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Biodegradation of N-Methylpyrrolidone by *Paracoccus* sp. NMD-4 and its degradation pathway

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ABSTRACT

N-Methylpyrrolidone (NMP), a kind of nitrogen-containing heterocyclic pollutant, is widely used in chemical industry. Microbial degradation is an important environmental fate process in soil and water, however, the microbial metabolic mechanism is still unknown. Strain NMD-4, capable of utilizing NMP as the sole source of carbon and nitrogen, was isolated from the activated sludge of a pesticide plant in Jiangsu, China, and identified as *Paracoccus* sp. based on its physiological–biochemical properties, as well as 16S rRNA gene sequence analysis. The degradation characteristic of NMP by strain NMD-4 was studied in a liquid culture, and the metabolic pathway of NMP by the strain was investigated. Two metabolites, 1-methyl-2,5-pyrrolidinedione and succinic acid, were detected and identified by liquid chromatography-mass spectrometry analysis, and a plausible microbial degradation pathway of NMP was proposed by the first time.

1. Introduction

N-Methylpyrrolidone (NMP) is a nitrogen-containing heterocyclic compound with a five-membered lactam structure. Its advantages include a strong polarity, good inertia, low viscosity, non-corrosiveness and low volatility (Lane DJ, 1991). The high production of NMP wide usage as a solvent and intermediate in chemical industry may result in its release to the environment through various waste streams (Rhee et al., 1997). NMP is difficult to remove because of its high solubility in water. According to the Toxics Release Inventory Data by American Conference of Governmental Industrial Hygienists (2008), NMP is released into the environment by chemical industries at a volume of 2450 tons. NMP shows certain toxic to human's health. E.g., it causes headache and irritation to the respiratory system and eyes (Beaulieu et al., 1993); skin contact with NMP may also cause abortion (Solomon et al., 1996; Bower et al., 1997). The Environmental Protection Agency of the USA and the European Union have recognized NMP as causing developmental toxicity. The Food and Drug Administration of the USA placed NMP into class 2 of residual solvents that should be limited in pharmaceutical products due to their inherent toxicities. Therefore, it is necessary to investigate its behavior and degradation mechanism in the environment, and a lot of studies concerned the absorption, migration, volatilization and conversion of NMP in soil and water have been reported. The Koc of 1-methyl-2-pyrrolidone is estimated as 5(SRC), using a log Kow of −0.38 (Sasaki H et al., 1988) and a regression-derived equation (US EPA; Estimation Program Interface (EPI) Suite. Ver. 4.1. Jan, 2010). According to a classification scheme (Swann RL et al., 1983), this estimated Koc value suggests that 1-methyl-2-pyrrolidone is expected to have very high mobility in soil. 1-Methyl-2-pyrrolidinone had Rf values of 0.74, 0.65, 0.67, and 1.0 in silt, loam, clay and sand, respectively, in laboratory soil thin layer chromatography (TLC) experiments (Shaver TN, 1984) which is consistent with significant mobility in soil (SRC). The Henry's Law constant for 1-methyl-2-pyrrolidone is 3.20×10^{-9} atm-cu m/mole (SRC) using a fragment constant estimation method (Kim et al., 2000). This Henry's Law constant indicates that 1-methyl-2-pyrrolidone is expected to be essentially nonvolatile from water surfaces (Lyman et al., 1990). 1-Methyl-2-pyrrolidone's Henry's Law constant indicates that volatilization from moist soil surfaces may not occur (SRC).

In soil, NMP is expected to have a very high mobility with an estimated Koc of 4.6, and a low volatilization from soil surfaces based upon its vapor pressure. According to Japanese MITI (Ministry of International Trade and Industry) data, 73 percent of the Theoretical BOD was reached in 4 weeks in a static die-away system using an activated sludge with the initial NMP present at 100 mg/L, suggesting that microbial degradation is an important

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environmental fate process in soil and water (Chem Inspect Test Inst., 1922). However, to the best of our knowledge, there is no report about the degradation of NMP by a pure culture and its microbial metabolic mechanism.

In this study, an NMP-degrading strain, NMD-4, was isolated from the activated sludge of a pesticide plant in Jiangsu, China. Strain NMD-4 was identified as *Paracoccus* sp. This strain could use NMP as the sole carbon and nitrogen sources for growth and completely degrade 500 mg L^{-1} NMP within 24 h. The degradation characteristic of NMP by strain NMD-4 was studied in a liquid culture, and the degradation pathway of the NMP by the strain was also investigated.

2. Materials and methods

2.1. Materials and media

The activated sludge used for enrichment was collected from a pesticide factory in Jiangsu, China. NMP and chromatographic-grade dichloromethane was purchased from Sinopharm Chemical Reagent Co. Ltd. Molecular biology reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). All other reagents used in this study were of analytical grade. A mineral salts medium (MSM) (1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_47H_2O$, 1.0 g NaCl per liter water, pH 7.0) was used in this study for enrichment, and a Luria–Bertani (LB) medium (10.0 g NaCl, 10.0 g peptone and 5.0 g yeast extract per liter water, pH 7.0) was used for strain culture. For solid media, 15 g per liter of agar powder was added. When necessary, NMP was added to the media at an appropriate concentration. All media were sterilized by autoclaving at 121 °C for 20 min.

