Ecological Engineering 52 (2013) 270–277

Contents lists available at SciVerse ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Aerobic and anaerobic biodegradation of TNT by newly isolated *Bacillus mycoides*

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 24 July 2012
Accepted 13 November 2012
Available online 7 December 2012

Keywords:
Biodegradation
TNT
Bacillus mycoides
Kinetic equation
Pathway

ABSTRACT

A TNT-degrading strain, which was isolated from Fe-reducing bacterial consortia and was identified as *Bacillus mycoides* based on its 16S rDNA sequence, was first used to degrade TNT under aerobic or anaerobic conditions. The results shows that 93% of the initial TNT was degraded after 16 h under aerobic environment, while 94% of TNT was reduced after 24 h under anaerobic environment. This is attributed to slower microorganism growth under anaerobic conditions and was confirmed in a biokinetic study, where lower degradation rates were obtained under anaerobic condition. Two TNT degradation metabolites, 4-amino-2,6-dinitrotoluene and 6-amino-2,4-dinitrotoluene, were identified by GC–MS and the degradation pathways of TNT by *B. mycoides* were proposed. Finally, *B. mycoides* was used to degrade TNT in industrial wastewater, where more than 88% of the TNT was removed, irrespective of the aerobic or anaerobic conditions.

1. Introduction

2,4,6-Trinitrotoluene (TNT) is an explosive, which has been used extensively in the military (Nyanhongo et al., 2009). Since both TNT and its metabolites exhibit toxicity to human beings, fish, algae and microorganisms, there have been considerable interest in the development of effective remediation technologies for removal of TNT from groundwater and wastewater (Kulkarni and Chaudhari, 2007). Bioremediation is one remediation technique attracting worldwide attention since it is usually a less expensive way of destroying organic pollutants than traditional engineering based technologies (Kulkarni and Chaudhari, 2007). Generally, biological processes for degradation of nitroaromatics can be classified as being either aerobic or anaerobic. Anaerobic transformation of nitroaromatics involves reduction of nitro groups to aromatic amines through a six-electron transfer mechanism, where the reduction of nitro-groups to nitroso derivatives, hydroxylamines or amines is catalyzed by nitroreductases through successive addition of electron pairs donated by co-substrates (Razo-Flores et al., 1997). Hence, synergistic participation of a consortium of bacteria is required for simultaneous partial degradation of several nitroaromatic compounds: viz. dinitrotoluene, 3,5-dinitrobenzoic acid, 2-, 3- and 4-nitrophenol and TNT (Kulkarni and Chaudhari, 2007). *Desulfovibrio* sp. *StrainB* (Boopathy and Kulpa, 1993) and *Pseudomonas* sp. *JLR11* (Esteve-Nunez et al., 2000) are capable of degrading TNT anaerobically. In contrast, under aerobic degradation, nitroaromatic compounds are used mainly as carbon, nitrogen and energy sources for bacteria and can be completely mineralized. Current research has focused on isolation of various microbes to degrade/mineralize nitroaromatics aerobically, and deciphered the catabolic pathways and enzymes involved in aerobic degradation (Kulkarni and Chaudhari, 2007). Various aerobic bacteria and fungi, such as *Enterobacter cloacae PB2* (French et al., 1998), *Phanerochaete chrysosporium* (Hawari et al., 2000) and *Pseudomonas putida HK-6* (Cho et al., 2008) have been reported to degrade TNT. However, these reports focused on either aerobic or anaerobic degradation of TNT, and there were few reports to discuss microbes, which can degrade TNT under both aerobic and anaerobic conditions.

To date there is no reported instances of *B. mycoides*, isolated from Fe-reducing bacterial consortia, used to biodegrade TNT. Hence, the purpose of the current study was to investigate the environmental conditions affecting the biodegradation of TNT by *B. mycoides* under aerobic and anaerobic conditions in order to achieve optimal biodegrading efficiency. In addition, to understand the mechanism of biodegradation of TNT using *B. mycoides*, the biokinetic model and the metabolites of TNT biodegradation were also investigated under the optimized conditions. Finally, the strain was used for the degradation of TNT in real TNT-containing wastewater to determine whether or not *B. mycoides* can be used as a potential biodegradation of TNT in real applications.

