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Selection and identification of a bacterial community able to degrade and detoxify *m*-nitrophenol in continuous biofilm reactors

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ABSTRACT

Nitroaromatics are widely used for industrial purposes and constitute a group of compounds of environmental concern because of their persistence and toxic properties. Biological processes used for decontamination of nitroaromatic-polluted sources have then attracted worldwide attention. In the present investigation *m*-nitrophenol (MNP) biodegradation was studied in batch and continuous reactors. A bacterial community able to degrade the compound was first selected from a polluted freshwater stream and the isolates were identified by the analysis of the 16S rRNA gene sequence. The bacterial community was then used in biodegradation assays. Batch experiments were conducted in a 2 L aerobic microfermentor at 28 °C and with agitation (200 rpm). The influence of abiotic factors in the biodegradation process in batch reactors, such as initial concentration of the compound and initial pH of the medium, was also studied. Continuous degradation of MNP was performed in an aerobic up-flow fixed-bed biofilm reactor. The biodegradation process was evaluated by determining MNP and ammonium concentrations and chemical oxygen demand (COD). Detoxification was assessed by *Vibrio fischeri* and *Pseudokirchneriella subcapitata* toxicity tests. Under batch conditions the bacterial community was able to degrade 0.72 mM of MNP in 32 h, with efficiencies higher than 99.9% and 89.0% of MNP and COD removals respectively and with concomitant release of ammonium. When the initial MNP concentration increased to 1.08 and 1.44 mM MNP the biodegradation process was accomplished in 40 and 44 h, respectively. No biodegradation of the compound was observed at higher concentrations. The community was also able to degrade 0.72 mM of the compound at pH 5, 7 and 9. In the continuous process biodegradation efficiency reached 99.5% and 96.8% of MNP and COD removal respectively. The maximum MNP removal rate was 37.9 g m^{-3} day^{-1}. Toxicity was not detected after the biodegradation process.

1. Introduction

The presence of nitroaromatic compounds in the environment is a consequence of anthropogenic activities (Schenzle et al., 1997), as they are some of the most widely used industrial organic compounds. Among these xenobiotic compounds, nitrophenols are frequently used as raw materials or intermediates in the manufacture explosives, pharmaceuticals, pesticides, dyes, wood preservatives and rubber chemicals (She et al., 2005). Due to their intense yellow color and pH reactivity nitrophenols are often used directly in titrations as indicators. The isomers *p*-Nitrophenol (PNP) and *o*-nitrophenol (ONP) coupled to others substrates are also used in monitoring enzyme activity. Pharmaceutical and agricultural uses of PNP include the synthesis of acetaminophen and the organophosphorus pesticides parathion and methylparathion (Zylstra et al., 2000). Because of their widespread use these compounds have been detected in aquatic environments, including river waters and industrial effluents (Iwaki and Hasegawa, 2007), and pesticides-treated soils (Marvin-Sikkema and de Bont, 1994). However, the stability, persistence and toxicity that make these compounds valuable to industry also make them hazardous when they are released into the environment (Nishino et al., 2000). Increased public concern about toxicity and the risks associated with nitroaromatic compounds encouraged the development of cost-effective technologies for their remediation (Kulkarni and Chaudhari, 2007). Biological processes used for decontamination of nitroaromatic-polluted sources have then attracted

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worldwide attention (Alexander, 1999). While ONP and PNP biodegradation processes have been exhaustively studied, information about *m*-nitrophenol (MNP) biodegradation is scarce. In fact, several bacterial strains able to degrade aerobically ONP and PNP have been previously reported and the biodegradation pathways characterized, such as *Alcaligenes* sp. NyZ215 (Xiao et al., 2007), *Arthrobacter protophormiae* RKJ100 (Chauan et al., 2000), *Burkholderia cepacia* RKJ200 (Prakash et al., 1996), and *Pseudomonas* sp. (Zhang et al., 2012), among others. Although some MNP-degrading strains such as *P. putida* (Meulenberg et al., 1996) and *Cupriavidus necator* JMP134 (Schenzle et al., 1997) have been previously reported, there is a lack of information about the use of these microorganisms for the degradation of this compound in liquid effluents. Moreover, considering that the high toxicity of MNP to aquatic organisms such as algae, invertebrates and fishes, has been demonstrated by several studies (WHO, 2000), detoxification of the compound after biodegradation processes should be evaluated. The aims of this investigation are: (a) to select MNP-degrading microorganisms from a polluted source, (b) to study the MNP biodegradation kinetic in batch reactors, (c) to evaluate the influence of abiotic factors in the biodegradation process and (d) to use the selected degrading microorganisms for the degradation and detoxification of MNP in a continuous-flow biofilm reactor.

