Journal of Hazardous Materials 267 (2014) 119–127

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Biodegradation of 3,4 dichloroaniline by fungal isolated from the preconditioning phase of winery wastes subjected to vermicomposting

Jean Manuel Castillo*, Rogelio Nogales, Esperanza Romero

Department of Environmental Protection, Estación Experimental del Zaidín-Consejo Superior de Investigaciones Científicas (EEZ-CSIC), C/Profesor Albareda 1, 18008 Granada, Spain

HIGHLIGHTS

- Fungi from vermicomposting degraded 3,4-dicholoraniline (DCA).
- *Fusarium* sp. and *A. niger* grew on DCA as nitrogen source.
- Enzymes involved in the DCA biodegradation pathway.
- *A. niger* has a high detoxification potential for DCA.
- New metabolites indicate different strategies of DCA biodegradation by fungi.

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
10 December 2013
Accepted 24 December 2013
Available online 3 January 2014

Keywords:
3,4-Dichloroaniline
Biodegradation pathway
Fungi
Winery wastes-vermicompost
SPME-GC/MS

ABSTRACT

A hazardous contaminant, 3,4-dichloroaniline (DCA) is widespread in the environment due to its extensive use in the manufacture of chemicals and its application in different sectors. The ability of fungi grow on in winery wastes in the preconditioning period of vermicomposting to degrade DCA was investigated. Three filamentous fungi (F1, F2, and F3) were isolated and one identified as *Aspergillus niger* and two as *Fusarium* sp. strains. The culture media with the fungus alone or in consortium (Fmix) with DCA as the nitrogen source were analyzed by solid-phase microextraction and gas chromatography–mass spectrometry (SPME-GC/MS). The fastest degradation rate was measured in Fmix with a DT_{50} of 0.85 day^{-1}. *Fusarium* sp. and *A. niger* differed in the metabolism of DCA. Five metabolites were identified as a result of oxidation, co-denitrification, *N*-acetylation, and polymerization reactions. The major metabolites were 3,4-dichloroacetanilide and dichloroquinolines. The azo-metabolites tetrachloroazobenzene and tetracloroazoxybenzene and 3,4-dichloronitrobenzene were found in minor amounts but appeared to be the most persistent in the *Fusarium* cultures (half-lives ranging from 8.3 to 30.9 days). This study highlights the metabolic potential of microorganisms in the preconditioning period of the vermicomposting process and its possible application for *in situ* bioremediation strategies.

1. Introduction

The compound 3,4-dichloroaniline (DCA) is used in the synthesis of a wide range of substances. DCA residues from the chemical industries are released *via* wastewater into the hydrosphere and are again incorporated into soil or water compartments when pesticides such as vinclozolin or phenylureas are biodegraded by microorganisms [1–3]. This compound remains highly persistent in soils and waters [4–6], affecting the soil microbial population [7] and aquatic species [8]. Tetrachloroazobenzene (TCAZB) and tetrachloroazoxybenzene (TCAXB) are also formed as unwanted by-products from compounds containing the DCA moiety [3]. Martins et al. [9] have reported that aromatic amines (AA) have genotoxic and cytotoxic potential towards most living organisms. Therefore, exposure to this toxic contaminant has raised increasing concern over its fate and persistence in the environment.

Fungi, in contrast to bacteria, are capable of degrading a wide range of organic pollutants such as pesticide residues even at high concentrations because they have a complex enzymatic system which is usually induced by nutrient depletion, not by a particular pollutant. Basidiomycetes, white rot fungi (*Chrysosporium lignorum*, *Trametes versicolor*, *Phanerochaete chrysosporium*) have physiological capacity to degrade lignin and many xenobiotic compounds with a variety of structures due to its ligninolytic system

* Corresponding author. Tel.: +34 958 181600/126; fax: +34 958 129600.
E-mail address: jeanmanuel.castillo@eez.csic.es (J.M. Castillo).

0304-3894/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jhazmat.2013.12.052

[10]. However, the effectiveness of these fungi in xenobiotic degradation under environmental conditions is questionable, because the pH and C:N ratio are not always optimal for growth [11]. To overcome these disadvantages, the isolation of imperfect fungi of lignocellulose wastes may be an excellent alternative. Imperfecti fungi have ability to grow and occupy high volumes of wood and tolerate water stress [10]. Moreover, their lignin-degradation systems are not very substrate-specific and therefore can oxidize a great variety of compounds, including environmental pollutants such as dye or anthracene [12,13]. Imperfecti fungi play an important role in the pre-composting period from vermicomposting of lignocellulose organic substrates, contributing to the optimal conditions for earthworm growth [14,15]. However, no information is available on the biocatalyst potential of these imperfecti fungi during the first step of vermicomposting despite that they may offer an efficient alternative for bioremediation techniques meant to degrade and minimize the environmental impact of DCA, providing a low-cost environmentally friendly technology.

The objectives of this study were: (a) to isolate fungi that often grow in winery wastes submitted to preprocessing before vermicomposting, selecting and identifying those able to survive in DCA media; (b) to evaluate their growth capacity, tolerance, and biodegradation potential for DCA, determining the presence of metabolites of this compound; and (c) to elucidate the mechanisms responsible for DCA degradation.