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http://dx.doi.org/10.1016/j.ecoleng.2012.11.004

2. Materials and methods

2.1. Isolation and identification of the microorganisms

The strain used in this study was isolated from a Fe-reducing bacterial consortia used to increase the whiteness of Kaolin in our laboratory (Guo et al., 2010). The Fe-reducing bacterial consortia were inoculated in a heterotrophic medium containing the following chemicals (g/L of distilled water): K_2HPO_4, 2; NaH_2PO_4, 1; $FeSO_4{\cdot}7H_2O$, 0.01; $MgSO_4{\cdot}7H_2O$, 0.020; $CaCl_2$, 0.005; $ZnSO_4$, 0.001; TNT, 0.1; glucose 1 and $(NH_4)_2SO_4$ 1. The pH of the culture medium was adjusted with HCl or NaOH to 7.0 ± 0.1.

The capability of microorganisms to degrade TNT was initially screened by adding TNT (50 mg/L) at the beginning of enrichment culture. Sub-cultivation was conducted by transferring one-tenth of the culture into fresh medium. After the culture was adapted to the TNT concentration, and the growth rate of the bacteria increased, the concentration of TNT was raised gradually. After five transfers, the concentration of TNT in the culture was 100 mg/L and the culture was plated for isolation on tryptic soy agar (TSA) plates with TNT (100 mg/L) (Boopathy et al., 1994). The isolated colonies were restreaked and transferred to liquid cultures.

The purified isolate was identified by Gram staining and the 16S rDNA sequence. The morphologic and microbial properties of the isolate were also investigated. The fragment of 16S rDNA sequence was amplified using a Gene Amp PCR System (PE, USA) under the following condition: template DNA (1 μL), 10 × PCR buffer (2.5 μL), 1.5 mM $MgCl_2$, 500 mM KCl, and 0.1% (w/v, glutin), 0.25 U of rTaq polymerase (Takara, Dalian, China), included 1′PCR buffer containing 1.5 mM $MgCl_2$ (Takara, Dalian, China), 200 μM of each deoxynucleoside triphosphate (Takara, Dalian, China), 10 pmol of each primer, 0.25 U of rTaq polymerase (Takara, Dalian, China), 1 μL bacteria liquid, 0.5 mM upstream primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), 0.5 mM downstream primer 1492R (5′-TACCTTGTTACGACTT-3′) (Jiang et al., 2007), 2.5 mM dNTPs and Mili-Q water contained a total volume of 50 mL. The tubes were incubated at 95 °C for 10 min and then subjected to the following thermal cycling programme: denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and chain extension at 72 °C for 1 min 30 s and a final extension at 72 °C for 30 min, for a total of 30 cycles. Sequencing on both strands of PCR-amplified fragments was performed by the dideoxy chain termination method in an ABI 3730 automated sequencer (Invitrogen, Shanghai, China). 16S rDNA sequence homology searches against the NCBI GenBank database were carried out using the basic local alignment search tool (BLAST) program (Chen et al., 2005) to search 16S rDNA database for similar sequences.

