Biodegradation of organochlorine pesticides by bacteria grown in microniches of the porous structure of green bean coffee

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Received 3 March 2005; received in revised form 13 October 2005; accepted 1 November 2006
Available online 5 January 2007

Abstract

In this paper, the authors propose a model for DDT biodegradation by bacteria grown in microniches created in the porous structure of green bean coffee. Five bacteria isolated from coffee beans, identified as \textit{Pseudomonas aeruginosa}, \textit{P. putida}, \textit{Stenotrophomonas maltophilia}, \textit{Flavimonas oryzihabitans}, and \textit{Morganella morgani}. \textit{P. aeruginosa} and \textit{F. oryzihabitans}, were selected for pesticide degradation. Bacteria were selected according to their ability to grow on mineral media amended with: (a) glucose (10 g l\textsuperscript{-1}), (b) peptone (2 g l\textsuperscript{-1}), and (c) ground coffee beans (2 g l\textsuperscript{-1}). These three media were supplemented with 50 mg l\textsuperscript{-1} of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) and endosulfan. GC/MS analysis demonstrated that the greatest DDT removal was obtained in the medium supplemented with coffee beans, where 1,1-dichloro-2,2\textsuperscript{0}-bis (4-chlorophenyl)ethylene (DDE), 1-chloro-2,2-bis (4-chlorophenyl) ethane (DDMU) and 2,2\textsuperscript{0}-bis (\textit{p}-chlorophenyl)ethanol (DDOH) were detected. DDMU is a product of the reductive dechlorination of DDE, which in this system could be carried out under the anaerobic conditions in microniches present in the porous structure of the coffee bean. This was supported by scanning electron microscopy. Green bean coffee could be used as a nutrient source and as a support for bacterial growth in pesticide degradation.

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Keywords: Coffee; DDT; Flavimonas; Pseudomonas; Microniche

1. Introduction

Although some persistent organochlorine pesticides have been banned from agricultural and public health use during the past few decades, high concentrations of DDT and its metabolites have been found in soil, water, and sediment samples (Shen et al., 2005; Miersma et al., 2003; Yanénez et al., 2002; Bould, 1994). Furthermore, other insecticides, such as endosulfan and lindane, are currently in use throughout the world (EPA, 2002) and their presence in air, water, and soil is a problem of great concern. Reducing their levels in the environment has therefore become an important goal.

The biochemical and molecular modes of pesticide degradation by microorganisms have been well documented (Singh et al., 1999; Kumar et al., 1996). Organochlorine pesticides possess halogen electron withdrawing groups that generate electron deficiency in the molecule, which thus resists aerobic degradation (Rieger et al., 2002). However, these compounds can be attacked more readily under reductive conditions, which could be enhanced by the addition of auxiliary electron donors. For example, a number of studies have reported a rapid rate of DDT to DDD reduction in soil under reducing conditions when a readily available energy source, such as alfalfa, barley straw, or glucose, is present (Aislabie et al., 1997).

Dechlorination of \textit{p,p}'-DDT to 1,1-dichloro-2,2\textsuperscript{2}-bis (4-chlorophenyl)ethylene (DDE), DDD, 1-chloro-2,2-bis (4-chlorophenyl) ethane (DDMU), DDMS, and DDNU,
by *Enterobacter aerogenes*, *P. fluorescens*, *Escherichia coli*, and *Klebsiella pneumoniae* under anaerobic conditions has been reported (Lal and Saxena, 1982).

Among these compounds, DDE has traditionally been considered as a dead-end DDT-metabolite, which is metabolized in co-metabolism with biphenyl by *P. acidivorans* and *Terrabacter* sp. (Hay and Focht, 1998; Aislalbie et al., 1999). Thus, a complex set of environmental conditions (redox potential, pH, co-substrate, pollutant concentration, etc.), is required for DDT mineralization (Bidlam and Manonmanni, 2002; Aislalbie et al., 1997; Nadeau et al., 1994).

Recently, agro-industrial wastes have been used to enhance toxic recalcitrant organopollutant biodegradation (Chávez-Gómez et al., 2003; Molina-Barahona et al., 2005; Pérez-Armendáriz et al., 2004; Aslan and Turkman, 2005). The use of agricultural wastes in bioremediation processes is highly advantageous, as these wastes can provide a nutrient source as well as a support for microorganisms. The reuse of these wastes could also solve problems related to their disposal.