2. Materials and methods

2.1. Chemicals

The chemical compound 3,4-dichloroaniline (DCA, 99.5% purity) was supplied by Chem Service. The metabolite standards, such as 3,4-dichloronitrobenzene (DCNB, 95% purity) was from ACROS Organics (Geel, Belgium), 3,4-dichloroacetanilide (DCAN) from MP Biomedicals, LLC (Illkirch, France), 4,7-dichloroquinoline (DCQ, 99% purity) was from Sigma–Aldrich, 3,3′4,4′-tetrachloroazobenzene (TCAZB, 98% purity) and 3,3′,4,4′-tetrachloroazoxybenzene (TCAXB, 99% purity) were supplied by Dr. Ehrenstorfer GmBH (Ausburg, Germany). 2,4,5-Trichloroaniline (99.0% purity) from Fluka (Steinheim, Germany) was used as internal standard. HPLC-grade acetonitrile from Scharlau Chemie, S.A (Barcelona, Spain) was used. Water was purified using the Milli-Q water-purification system (Millipore, MA, US).

2.2. Fungal Isolation and culture media

One gram of a representative organic sample from the preprocessing period of the vermicomposting of vine-shoot (Appendix A) was inoculated into 9 ml of peptone water and homogenized for 1 min by vortexing. Serial dilutions were made (10^{-1}, 10^{-3}, and 10^{-5}). Aliquots of 1 ml from each dilution were inoculated on potato dextrose agar (PDA) medium supplemented with streptomycin (30 mg l^{-1}) and incubated at 28 °C for 4 days. Colonies with the presence of mycelia were isolated from PDA medium. For degradation studies, a minimal salts medium (MM) was used, composed of (g l^{-1}) glucose 5.0, NH_4SO_4 7.5, K_2HPO_4 4.8, and $MgSO_4{\cdot}7H_2O$ 0.2, and supplemented with streptomycin (30 mg l^{-1}) and trace elements (mg l^{-1}): $MnSO_4$ 20, $FeSO_4{\cdot}7H_2O$ 1, $CuSO_4{\cdot}7H_2O$ 0.7, and NaCl 0.7. All media were adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 20 min.

2.3. Tolerance of the isolated fungi to DCA

For an assessment of the ability of the strains to grow on MM with DCA, the isolated fungi were inoculated into 50 ml of MM and incubated at 28 °C at 110 rpm for 4 days. For the elimination of any trace of C and N, cultures were centrifuged at 4500 rpm for 15 min and washed twice with phosphate buffer at pH 6.5 (KH_2PO_4 6.8 g l^{-1} + K_2HPO_4 8.8 g l^{-1}). Subsequently, 0.5 ml of the suspension was inoculated into solid MM (agar 15 g l^{-1}) that contains DCA at 20 mg l^{-1} as a carbon source (MMCS). DCA was also included in the solid MM as nitrogen source using in this case glucose (5 g l^{-1}) as a carbon source (MMNS). These were maintained at 28 °C under darkness for 5 days. The strains that grew were selected for degradation studies.

2.4. Identification of fungal strains

Chromosomal DNA was extracted from 20 mg of mycelium from each fungus according to Sambrook and Russell [16] with some modifications as an increment of 100 μl in SDS (20%) and 30 μl in proteinase K (20 mg ml^{-1}). Due to PCR inhibition factors, DNA was diluted to 10 ng μl^{-1}. The small subunit (SSU) 18S ribosomal RNA gene was amplified by PCR using 5 ng of DNA as template, 10 μM concentration of each primer un-SSU-0817F (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536R (ATTGGCAATGCYCTATCCCCA) described by Borneman and Hartin [17] and 1 U of Taq DNA polymerase (BioCat GmbH, Germany) in a 25 μl final reaction volume. Amplifications were performed in a Multigene gradient cycler (Labnet International, USA) under the following conditions: 5 min at 95 °C, 30 cycles of 45 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, plus an additional 7 min cycle at 72 °C. The amplicon size was 762 bp. The PCR-amplified fragments were cloned in pGEMT-Easy vector (Promega) and sequenced using ABI PRISM 3130x l Genetic Analyzer. Sequences were compared with those available on the database using the BLAST programme at the National Center for Biotechnology Information (NCBI). Molecular-sequence data were analyzed using MEGA 5.05 package [18]. Those sequences were aligned and phylogenetically analyzed with the 18S SSU ribosomal DNA sequences from other DCA-degrading fungi using the MUSCLE programme (multiple sequence comparison by log-expectation) and choosing Neighbour Joining as clustering method to produce the distance matrices. The phylogenetic trees were plotted using likelihood as the statistical method. The alignment was bootstrapped using 100 replications. The model of nucleotide substitution was selected [19]. The consensus tree was constructed following the distance tree.

2.5. Pre-inoculums preparation and degradation experiments

Three fungi (F1, F2, and F3) selected by their tolerance to DCA were inoculated in Erlenmeyer flasks of 500 ml with 100 ml of MM for 4 days to grow abundant mycelia. Fungal biomass was washed twice with phosphate buffer as was described above.

Fungal biomass of F1, F2 and F3 (0.30 g l^{-1} dry weight) individually and three-fungi mixture (Fmix; 0.1 g l^{-1} d.w. each) were inoculated in Erlenmeyer flasks of 250 ml containing 50 ml of the MMNS and supplemented with 15 μg ml^{-1} of DCA, respectively. Controls (F1C, F2C, F3C, and FmixC) without DCA and heat-killed inoculum (121 °C by 15 min) were set up to control the abiotic degradation and sorption effect. The treatments were incubated in triplicate at 28 °C on a rotary shaker at 130 rpm in darkness. The fungal growth, pH, and concentrations of DCA were analyzed at 0, 3, 5, 7, 15, 29, and 42 days.

2.5.1. Evaluation of growth of fungal strains on DCA

For the determination of the growth of these fungal strains on DCA, the fungal culture from each flask was harvested by using a pre-weighted filter paper (Whatman GP filters) and dried for 24 h at 105 °C. Fungal growth was quantified as a gram of dried mycelium per litre of culture medium. Different parameters were measured in the exponential phase [20]: biomass productivity (BP), specific