2. Materials and methods

2.1. Selection and identification of MNP-degrading microorganisms

Enrichment was performed in an aerated batch reactor with an effective volume of 1 L, by exposing a water sample from the Reconquista River (located in Buenos Aires, Argentina) to 0.36 mM of MNP as the sole source of carbon and nitrogen. Once a consistent depletion of the compound was achieved, an aliquot of 1 mL of this enrichment was transferred to a 250 mL Erlenmeyer flask containing 100 mL of a synthetic minimal medium (pH 7) described by Korol et al. (1989) with MNP (0.36 mM) as the sole carbon and nitrogen source, and successively subcultured in a rotatory shaker at 200 rpm and 28 °C. The medium was modified by removing the ammonium salt to obtain a mineral medium with no nitrogen source other than the compound under study. The bacterial community obtained by this selection procedure was then employed as inoculum in biodegradation tests in batch and continuous reactors.

Individual strains from the bacterial community were isolated by streaking onto tryptone soy agar medium (Merck, Darmstadt, Germany) supplemented with 0.36 mM of MNP for further identification. In order to identify the individual strains Gram staining and molecular techniques were employed. Molecular techniques were described previously (González et al., 2012). They consisted in the amplification of the 16S ribosomal RNA (rRNA) gene by the polymerase chain reaction (PCR) and the sequencing of the amplified fragments. For amplification the following primers (5′–3′) were used: 16SR: GYTACCTTGTTACGACTT and 16SF: AGAGTTTGATCMTGGCTCAG. Amplified fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Duesseldorf, Germany) according to manufacturer's instructions, and sequenced using an ABI Prism DNA 3700 sequencer (Applied Biosystems, California, USA). Finally, nucleotide sequences were compared with databases using the NCBIs' Basic Local Alignment Search Tool (BLAST).

2.2. Chemicals

MNP (99% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). All the other chemicals were of analytical reagent grade and purchased from Mallinckrodt Chemical (St. Louis, USA) and Merck (Darmstadt, Germany). The MNP solution was aseptically prepared by dissolving the necessary amount in sterile 0.1 N NaOH.

2.3. Preparation of stock cultures for biodegradation assays

In order to obtain stock cultures for batch and continuous experiments, the bacterial community was pre-exposed to the compound by inoculating 100 mL of the synthetic minimal medium described in Section 2.1. supplemented with 0.72 mM of MNP as the sole source of carbon and nitrogen. Incubation was carried out in a rotatory shaker at 200 rpm and 28 °C until the compound was not detected in the medium.

2.4. Degradation of different nitroaromatic compounds by the bacterial community

Several nitroaromatic compounds were tested as the sole carbon source for the bacterial community. These experiments were carried out in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 20 mg L^{-1} of *p*-nitrobenzoate, *o*-nitrophenol, *p*-nitrophenol, 2,4-dinitrophenol or picric acid. An aliquot of 1 mL of the stock culture was used to inoculate the flasks. Incubation was performed in a rotatory shaker at 200 rpm and 28 °C. After 7 days of incubation degradation of each substrate was evaluated.

2.5. Degradation of MNP by the individual strains

The ability of the individual strains to degrade MNP was also study. The assays were performed in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 0.36 mM of the compound and inoculated with 1 mL of a cell suspension of each individual strain. The flasks were incubated in a rotatory shaker at 200 rpm and 28 °C, for 7 days. At the end of the experiment the remaining concentration of MNP was determined.

2.6. Biodegradation assays in batch reactors

MNP biodegradation kinetic was studied in a New Brunswick Multigen™ microfermentor aerobically operated at 28 °C, with agitation (200 rpm), and an effective volume of 1250 mL of the same medium supplemented with 0.72 mM of MNP as carbon and nitrogen source. An aliquot of 12.5 mL of the stock culture was used to inoculate the system, so as to obtain a cell concentration of 10^6 cells mL^{-1}. During the incubation period samples of 10 mL were aseptically removed from the microfermentor at appropriate intervals in order to determine the remaining MNP concentration and evaluate microbial growth.

In order to evaluate the influence of abiotic factors on the biodegradation process two experiments were performed, with different concentrations of the compound and pH values. These experiments were carried out in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 0.72, 1.08 or 1.44 mM of MNP, or 100 mL of minimal medium at pH 5, 7 or 9, supplemented with 0.72 mM of MNP. The initial pH of the medium was adjusted to 5 and 9 with concentrated phosphoric acid or 0.1 N NaOH respectively. An aliquot of 1 mL of the stock culture was used to inoculate each Erlenmeyer flask, so as to obtain a cell concentration of 10^6 cells mL^{-1}. Incubation was performed in a rotatory shaker at 200 rpm and 28 °C. Samples were aseptically removed from the flasks at appropriate intervals in order to determine the remaining MNP concentration and evaluate microbial growth.

Two replicates of each biodegradation assay were carried out. Average values of MNP concentration and bacterial count were