Due to a surplus of coffee on the world market, coffee producers have committed themselves to removing the lowest quality coffee, i.e., “defective” coffee or triage, from the market to stabilize prices. In Mexico, 5% of total coffee exports (about 12,000 tons annually) is withheld from the market (Federal Official Gazette (FOG), 2001). In contrast with other agricultural wastes, green bean coffee is rich in nutrients such as carbohydrates, vitamins, minerals, proteins, and alkaloids (Belitz and Grosch, 1999; Mazzafera, 1999; Rogers et al., 1999; Nunes and Coimbra, 2001; Kilmartin and Hsu, 2003; Oosterveld et al., 2004; Campa et al., 2004; Franca et al., 2005), which would be advantageous to bacterial growth in bioremediation processes.

For these reasons, the goal of this research was to study the role of microniches in the porous surface of the green coffee bean in DDT and endosulfan biodegradation by bacteria associated with the beans. Changes in the surface of the coffee beans during cultivation were investigated by SEM analysis.

2. Materials and methods

2.1. Reagents and chemicals

Technical-grade DDT and endosulfan were supplied by Teckchem and Velsimex Company (Mexico). The compositions of the following pesticides were determined by GC–MS: *p,p*'-DDT (62%), *o,o*'-DDT (30%), DDE (8%), endosulfan I (65%), and endosulfan II (35%).

Green bean coffee (*Coffea arabica* var. Veracruz) was supplied by the Mexican Coffee Council (Consejo Mexicano del Café). Beans were air-dried, ground, and sieved (10–20 mesh). All chemicals used were reagent-grade and solvents were HPLC-grade (Merck).

2.2. Culture medium

The composition of the mineral salts medium (MSM) used was (g l\(^{-1}\)): Na\(_2\)HPO\(_4\), 2.4; KH\(_2\)PO\(_4\), 2.0; NH\(_4\)NO\(_3\), 0.1; MgSO\(_4\)·7H\(_2\)O, 0.01; CaCl\(_2\), 0.01. The pH was adjusted to 6.5 (Radehaus and Schmidt, 1992). The medium was sterilized by autoclaving at 121°C for 20 min.

2.3. Isolation of organochlorine pesticide degrading bacteria from coffee beans

The procedure to isolate bacteria consisted of the addition of 0.1 g of ground coffee beans to a flask containing 50 ml of MSM medium, with 200 mg l\(^{-1}\) of pentachloronitrobenzene (PCNB) as the organochlorine pesticide model and as a fungal inhibitor. Cultures were incubated at 30°C and shaken at 100 rpm for 7 days. Two milliliters were then transferred to a fresh MSM medium containing 200 mg l\(^{-1}\) of PCNB and incubated under the same conditions. Cultures from the fifth transfer were plated on nutrient agar and incubated for 24 h at 30°C. Colonies were isolated on the basis of morphological, culture, and biochemical characteristics using BBL Crystal GP System (Becton, Dickinson and Co., 2005).

Bacterial isolates were plated separately on MSM medium containing 50 ppm DDT, 50 ppm endosulfan, or 200 ppm PCNB. Cultures that were able to grow in all pesticides tested were used for further studies.

2.4. Preparation of bacterial inoculum for biodegradation studies

The strains selected were pre-grown on liquid MSM amended with 2 g l\(^{-1}\) of peptone and incubated at 30°C at 100 rpm for 48 h. The inoculum was then centrifuged (5000 rpm, 20 min). To remove residual nutrients, cells were washed twice by centrifugation (5000 rpm, 20 min) using 15 ml of 0.85% NaCl. Washed cells were resuspended in 0.85% NaCl (Okcete et al., 2002). A standard curve of Abs\(_{600}\) vs. dry biomass l\(^{-1}\) was obtained.

2.5. Effect of carbon source on bacterial growth and pesticide biodegradation

Assays were performed in 125-ml conical flasks containing 50 ml of MSM medium amended with glucose (10 g l\(^{-1}\)), peptone (2 g l\(^{-1}\)), and ground coffee beans (2 g l\(^{-1}\)). Sterilized coffee grains (γ-radiation) were added after autoclaving the MSM medium. Media were spiked with technical DDT or endosulfan (40 μl of acetone/ethanol) to give 50 mg l\(^{-1}\) final concentration (Siddique et al., 2003).