2.2. Biodegradation of TNT experiments

In order to enhance degradation efficiency, the factors affecting the biodegradation of TNT in aqueous solution were investigated. Experiments under aerobic conditions were conducted in a gyratory shaker operated at 150 rpm, while experiments under anaerobic condition were conducted in culture bottles where the headspace had been purged with N_2 and sealed tightly with a butyl rubber stopper under static condition. Culture bottles were incubated in thermostatic incubator. Batch experiments under both aerobic and anaerobic conditions were conducted in a series of 50 mL culture bottles containing media (30 mL) in the dark under various conditions: glucose concentration of 0.5, 1, 1.5, 2 and 2.5 g/L; $NH_4(SO_4)_2$ concentration of 0.5, 1, 1.5, 2 and 2.5 g/L; inoculum concentration of 0.005, 0.01, 0.0H40.03 and 0.04 g/L (dry weight); autoclaved medium pH of 5–9 adjusted with 1 N HCl or 1 N NaOH; incubation temperature of 20 °C, 25 °C, 30 °C, 35 °C and 40 °C. The initial TNT concentration in these assays was 100 mg/L and concentrations of TNT were measured after 12 h for aerobic condition and after 18 h for anaerobic condition.

2.3. TNT biodegradation kinetics

All flasks were incubated in the dark under the optimum conditions obtained from above-mentioned batch experiments (aerobic condition: agitation speed 150 rpm; glucose concentration 1 g/L; $NH_4(SO_4)2$ concentration 1 g/L; inoculum concentration 0.01 g/L; pH 7; temperature 30 °C. Anaerobic condition: agitation speed 150 rpm; glucose concentration 1.5 g/L; $NH_4(SO_4)_2$ concentration 1.5 g/L; inoculum concentration 0.01 g/L; pH 7; temperature 30 °C.). Experiments examining TNT degradation were conducted in a series of 50 mL culture bottles containing media (30 mL) and different concentrations of TNT ranged from 23.2 to 120.3 mg/L. Sterile controls were prepared by autoclaving cells before the introduction of TNT. Samples were collected periodically from growing cultures and the TNT concentration was analyzed immediately. All experiments were performed in triplicate.

2.4. Removal of TNT from industrial wastewater

The TNT-containing wastewater was collected from an industrial sewage treatment plant of Fuzhou. Samples collected (TNT concentration 40.6 mg/L; pH = 7.39) were stored at 4 °C for 4 h before subsequent experimentation. Since the wastewater collected only contained 40.6 mg/L TNT, additionally TNT was spiked into the wastewater to a concentration of 100 mg/L. Inoculum and substrate (1 g/L glucose and 1 g/L $NH_4(SO_4)_2$ in aerobic condition; 1.5 g/L glucose and 1.5 g/L $NH_4(SO_4)_2$ in anaerobic condition) was added to wastewater (30 mL) in a 50 mL culture bottle under the optimized conditions (those indicated in the previous section) in the dark for 72 h. Sterile controls were also used. Liquid samples collected over time under aerobic and anaerobic conditions were analyzed for TNT concentrations.

2.5. Analysis procedure and methodology

Samples were centrifuged at 6000 rpm for 10 min and the supernatants were collected for analysis of TNT concentrations using a spectrophotometer at 420 nm (Xue et al., 2005) (722N visible spectrophotometer, Shanghai Precision & Scientific Instrument Co., Ltd.).

Dry weight of biomass was obtained after the cultured samples were centrifuged for 20 min at 5000 rpm, washed twice with distilled water, and oven dried to a constant weight at 100–105°C for 24 h (Weng and Sun, 2006). Chemical oxygen demand (COD_{cr}) was measured by a closed reflux titration method (APHA, 1999).

Metabolites of TNT were extracted from the samples by dichloromethane and detected using gas chromatography–mass spectrometry (GC–MS) (Boopathy et al., 1994). Gas chromatographic analysis of the extracts was performed on a Thermo Trace DSQ, using a 30 m × 0.25 mm TR-35MS capillary column with a film thickness of 0.25 μm. The carrier gas was helium at a pressure of 105 kPa. A sample of the extract (0.1 μL) was injected into the column following the temperature program: hold at 60 °C for 2 min; ramp from 60 °C to 150 °C at 10 °C/min; hold at 150 °C for 5 min, ramp from 150 °C to 240 °C at 10 °C/min; hold at 240 °C for 1 min, ramp from 240 °C to 290 °C at 25 °C/min; hold at 290 °C for 2 min.