agar	Ash gray	Light brown	Clove-brown
Gaoshi No.1 agar	Milk yellow	White	None
Sauton's agar	Lotus seed white	Dull green	Light cyan

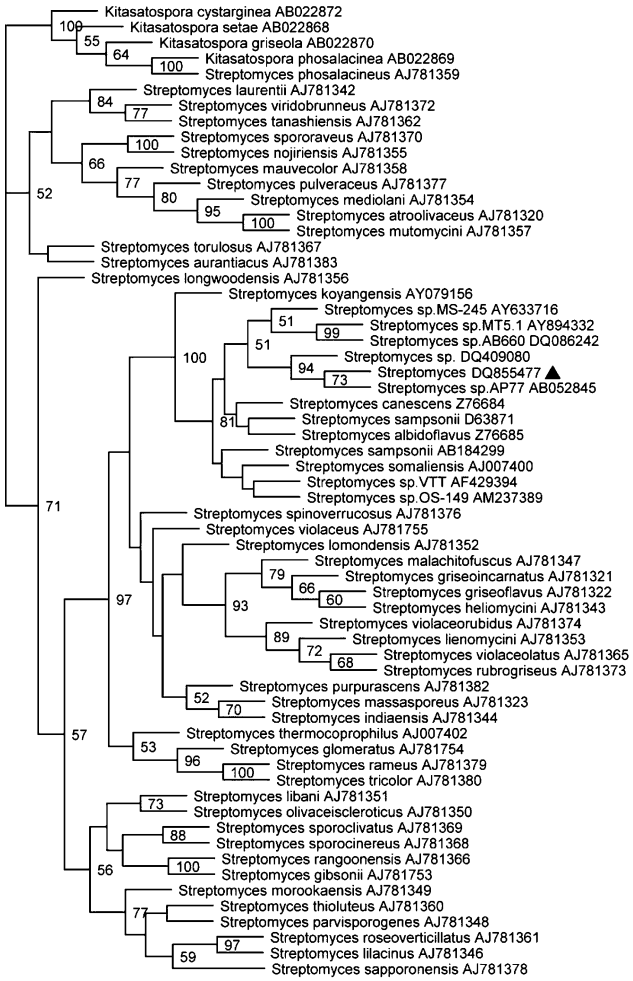

Fig. 2 Phylogenetic tree of strain Z2 based on 16S rDNA sequence comparison

carbon was transformed to carbon dioxide. As shown in Figure 3, although the removal of nitrobenzene was high, the production of new biomass was low. This result showed that most of the nitrobenzene was utilized by strain Z2 for the generation of energy to drive metabolic reactions, not for the production of new biomass. The characteristics of higher degrading efficiency with less biomass suggests that strain Z2 may be used in activated sludge to remove nitrobenzene from industrial wastewater. The maximum specific growth rates (μ_{max}) of strain Z2 in LB and MS media were calculated (Qi and Xia, 2004). The μ_{max} value in LB medium was 0.23 h^{-1}. In MS medium with 200 mg·L^{-1} or 400 mg·L^{-1}nitrobenzene, the μ_{max} value was 0.075 h^{-1} or 0.048 h^{-1}. These results demonstrated that strain Z2 grew better in LB medium than in MS medium with nitrobenzene as the source of carbon, nitrogen, and energy. The growth rate of strain Z2 decreased with the increase of nitrobenzene concentration in MS medium.

Nitrobenzene industrial wastewater usually contains inorganic salts and multiorganic compounds. The existence of these compounds can influence the performance of biological process. So the nitrobenzene degradation by strain Z2 in high salinity was tested (Fig. 4). Strain Z2 degraded, over 98%, nitrobenzene with 200 mg·L^{-1} in 2% NaCl after 72 h. This indicated that strain Z2 grew well in 2% NaCl. At higher concentrations of NaCl (3%–5%), the nitrobenzene degradation was less effective within 72 h. However, over a longer period, 120 h, strain Z2 degraded nitrobenzene effectively. It has been generally accepted that conventional microbes could not be used to treat wastewater containing salts over 3% (specifically NaCl) (Woolard and Irvine, 1995). In this article, strain Z2 degraded, up to 81% and 61%, nitrobenzene with 200 mg·L^{-1} in 3% and 5% NaCl after 120 h, respectively. Strain Z2 had a high salt tolerance.

