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Degradation of piperazine by *Paracoccus* sp. TOH isolated from activated sludge

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HIGHLIGHTS

- A novel bacterium was isolated from activated sludge for piperazine biodegradation.
- The optimal pH and temperature for degradation were 8.0 and 30 °C, respectively.
- Glucose as co-substrate plays a role in enhancement of degradation.
- The metabolic pathway for piperazine was proposed by the first time.

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ABSTRACT

Piperazine is widely used as an intermediate in the manufacture of insecticides, rubber chemicals, corrosion inhibitors, and urethane. In this study, a highly effective piperazine-degrading bacteria strain, TOH, was isolated from the acclimated activated sludge of a pharmaceutical plant. This strain, identified as *Paracoccus* sp., utilises piperazine as the sole source of carbon, nitrogen and energy for growth. The optimal pH and temperature for the growth of TOH were 8.0 and 30 °C, respectively. The effects of co-substrates and heavy metals on the degradation efficiency of piperazine were investigated. The results indicated that exogenously supplied glucose promoted the degradation of piperazine, while the addition of ammonium chloride slightly inhibited piperazine degradation. Metal ions such as Ni^{2+} and Cd^{2+} inhibited the degradation of piperazine, whereas Mg^{2+} increased it. In addition, metabolic intermediates were identified by mass spectrometry, allowing a degradation pathway for piperazine to be proposed for the first time.

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1. Introduction

Alicyclic amines, also known as *N*-heterocyclic alkanes, are frequently detected in the environment (Kim et al., 2006). Piperazine (diethylenediamine) is an important alicyclic amine that is commonly used in the pharmaceutical industry in the synthesis of methyl and hydroxyl derivatives and as an intermediate in the synthetic chemical industry (Gift and Jeremy, 1994; Renata, 2005). The widespread use of piperazine has resulted in the discharge of large amounts of the compound into the environment, which eventually reach the biosphere. Several studies have demonstrated that piperazine and its derivatives are toxic and carcinogenic to humans and other living organisms (Gift and Jeremy, 1994). Therefore, the degradation of piperazine in the environment is of great concern.

The biodegradation of heterocyclic amines has drawn particular interest from an environmental point of view (Bae et al., 2009). While some amines are readily biodegradable, others (often structurally similar) are not (Bae et al., 2002). The biodegradation of secondary amines (pyrrolidine, piperidine, piperazine and morpholine) is extremely important due to their propensity for conversion (either chemically or microbiologically) to *N*-nitrosamines, many of which are potent carcinogens (Gift and Jeremy, 1994). Heterocyclic amines can be degraded by several species of bacteria belonging to the genera *Pseudomonas*, *Mycobacterium* (Kim et al., 2006; Adjei et al., 2007) and *Arthrobacter* (Bae et al., 2009). Pyrrolidine and piperidine are readily degradable over a long period of time, but morpholine and piperazine are considered recalcitrant to biodegradation (Poupin et al., 1999). Bae et al. (2009) isolated a total of 30 strains capable of degrading pyrrolidine and piperidine under denitrifying conditions; however, no isolate was able to degrade piperazine or morpholine. Kim et al. (2006) reported that *Mycobacterium* sp. strain THO100, which was isolated from a morpholine-containing culture, was able to utilise pyrrolidine, morpholine, piperidine, and piperazine as sole sources of carbon and nitrogen, but the degradation of piperazine was much lower than

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that of the other compounds. In an earlier report, piperazine was considered the most recalcitrant to degradation (Gift and Jeremy, 1994). Lag periods before the onset of piperazine degradation ranged from 30 to 60 days, and complete degradation times ranged from 40 to 68 days (Gift and Jeremy, 1994). Under the same conditions, pyrrolidine and piperidine were always rapidly degraded with little or no lag period. In an article on the treatment of drinking water, Jőrg et al. (2001) reported that the order of biodegradation of selected compounds was as follows: ethanolamine > dimethylamine > pyrrolidine > ethylenediamine ≈ piperidine > diethylamine > morpholine > piperazine > cyclohexylamine. In summary, piperazine is difficult to biodegrade, and there have been few reports of bacteria that can degrade piperazine rapidly. In addition, the metabolic pathway by which piperazine is biodegraded has not been described.

In this report, a highly effective piperazine-degrading strain, designated TOH, was isolated from the activated sewage sludge of a pharmaceutical plant and identified as *Paracoccus* sp. Members of this genus are saprophytic soil and water bacteria, and isolates of this genus are responsible for the biodegradation of a wide variety of toxic organic pollutants, such as amide pesticides (Zhang et al. 2012), dimethoate (Li et al., 2010) and acetochlor (Zhang et al. 2011). Strain TOH was able to use piperazine as the sole source of carbon and nitrogen for growth and completely degraded 100 mg L^{-1} piperazine within 24 h. The effects of several parameters, including pH, heavy metal ions, initial substrate concentration and the addition of other carbon/nitrogen sources, on the biodegradation of piperazine by the isolated strain were investigated. Moreover, the pathway of piperazine biodegradation by strain TOH is proposed for the first time. This paper highlights a significant potential use of pure cultures of microbial cells for the cleanup of piperazine-contaminated wastewater.