Media were inoculated with 5 × 10\(^{8}\) biomass (Abs\(_{600}\) = 0.6; 0.3 g dry biomass l\(^{-1}\) and incubated in the dark on an orbital shaker (100 rpm) at 30°C for 7 days. The addition of 17 g NaCl to the medium was used to stop bacterial growth. Two experimental sets (for cell growth and degradation tests) were prepared in duplicate.

A biomass (g l\(^{-1}\)) versus time curve was determined for a 5-ml aliquot of culture broth as described above. A control for the determination of pesticide recovery efficiency was carried out under the same culture conditions, but pesticides were added after bacterial inactivation.

The experimental and control samples were extracted with dichloromethane. The extracts were dehydrated by passing them through anhydrous Na\(_2\)SO\(_4\) and concentrated using a rotary evaporator. The residue was re-dissolved in 5 ml of methanol and 2 μl were injected into the GC/MS. The GC/MS consisted of a Varian Saturn 3 with an electron multiplier as detector and an SPI injector. A DB-5 fused silica capillary column (30 m × 0.25 mm) was used. The temperatures in the injector and the transfer line were 250 and 280°C, respectively, and the operating conditions were: flow rate 1 ml min\(^{-1}\), column temperature 140°C for 2 min followed by ramping at 5°C min\(^{-1}\) to 240°C and then maintained at 240°C for 27 min. Ionization was carried out at 70 eV. Quantification of the chromatographic peaks was performed using external standards. Metabolites were identified by comparison with MS spectra library records (NIST/EPA/NIH, 1998).
2.6. Statistical analysis

Data were interpreted through analysis of variance ($p < 0.05$), multiple range analysis (least significant difference), and correlation analysis. Each experiment was performed in duplicate. Treatments were considered significant when $p < 0.05$. Statistical Analysis System Software (SAS, v 6.08 (8), SAS Institute S.A de C.V, Mexico) was used.

3. Results and discussion

Green bean coffee is a material rich in nutrients that promotes the growth of microorganisms (Avallone et al., 2001). The magnitude and diversity of the microbial populations associated with the natural processing of coffee (C. arabica) have previously been assessed, and members of the genera Aeromonas, Pseudomonas, Enterobacter, Serratia, Cellulomonas, Arthobacter, Microbacterium, Dermabacter, and Lactoballis (Silv et al., 2000) have been identified. The fungi that have been isolated include Cladosporium, Fusarium, Penicillium, and Aspergillus (Silv et al., 2000).

Enrichment techniques were used to isolate pesticide-degrading bacteria with PCNB as an inducer; only five bacteria were isolated, identified as: Pseudomonas aeruginosa, Flavimonas oryzihabitans, P. putida, Stenotrophomonas maltophilia, and Morganella morganii. Only F. oryzihabitans and P. aeruginosa were able to grow on plates with DDT or endosulfan as the sole carbon source.

It has been reported that certain pesticides have inhibitory effects on bacterial growth (Nawab et al., 2003). Complete degradation of DDT at concentrations up to 15 ppm in flasks, with shaking, has been achieved, but inhibitory effects were observed at 50 ppm. A carbon source other than the target chemical has been found to influence the degradation rate of organic toxins. It has been reported that the addition of sucrose, glucose, sodium acetate, sodium succinate, and sodium citrate inhibits DDT biodegradation (Bidlam and Manonmani, 2002) and also that the presence of sodium acetate and sodium succinate inhibits endosulfan degradation (Awasthi et al., 2000).

In this research, F. oryzihabitans and P. aeruginosa were able to grow with different carbon sources when supplemented with 50 ppm of DDT or endosulfan (Figs. 1 and 2). In all cases, biomass production was higher than 0.3 g l$^{-1}$ and reached a maximum between 24 and 48 h of incubation at 30 °C and 100 rpm. Bacterial growth of both strains in peptone supplemented with DDT and endosulfan, where
the biomass production was between 0.34 and 0.44 g l\(^{-1}\),
did not show significant differences (\(p > 0.05\)). In contrast,
biomass production by \textit{P. aeruginosa} in medium with green
bean coffee was higher in medium supplemented with
endosulfan (0.53 g l\(^{-1}\)) than DDT (0.36 g l\(^{-1}\)). In addition,
\textit{F. oryzihabitans} and \textit{P. aeruginosa} biomass (0.26–0.6 g l\(^{-1}\)),
grown on glucose with 50 ppm of DDT or 50 ppm of
endosulfan, showed significant differences (\(p < 0.05\)). The
highest biomass production (0.6 g l\(^{-1}\)) was obtained with \textit{F.
oryzihabitans} in medium with glucose and endosulfan at
24 h.

