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Enzymatic degradation of aliphatic nitriles by *Rhodococcus rhodochrous* BX2, a versatile nitrile-degrading bacterium

Shumei Fang [a,b], Xuejiao An [a], Hongyuan Liu [a], Yi Cheng [a], Ning Hou [a], Lu Feng [a], Xinning Huang [a], Chunyan Li [a,*]

[a] College of Resource and Environment, Northeast Agricultural University, Harbin 150030, Heilongjiang, PR China
[b] College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 16339, Heilongjiang, PR China

HIGHLIGHTS

- Three aliphatic nitriles were degraded efficiently by *Rhodococcus Rhodochrous* BX2.
- BX2 exhibited much different biodegradability for cis- and trans-crotononitrile.
- BX2 harbors two enzyme systems for aliphatic nitrile degradation.
- NHase/amidase system was dominant in BX2 based on mRNA expression and enzyme assay.
- NHase/amidase system was inducible while nitrilase was constitutive in BX2.

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ABSTRACT

Nitriles are common environmental pollutants, and their removal has attracted increasing attention. Microbial degradation is considered to be the most acceptable method for removal. In this work, we investigated the biodegradation of three aliphatic nitriles (acetonitrile, acrylonitrile and crotononitrile) by *Rhodococcus rhodochrous* BX2 and the expression of their corresponding metabolic enzymes. This organism can utilize all three aliphatic nitriles as sole carbon and nitrogen sources, resulting in the complete degradation of these compounds. The degradation kinetics were described using a first-order model. The degradation efficiency was ranked according to $t_{1/2}$ as follows: acetonitrile > trans-crotononitrile > acrylonitrile > cis-crotononitrile. Only ammonia accumulated following the three nitriles degradation, while amides and carboxylic acids were transient and disappeared by the end of the assay. mRNA expression and enzyme activity indicated that the tested aliphatic nitriles were degraded via both the inducible NHase/amidase and the constitutive nitrilase pathways, with the former most likely preferred.

1. Introduction

Nitriles are a group of highly poisonous organic compounds bearing cyano group that are extensively used in the manufacture of solvents, plastics, rubbers and pharmaceuticals (Ramteke et al., 2013). The large amounts of production and persistent applications of these compounds have inevitably caused serious environmental pollution. Nitriles have been detected in samples from diverse environments, such as sewage sludge from a water-treatment plant and samples collected from marinas and coastal areas (Barrio et al., 1996; Baxter and Cummings, 2006; Sakkas et al., 2002). Moreover, the majority of nitriles are highly toxic, mutagenic, and carcinogenic (Ramteke et al., 2013). Studies have indicated that the toxicity of aliphatic nitriles (e.g., acetonitrile, acrylonitrile and crotononitrile) leads to neurobehavioral abnormalities in rats and some human sicknesses (Boadas-Vaello et al., 2007; Khan et al., 2009; Ramteke et al., 2013; Seoane et al., 2005). Such studies have heightened the urgent demand for efficient restoration techniques for this class of contaminants.

The elimination of nitrile compounds can be accomplished using physical, chemical and biological methods. Of these, the microbial method is the most accepted due to its mild, low-cost and environmentally friendly characteristics (Santoshkumar et al., 2011). Generally, microbial degradation of nitrile compounds occurs via the hydrolytic route, which consists of two enzymatic systems: (1) nitrile hydratase (NHase, EC 4.2.1.84) catalyzes the

* Corresponding author. Tel.: +86 0451 55190034.
E-mail address: chunyanli@neau.edu.cn (C. Li).

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formation of amides from nitriles, which are subsequently converted to carboxylic acids and ammonia by amidase (EC 3.5.1.4); and (2) nitrilase (EC 3.5.5.1) catalyzes the direct conversion of nitrile compounds into corresponding carboxylic acids and ammonia (Ramteke et al., 2013). To date, a number of nitrile-degrading microorganisms harboring NHase/amidase or nitrilase activity have been isolated (Banerjee et al., 2002; Feng and Lee, 2009; Kao et al., 2006; Prasad et al., 2007; Vesela et al., 2012). Unfortunately, only a few organisms have multiple enzymatic systems (e.g., *Amycolatopsis* sp. IITR215 (Babu et al., 2010), *Nocardia globerula* NHB-2 (Bhalla and Kumar, 2005) and *Bacillus subtilis* ZJB-063 (Zheng et al., 2008)). Furthermore, although two enzyme systems were detected in these organisms, only one enzyme system functioned for each type of nitrile compound (i.e., NHase/amidase or nitrilase). For example, *Amycolatopsis* sp. IITR215, possessing both NHase/amidase and nitrilase activities, degraded polyacrylonitrile through the NHase/amidase system and, hexanenitrile through the nitrilase system (Babu et al., 2010). Up to now, there have been few reports in the literature demonstrating that when the two enzyme systems are expressed in one strain, both systems catalyze the degradation of a given type of aliphatic nitriles, and the identification of the dominant pathway has not been discussed (Bhalla and Kumar, 2005; Babu et al., 2010).