2. Methods

2.1. Media and culture conditions

The activated sludge used for bacterial isolation was collected from a pharmaceutical plant in Zhejiang, China. Piperazine anhydrous (99.5% purity) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai). Chromatographic grade methanol was purchased from Sigma–Aldrich (St. Louis, USA). Molecular biology reagents were purchased from TaKaRa Biotechnology Co., Ltd (Dalian). All other reagents used in this study were of analytical reagent quality. Luria–Bertani (LB) medium (10.0 g NaCl, 10.0 g peptone and 5.0 g yeast extract per litre water, pH 7.0) and mineral salts medium (MSM) (1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl per litre water, pH 7.0) were utilised in this study. For solid medium, agar powder was added at a concentration of 1.8%. When necessary, piperazine was added to the media at an appropriate concentration. All media used in this study were prepared using Milli-Q water (>18.2 MΩ) and sterilised by autoclaving at 121 °C for 20 min.

2.2. Enrichment and isolation

An activated sludge sample (5 g) was added to 100 mL of MSM containing 100 mg L^{-1} piperazine as the sole carbon and nitrogen source. The sample was incubated on a rotary shaker at 160 rpm at 30 °C. After 7 days (d), the supernatant became obviously turbid, and 5 mL of the supernatant was sub-cultured into fresh MSM containing 200 mg L^{-1} piperazine. The decrease in piperazine was monitored by HPLC analysis as described below. The ammonium content of the medium was also measured to determine whether piperazine was completely degraded. Enrichment cultures capable of degrading piperazine were diluted and spread onto MSM agar containing 300 mg L^{-1} piperazine. Colonies grown on these plates were evaluated for their piperazine-degrading capabilities. One strain, designated as TOH, which possessed the highest piperazine-degrading ability and could utilise piperazine as the sole carbon and nitrogen source for growth, was purified and selected for further investigation.

2.3. Identification of the strain

TOH was identified based on its morphological, physiological and biochemical properties according to Bergey's Manual of Determinative Bacteriology (Holt et al., 2004) and by 16S rRNA gene sequence analysis. Genomic DNA was extracted, and the 16S rRNA gene was amplified by PCR as described previously (Li et al., 2010). The nucleotide sequence coding for the 16S rRNA gene of TOH was sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd. Alignment with related 16S rRNA gene sequences from GenBank was performed using Clustal X 1.8.3 with default settings (Thompson et al., 1997). Phylogeny was analysed with MEGA version 4.0 software (Tamura et al., 2007), and evolutionary distance was calculated using the Kimura 2-parameter distance model. A phylogenetic tree was built using the neighbour-joining method. Each dataset was bootstrapped 1000 times.

2.4. Analytical methods

Cell growth was monitored by measuring the optical density of culture samples at 600 nm (OD_{600}). The concentration of piperazine was determined by HPLC using precolumn derivatisation with *p*-tosyl chloride (ptsc), as described by Lin et al. (2010). All liquid culture samples were filtered through a microporous membrane (0.22 μm) and analysed by HPLC on a reverse-phase C18 column at 240 nm. The mobile phase was methanol:water (60:40, v:v), and the flow rate was 1 mL min^{-1}. A calibration curve for the peak area versus the concentration of piperazine was used to calculate the concentrations of piperazine. The piperazine concentration in the enrichment protocol was determined by measuring chemical oxygen demand (COD), as described by the American Public Health Association (American Public Health Association, 1976). Ammonium was quantified using Nessler's reagent. A yellow colour formed in proportion to the ammonium concentration and was measured at 420 nm with a 752 UV–visible spectrophotometer.

Liquid chromatography/electron spray ionisation tandem mass spectrometry (LC/ESI-MS/MS) analyses were performed on an Agilent G6410B Triple Quad Mass Spectrometer equipped with an Agilent Technologies 1200 Series HPLC. The HPLC system was composed of an autosampler, a binary high-pressure gradient pump, and a UV–visible detector. The system was also equipped with a column oven and an on-line vacuum degasser. The mass spectrometer was operated in positive-ion ESI mode. Other ESI conditions were as follows: a gas temperature of 350 °C, a capillary voltage of 4.0 kV, a nebulisation pressure of 30.0 psi, and a gas flow rate of 10.0 V/min. MS/MS conditions were as follows: a fragmentor voltage of 90 V and a collision energy of 10–25 eV. The chromatography runs were performed on an Agilent XDB-C18 (50 mm × 4.6 mm) analytical column at 30 °C. The isocratic mobile phase was composed of a mixture of water:methanol (40:60, v:v), which was pumped through the column at a flow rate of 0.2 mL min^{-1}.

2.5. Inoculum preparation for degradation studies

TOH was pre-cultured in 5 mL of LB medium at 30 °C with shaking at 160 rpm for 20 h until late-exponential growth. All cultures

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