The nitrobenzene degradation by strain Z2 was also tested in the presence of aniline or phenol, since these toxicants were present along with nitrobenzene in industrial wastewaters. Strain Z2 degraded 200 mg·L^{-1} nitrobenzene in the presence of 25 mg·L^{-1} aniline without inhibition (Fig. 5). Over 98% of nitrobenzene was removed after 72 h. At 50 mg·L^{-1} of aniline, the nitrobenzene degradation rate was reduced. Compared to aniline, phenol concentrations of up to 150 mg·L^{-1} did not inhibit the nitrobenzene degradation by strain Z2. These results

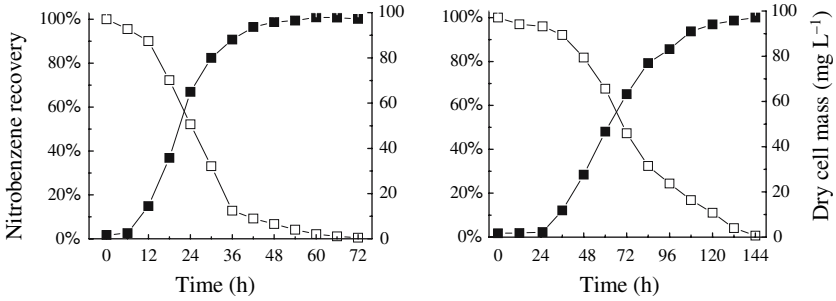

Fig. 3 Degradation of 200 mg·L^{-1} nitrobenzene (Left) and 400 mg·L^{-1} nitrobenzene (Right) by strain Z2. □, nitrobenzene recovery; ■, cell growth

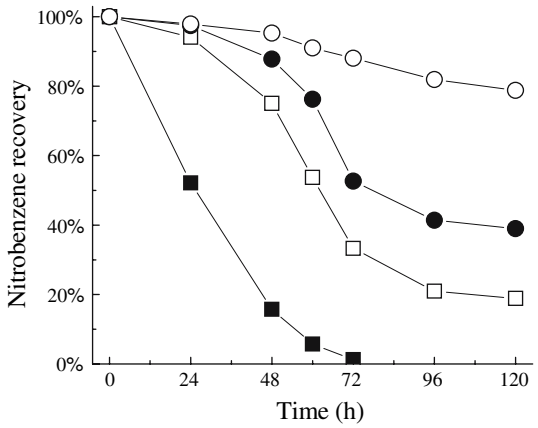

Fig. 4 Degradation of 200 mg·L^{-1} nitrobenzene in high salinity by strain Z2. ■, 2% NaCl; □, 3% NaCl; •, 5% NaCl; ○, 7% NaCl

demonstrated that strain Z2 degraded nitrobenzene effectively even in the presence of these toxicants.

Table 2 shows the growth of strain Z2 on various aromatic compounds. Z2 grew on nitrobenzene, phenol, 2-aminophenol, and picolinic acid. Many studies have described the catabolism pathways of nitrobenzene by bacterial strains under aerobic conditions. The catabolism pathways have been classified as two kinds: the oxidative pathway or the partial reductive pathway, with the partial reductive pathway seeming to be more common (Nishino and Spain, 1999). In the partial reductive pathway, nitrobenzene was degraded to 2-aminomuconic 6-semialdehyde via 2-aminophenol, and 2-amino- muconic 6-semialdehyde was further degraded to pyruvate and acetaldehyde. Due to the instability of 2-aminomuconic 6-semialdehyde, picolinic acid was abiotically formed simultaneously. Picolinic acid was a dead-end product and could not be utilized by nitrobenzene-assimilating bacteria (Nishino and Spain, 1993). In this article, a new nitrobenzene-degrading strain, belonging to the genus *Streptomyces*, was isolated. This indicates that nitrobenzene degradation can be achieved with *Streptomyces* strains, not only with bacterial strains and white rot fungi. Moreover, picolinic acid is a growth substrate for *Streptomyces sp.* strain Z2. A significant new finding of this research is that picolinic acid can be utilized by a nitrobenzene-assimilating *Streptomyces* strain. The catabolism pathway of nitrobenzene by strain Z2 is now investigated.

