International Biodeterioration & Biodegradation 74 (2012) 36–47

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

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

Lindane biodegradation by the *Fusarium verticillioides* AT-100 strain, isolated from *Agave tequilana* leaves: Kinetic study and identification of metabolites

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ARTICLE INFO

Article history:
Received 21 July 2011
Received in revised form
2 April 2012
Accepted 7 April 2012
Available online 9 August 2012

Keywords:
Lindane
Agave tequilana
Fusarium verticillioides
Aerobic carboxylation

ABSTRACT

A fungal strain able to use lindane as a carbon and energy source under aerobic conditions was isolated from *Agave tequilana* leaves by enrichment techniques. With molecular techniques, it was identified as *Fusarium verticillioides*. The effect of a number of nutritional and environmental factors on the pesticide biodegradation was investigated using a Plackett–Burman design. Lindane biodegradation was higher in the presence of limited amounts of nitrogen and phosphorus. The addition of agave leaves to the culture medium and the use of higher concentrations of lindane, copper, and yeast extract improved the efficiency of the biodegradation process. The analysis of the metabolites using GC–MS identified γ-pentachlorocyclohexene and benzoic acid derivatives. This finding suggests that there is an aerobic carboxylation step, reported for the first time, in the lindane biodegradation pathway of *F. verticillioides*. Adding organic matter such as *Agave tequilana* leaves to the culture medium improved the fungal viability, eliminated the lag growth phase for fungal growth, and increased the pH value of the culture medium. The increased pH helped to overcome the toxicity of the benzoic acid derivatives that were released during lindane degradation.

1. Introduction

Technical lindane is a mixture of several isomers of hexachlorocyclohexane (HCH) that is produced industrially by the photochemical chlorination of benzene under a UV light (Okeke et al., 2002). As the γ-isomer of HCH, commonly known as lindane or γ-HCH, is the only one of these isomers that exhibits effective insecticidal properties (Quintero et al., 2005), it has been used extensively for agricultural applications and in the public health service.

Because of its high toxicity, persistence, mobility in the environment, and bioaccumulation in the food chain, the production and use of lindane is prohibited or severely restricted in many countries (Barriada-Pereira et al., 2005; Mertens et al., 2005). Nevertheless, the problem of lindane residues still exists in these countries, possibly due to the widespread distribution of this isomer, which is caused, in part, by its continued use in some developing countries for economic reasons. Another potential source of lindane pollution is the large unused stockpiles of pure and/or technical lindane in many areas (Okeke et al., 2002).

Many studies have described the adverse effects of lindane, which primarily affects the central nervous system and appears to cause renal and hepatic toxicity (U.S. EPA, 2006). In mammals, acute lindane intoxication may cause respiratory dysfunction, generalized trembling, hyper-salivation, and convulsions, which can lead to death in extreme cases (Pesce et al., 2008). It has been shown that lindane may be an endocrine disrupting chemical (EDC); this is evidenced by the fact that it induces reproductive impairment, including adverse effects on the male and female reproductive system, gestation, and fetal development (Traina et al., 2003).

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http://dx.doi.org/10.1016/j.ibiod.2012.04.020

To protect the environment and the health and wellbeing of the public, several treatment technologies have been proposed to remove lindane and its derivatives from contaminated sites. These methods include combustion, dehydrochlorination (DHC) using thermal- or base-assisted processes, catalytic reduction over metal catalysts (Mertens et al., 2007), and biodegradation by several bacterial and fungal species (Phillips et al., 2005). In contrast to non-biological processes, the microbial biodegradation of lindane has attracted increasing attention due to its increased effectiveness, lower cost, and more environmentally friendly nature (Nagpal and Paknikar, 2006).

Lindane biodegradation by bacterial consortia, pure bacterial cultures, and pure cultures of white-rot fungi has been studied extensively under aerobic and anaerobic conditions (Phillips et al., 2005). However, little information is currently available regarding the use of non-white-rot fungal strains for lindane biodegradation. Recently, three non-white-rot fungi, *Conidiobolus* 03-1-56 (Nagpal et al., 2008), *Fusarium poae*, and *Fusarium solani* (Sagar and Singh, 2011), were shown to aerobically degrade lindane. The use of non-white-rot fungal species has potential advantages in bioremediation applications because they grow quickly without the need for an oxygen-rich environment, and their mycelia provide deeper penetration and a larger surface area for absorption (Nagpal et al., 2008).

Agro-industrial waste products are often used in bioremediation processes because they provide large quantities of raw materials at a low cost, and at the same time, they solve disposal-related problems. These waste products provide nutrients, improve aeration or water retention, reduce toxicity, and stimulate the production of non-specific oxidative extracellular enzymes (Phillips et al., 2005). In some cases, the structure and composition of these waste products tend to create micro-environmental conditions that improve the biodegradation of xenobiotic compounds (Barragán-Huerta et al., 2007). In this context, *Agave tequilana* leaves are a waste product of tequila production processes. In recent years, the tequila market has grown and has gained international recognition, resulting in ever-increasing amounts of waste. These leaves, which represent approximately 46% of the total wet weight of the plant, are left behind in the field after the harvesting of the agave heads that are used for the tequila production process (Iñiguez-Covarrubias et al., 2001). The *A. tequilana* leaves are a waste product that offers considerable potential for bioremediation processes.