For all media tested, no significant endosulfan degrada-
tion by \textit{F. oryzihabitans} was observed. In contrast,
endosulfan degradation by \textit{P. aeruginosa} was shown at
51\% with coffee bean medium and only 30\% in glucose
and peptone media at 30°C, 100 rpm, and 7 days of
incubation. Detoxification of endosulfan by aerobic
microorganisms often results in the formation of a toxic
endosulfan sulfate (Sutherland et al., 2000). Also, other
metabolites such as endosulfan diol or endosulfan ether
can be produced by microbial metabolism; however,
either of these was identified by GC/MS in the culture
extracts. Removal of DDT was higher than that of
endosulfan, indicating that both bacteria preferentially
degrade aromatic compounds. A DDT concentration of
only 32–37\% remained in the coffee bean culture medium
after 7 days of incubation (Fig. 3). Changes in the DDE
concentration indicate its production during DDT biode-
gradation, as well as its degradation, in the medium with
coffee bean addition. DDMU and 2,2′-bis (\(p\)-chlorophenyl-
)ethanol (DDOH) metabolites were also identified by GC/
MS in the culture broth.

Masse et al. (1989) and Quensen et al. (1998) reported
the conversion under anaerobic conditions of DDE to
DDMU through reductive dechlorination by bacteria and
marine sediments, respectively. In this research, experi-
ments were run with shaking, where aerobic conditions are
expected. However, it is possible that inside the porous
structure of the coffee bean, anaerobic microniches could
be created, promoting the reductive dechlorination of DDE.
to DDMU. Also, the DDOH peak in the chromatogram indicates further degradation of DDMU (Singh et al., 1999). In the medium with coffee beans, caffeine was consumed by *F. oryzihabitans*, according to the GC/MS analysis.

Scanning electron microscopy of the coffee particles showed a regular structure with a smooth external region, rough zones, and open cavities (Fig. 4a). Micrographs at early stages of culture (Figs. 4b and c) revealed colonization primarily in cavities and other regions of green bean coffee sheltered from hydraulic shear forces. After 30 days of growth, the coffee surface was completely colonized by bacteria (Fig. 5b); at this time it was also possible to observe coffee bean biodegradation (Fig. 5a). This colonization pattern is probably the result of the adsorption properties, fluid dynamics, and system geometry, as in granular activated carbon (GAC) systems (Massol-Deya´et al., 1995). However, the coffee particles are a natural support, which allow bacterial growth because of the relatively high amount of carbon and other nutrients (Belitz and Grosch, 1999). Microaerophilic biological niches could be developed inside colonized coffee bean cavities, due to the metabolic activity of microorganisms (Beuninck and Rehm, 1988). This fact may explain the DDD and DDE biodegradation in this system, since their accumulation in the medium was not observed.

The combination of anaerobic and aerobic environments enhances the mineralization of many electrophilic aromatic contaminants such as organochlorine and azo compounds, for which several strategies have been proposed (Field et al., 1995). This is the first report to present evidence on the creation of micro-environmental conditions by way of structural microniches in natural waste, where it is possible for reductive reactions to occur.

4. Conclusions

Bacteria isolated from defective green bean coffee with traditional enrichment techniques using PCNB as an organochlorine pesticide model were able to grow on and to degrade DDT and endosulfan in liquid media. The highest pesticide biodegradation was obtained when coffee bean was added. The presence of certain metabolites such as DDMU and DDOH suggests the formation of anaerobic microniches in the porous structure of the coffee bean. SEM photomicrographs at 3 days incubation showed that colonization occurred mainly inside the coffee bean. Thus, defective green bean coffee can be used as both a nutrient source and a support for organochlorine pesticide degrading bacteria in liquid media.

Acknowledgments

We would like to thank Ma. Esther Sánchez for taking the microphotographs (ENCB-IPN), Dolores Díaz Cer- vantes (CINVESTAV-IPN) for her technical help, and Ing. Elvira García (Consejo Mexicano del Café). This study was supported by Consejo Nacional de Ciencia y Tecnología (Fondo Mixto TLAX-2003-C02-12416).

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