2.2. Enrichment and strain isolation

An activated sludge sample (5 g) was added to 100 ml of MSM containing 500 mg L^{-1} NMP as the sole carbon and nitrogen source. The sample was incubated on a rotary shaker at 160 rpm at 30 °C. After 3 days, the culture became turbid, and 5 ml of the culture was inoculated in fresh MSM containing 500 mg L^{-1} NMP and incubated for another 3 days. The decrease of NMP was monitored by HPLC as described below. Enrichment cultures capable of degrading NMP were diluted and spread onto MSM agar containing 500 mg L^{-1} NMP. Colonies grown on these plates were picked up, purified by repeated streaking, and tested for the ability to degrade NMP. In this study, four strains capable of degrading NMP were isolated. Among them, strain NMD-4, which processed the highest NMP-degrading ability, was selected for further investigation.

2.3. Identification of the strain

The NMP-degrading bacterium was identified according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Genomic DNA was extracted by high-salt precipitation (Sambrook and Russel, 2001), and the 16S rDNA sequence was amplified by PCR using standard procedures with a bacterial universal primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) (Lane, 1991; Marchesi et al., 1998). A fragment corresponding to *E. coli* 16S rDNA positions 8–1513 was amplified. The PCR product was purified using an Axy-Prep PCR Purification Kit (AxyGen) and determined by the Nanjing Jinsirui Biological Technique Company (Applied Biosystems, model 3730). Closely related 16S rDNA sequences from GenBank were aligned using Clustal_X, and phylogenetic analysis was performed with the software MEGA version 3.0 (Kumar et al., 2004). Phylogenetic trees were generated using the neighbor-joining method according to the Kimura 2-parameter model and evaluated by bootstrap analyses based on 1200 resamplings (Li et al., 2009a,b). DNA–DNA hybridizations were performed according to the method of Ezaki et al.

2.4. Inoculum preparation for degradation studies

Strain NMD-4 was precultured in 5 ml of LB medium at 30 °C with shaking at 160 rpm for 12 h. Cells were harvested by centrifugation at 6000 rpm for 3 min, washed twice, and resuspended in fresh MSM ($OD_{600nm} = 1.0$).

2.5. Degradation of NMP by strain NMD-4 in relation to the bacterial growth

An inoculum (1 percent, v/v) was inoculated into a 500 ml flask containing 200 ml MSM supplemented with 500 mg L^{-1} of NMP. Each treatment was performed in triplicate, and control experiments without inoculation or without substrate were carried out under the same condition. The cultures were incubated on a rotary shaker at 160 rpm and 30 °C. At regular intervals, 5 ml samples were collected from each flask. Bacterial growth was monitored by measuring the optical density of culture samples at 600 nm (OD_{600}). The disappearance of NMP was monitored by high-performance liquid chromatography (HPLC), and the metabolites were identified by HPLC–Mass spectrometry (MS) as described below.

2.6. Biodegradation kinetics

The NMP-degrading strain incubated in LB medium for 48 h was centrifuged at 6000 g for 10 min in 50 mL Eppendorf centrifuge tube. The cell pellet was washed twice with phosphate buffer saline (PBS, pH 7.4) and then suspended in NaCl solution (0.85 percent). Then the bacteria suspension was prepared for the next biodegradation experiments. 4.0 ml of bacteria suspension was added into 250 ml flasks containing 100 ml MSM with 500 mg L^{-1} NMP to make the final cell density 1.0×10^8–1.0×10^9 cfu mL^{-1} .The flasks was placed in rotary shaker (160 r min^{-1}) at 30 °C. At regular intervals, 5 ml samples were collected from each flask and the samples were used to determine the concentration of NMP. Cell counting was performed with LB plates by the plate dilution technique, and colonies were counted after 72 h of incubation at 28 °C.

2.7. Effects of the temperature, pH, metal ions, initial substrate concentration and dissolved oxygen concentration on NMP degradation

Two milliliter inoculums were incubated in 200 ml of MSM containing 500 mg L^{-1} NMP for 24 h at different temperatures (20, 25, 30, 35, 40 and 45 °C), under different pH conditions (5.0–10.0, in increments of 1.0 pH units) and with different concentrations (0.1 mM and 1 mM) of metal ions (Cd^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+}). The effects of initial NMP concentrations of 500 mg L^{-1}, 750 mg L^{-1}, 1000 mg L^{-1}, 1500 mg L^{-1}, and 2000 mg L^{-1} were investigated. The effects of oxygen level on NMP degradation was investigated by inoculating the strain into 50 mL, 75 mL and 125 mL MSM containing 500 mg L^{-1} NMP, respectively. The cultures were incubated on a rotary shaker at 160 rpm and 30 °C. At regular intervals, 2 ml samples were collected from each flask. The biomass was monitored by OD_{600}, and the concentration of the NMP was determined by HPLC. Experiments were conducted in three parallel flasks and the concentration of NMP in each flask was independently determined.