Table 2 Growth of strain Z2 on aromatic compounds under aerobic conditions

Aromatic compounds	Growth[a]	Aromatic compounds	Growth[a]
Benzene	–	Aniline	–
Toluene	–	2-Aminophenol	+
Chlorobenzene	–	3-Aminophenol	–
Catechol	–	4-Aminophenol	–
Phenol	+	Picolinic acid	+

[a] +, growth; -, no growth

Research to date has reported the capacities of bacterial strains and white rot fungi to degrade nitrobenzene. Wei et al. (2000) investigated the abilities of the three bacteria (*Bacillus subtilis*, *Pseudomonas mendocina*, *Klebsiella pneumoniae*) to degrade nitrobenzene individually and in combination. Of the three bacteria, *B. subtilis* manifested the highest nitrobenzene degradation ability. *B. subtilis* degraded 200 mg·L^{-1} nitrobenzene completely within 145 h, while the mixture of the three bacteria did so within 120 h. The nitrobenzene degradation by Laccase from white rot fungus was tested also (Zhang et al., 2006). At a concentration of 150 mg·L^{-1}, over 86% of nitrobenzene was removed by 72 h. At a concentration of 200 mg·L^{-1}, nitrobenzene was not degraded. Davis et al. (1981) reported on the cultures from industrial wastewater, which contained four bacterial genera (*Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas*) and one yeast (*Rhodotorula*). When the nitrobenzene concentrations were 100–200 mg·L^{-1}, biodegradation by the cultures was inhibited. Levin et al. (2003) investigated the nitrobenzene degradation by the white rot basidiomycete *Trametes trogii*. At concentrations of 250–500 mg·L^{-1}, more than 90% of nitrobenzene was removed within 12–24 days. Although the strain *Trametes trogii* tolerated nitrobenzene up to a concentration of 500 mg·L^{-1}, the degrading time was long. Also, the medium used for cultivation of the strain *Trametes trogii* was complex. In this article, the Z2 strain utilized nitrobenzene as a sole source of carbon, nitrogen, and energy. It degraded 200 mg·L^{-1} and 400

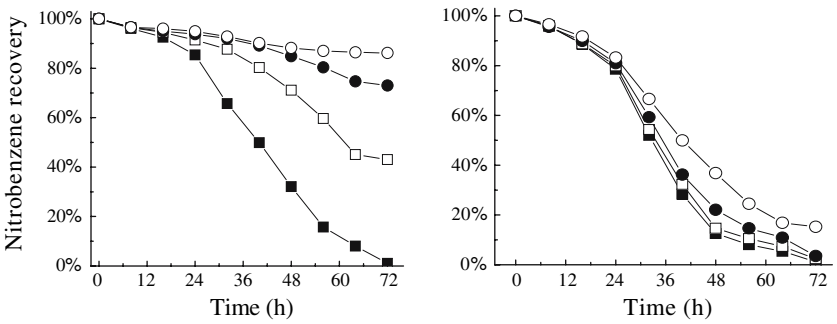

Fig. 5 Degradation of 200 mg·L^{-1} nitrobenzene in the presence of aniline (Left) and in the presence of phenol (Right) by strain Z2. ■, 25 mg·L^{-1} aniline (Left), 50 mg·L^{-1}phenol (Right); □, 50 mg·L^{-1} aniline (Left); 100 mg·L^{-1} phenol (Right); •, 75 mg·L^{-1} aniline (Left), 150 mg·L^{-1} phenol (Right); ○, 100 mg·L^{-1} aniline (Left), 200 mg·L^{-1} phenol (Right)

mg·L^{-1} nitrobenzene completely within 72 h and 144h, respectively. Even in 3%–5% NaCl or in the presence of aniline and phenol, the Z2 strain degraded 200 mg·L^{-1} nitrobenzene effectively. These results demonstrate that the Z2 strain has a higher potential for being applied to nitrobenzene industrial wastewater treatment.

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