In a previous study, our laboratory isolated *Rhodococcus rhodochrous* BX2 from contaminated soil. This bacterium was capable of utilizing acetonitrile as the sole carbon, nitrogen and energy source (Li et al., 2013). However, the capacity of this strain to degrade additional nitrile compounds and the characterization of its mechanism of nitrile degradation were not studied in detail. In this work, we showed that this organism could also efficiently degrade acrylonitrile and crotononitrile. The degradation kinetics of the three aliphatic nitriles (acetonitrile, acrylonitrile, and cis- and trans-crotononitrile) were investigated based on the first-order model. The metabolic products were determined by gas chromatography/mass spectrometry (GC/MS), and the dominant metabolic pathway was proposed by monitoring the mRNA expression and catalytic activity of the nitrile-degrading enzymes during nitrile biodegradation. To the best of our knowledge, this report is the first detailing the dominant pathway for aliphatic nitrile biodegradation and the distinctly different efficiencies of cis- and trans-crotononitrile biodegradation. All of these results strongly highlight the promising potential of *R. Rhodochrous* BX2 in the bioremediation of nitrile-contaminated environments and will contribute to further exploration of the application of these enzymes.

2. Methods

2.1. Chemicals, bacterial strain, and culture medium

Acetonitrile (CAS no. 75-05-8) was purchased from Fisher Scientific Co. (Shanghai, China). Acrylonitrile (CAS no. 107-13-1) and crotononitrile (CAS no. 4786-20-3) (cis:trans ratio of 60:40) were procured from Tokyo Chemical Industry (TCI) Co., Ltd. (Tokyo, Japan). All amides and carboxylic acids used in this study were obtained from Aladdin Industrial Corporation (Shanghai, China). All other chemicals used were of analytical grade and were available commercially.

R. Rhodochrous BX2 was isolated from contaminated soil (Li et al., 2013) and stored in our laboratory. Mineral salt (MS) medium devoid of carbon and nitrogen sources used for various nitrile utilization experiments consisted of KH_2PO_4 1700 mg L^{-1}, Na_2HPO_4 9800 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 100 mg L^{-1}, MgO 10 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 1.44 mg L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.9 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.25 mg L^{-1}, and H_3BO_3 0.06 mg L^{-1} at an initial pH of 7.4. Luria-Bertani (LB) medium contained tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, and NaCl 10 g L^{-1}. Fresh medium was sterilized immediately by autoclaving at 121 °C and 102.9 kPa for 20 min.

2.2. Bacterial growth, nitrile degradation and identification of metabolic products

Bacterium grown on agar plates containing acetonitrile, acrylonitrile or crotononitrile were inoculated in LB medium and cultured for 20 h at 30 °C and 180 rpm. After centrifugation of the cultures, the pellets were washed with MS medium three times and resuspended to a suitable density (OD_{600} = 2.0 ± 0.1). A 1-mL sample of this suspension (approximately 6.7×10^8 bacterial cells) was incubated in a 150-mL Erlenmeyer flask containing 50 mL MS medium supplemented with 19.51 mM acetonitrile, 7.54 mM acrylonitrile and 14.93 mM crotononitrile (cis:trans ratio of 60:40). Simultaneously, uninoculated flasks containing equal amounts of each nitrile were designated as controls. Then, the cultures were incubated at 30 °C in an orbital shaking incubator at 180 rpm, and samples were collected at regular intervals. Bacterial growth was monitored by measuring the absorbance at 600 nm. The concentrations of various nitriles, amides and carboxylic acids in the culture filtrate were determined by gas chromatography (GC2014, Shimadzu, Kyoto, Japan). The identification of metabolites was confirmed by GC/MS (Trace 1300 GC, ISQ MS, Waltham, MA, USA). The amount of ammonia released in the reaction was estimated colorimetrically using the phenol-hypochlorite method (Fawcett and Scott, 1960), and the pH was measured throughout the assay. All experiments were performed in triplicate.

2.3. Analysis of the mRNA expression levels of nitrile-degrading enzymes

2.3.1. RNA extraction

BX2 cells grown in MS medium containing acetonitrile, acrylonitrile or crotononitrile were harvested by centrifugation during different developmental periods. Total RNA was isolated using the pillar bacteria total RNA extraction kit (Sangon Biotech, Shanghai, China) following the directions of the manufacturer. The RNA concentration was calculated by measuring the absorbance at 260 nm, and the purity was evaluated by the ratios of 260/280 nm and 260/230 nm using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA).

2.3.2. Reverse transcription and quantitative PCR

Extracted total RNA (3 μg) was treated with 3 U of DNase I (RNase-free, Thermo, Shanghai, China) as described by the manufacturer. The removal of genomic DNA contamination was verified by PCR. Purified RNA (30 μg) was reverse transcribed using the PrimeScript® RT Perfect Real Time reagent kit (Takara Biotech, Dalian, China) with 6-mer random primers as recommended. The synthesized cDNA was subjected to PCR and quantitative real-time PCR (qPCR). Specific primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA) (see Table S1 in the supplementary document and Table 1).

The qPCR reaction was performed in a Bio-Rad iQ5 instrument in a 20 μL reaction mixture containing 0.8 μL cDNA, 0.4 μmol primers, and 9.0 μL SYBR® *Premix Ex Taq*™ II (Takara Biotech, Dalian, China). Thermal cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. A melting curve was analyzed at the end of the qPCR to verify specific amplification. 16S rDNA was used as a reference gene to normalize the amount of RNA in each sample (Lino et al., 2013). Each sample was measured in triplicate.