In this study, microorganisms that use lindane as a carbon and energy source were isolated from *A. tequilana* leaves, and the microbial strain that exhibited the highest biodegradation potential was identified by employing molecular techniques. Additionally, the metabolites produced during the lindane biodegradation process were determined using GC–MS, and the role of agave leaves (as part of the culture medium) in the removal of the pesticide was analyzed.

2. Materials and methods

2.1. Chemicals

The γ-1,2,3,4,5,6-hexachlorocyclohexane or lindane (analytical-grade, 97% pure) was obtained from Sigma–Aldrich (Mexico). The *A. tequilana* leaves were supplied by agave producers from Guanajuato, Mexico. The leaves were rinsed in water, air-dried, and cut into squares (0.5 × 0.5 cm). In every experiment, with the exception of the isolation step, the *A. tequilana* leaves were sterilized using gamma irradiation (^{60}Co) at a dose of 31.15 kGy. All chemicals were of analytical grade, and the solvents were HPLC-grade (Merck, Mexico).

2.2. Stock solution of lindane

A stock solution of lindane was prepared using 500 mg lindane dissolved in 7 ml acetone and 7 ml methanol.

2.3. Culture media

For the lindane degradation studies, three culture media were used. The first was a minimal salt medium (MSM) that is usually used for pesticide degradation studies (Kanaly et al., 2002; Kim et al., 2004; Madueño et al., 2011). The chemical composition of the medium was as follows: 1.5 g l^{-1} Na_2HPO_4, 2.0 g l^{-1} KH_2PO_4, 1.12 g l^{-1} NH_4NO_3, 0.02 g l^{-1} $Na_2S_2O_3 \cdot 5H_2O$, 0.02 g l^{-1} Tween 20, and the pH was adjusted to 6.5 ± 0.1 (Aitken et al., 1998).

A second culture medium (WA) was prepared using 12 g l^{-1} *A. tequilana* leaves, 0.944 g l^{-1} $(NH_4)_2SO_4$, 0.0346 g l^{-1} Na_2HPO_4, 0.0462 g l^{-1} KH_2PO_4, 0.0735 g l^{-1} Tween 20, 0.24 g l^{-1} $CuSO_4 \cdot 5H_2O$, and 0.5 g l^{-1} zero-valent iron (elemental iron). This medium composition corresponds to the treatment that attained the maximum percentage of lindane degradation in the Plackett–Burman design.

The third culture medium (WOA) was the same as the WA medium but without the addition of the *A. tequilana* leaves. The initial pH of the WA and WOA culture media was 4.5 ± 0.1. All media were sterilized by autoclaving at 121 °C for 15 min.

2.4. Isolation of lindane-degrading microorganisms from *A. tequilana* leaves

The microorganisms were isolated from the *A. tequilana* leaves using batch enrichment culture techniques in MSM. The assays were performed in 125-ml Erlenmeyer flasks containing 30 ml MSM medium, 50 mg l^{-1} lindane, and 3 g *A. tequilana* leaves, and were incubated at 30 ± 2 °C for 5 days. A 1-ml sample was transferred to fresh MSM medium containing 50 mg l^{-1} lindane as the carbon and energy source and was incubated under the same conditions.

The cultures from the fifth transfer were plated in duplicate on nutrient agar and on Rose Bengal chloramphenicol (RBC) agar and were incubated at 30 ± 2 °C for 24 and 48 h, respectively. The colonies were isolated based on their morphological characteristics. The stock cultures of isolated microorganisms (two bacterial strains and one fungal strain designated B1, B2, and AT-100, respectively) were maintained on solid MSM amended with 50 mg l^{-1} lindane. The cultures were stored at 4 °C and were routinely sub-cultured every two months.

2.5. Preparation of inocula for degradation studies

The B1, B2, and AT-100 strains were pre-grown in 50 ml MSM medium amended with 50 mg l^{-1} lindane, and the cultures were incubated at 30 ± 2 °C and 120 rpm for five days. The inocula were centrifuged at 4000 rpm for 15 min and were washed three times with sterilized deionized water to remove the cell debris and residual nutrients. The cell pellets were resuspended in sterilized deionized water.

For the lindane biodegradation studies performed with the pure bacterial cultures, 5% of the B1 or B2 cell suspensions were used to produce an initial concentration of 0.15 g dry biomass l^{-1}. For the AT-100 fungal strain, 5% of the inoculum was added to flasks to produce an initial concentration of 4.5 × 10^6 spores ml^{-1}. The inoculation of flasks with the combination of two microorganisms was performed using 2.5% of the bacterial and or fungal suspensions. For the treatments using three microorganisms, the