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Chlordane biodegradation under aerobic conditions by indigenous *Streptomyces* strains

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ABSTRACT

Four actinobacteria strains isolated from pesticide-contaminated soil were able to grow in the presence of 16.6 mg L^{-1} chlordane, an organochlorine pesticide. The strain that showed best growth after 96 h of incubation in synthetic medium with chlordane as the sole carbon source was identified as *Streptomyces* sp. A5. When *Streptomyces* sp. A5 was cultured in the presence of chlordane, the pesticide was degraded from the culture medium after 24 h of incubation. In soil assays, a reduction of 56% in γ-chlordane was observed after 28 days of incubation. This is the first report about chlordane degradation under aerobic conditions by a *Streptomyces* strain.

1. Introduction

Persistent organic pollutants (POPs) are organic compounds that remain intact in the environment for long periods, favoring their wide geographical distribution. They accumulate in the fatty tissue of living organisms and their toxicity has been proven in humans and wildlife (Hirano et al., 2007).

Chlordane ($C_{10}H_6Cl_8$, 1,2,4,5,6,7,8,8a-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) is an organochlorine pesticide that has been used worldwide, especially during the early 1980s, on farmlands, home lawns and in gardens, and also as a termiticide for house foundations (Dearth and Hites, 1990; Colt et al., 2009). Commercially available technical-grade chlordane (CLD) is a mixture of over 140 different but related compounds; the three most common components are α-(cis-) chlordane, γ-(trans-) chlordane and trans-nonachlor (Dearth and Hites, 1990).

Many of the chlordane components and their metabolites are ubiquitous and persistent and have a tendency for bio-magnification. They have been shown to be toxic in higher animals; they are suspected to be carcinogenic and may have estrogenic activities (Liu et al., 2010; Chia et al., 2010).

Technical chlordane, along with many other organochlorine pesticides, has been phased out from the market under the UNEP (United Nations Environmental Program) and has been included in the group of persistent organic pollutants (UNEP, 2000). Although chlordane ranks among the "dirty dozen" priority pollutants established during the Stockholm Convention, a global treaty to protect human health and the environment which came into force in May 2004 (Wong et al., 2005), this pesticide is still detected in foods and environmental samples around the world (Barber et al., 2006; Hageman et al., 2006; Yago et al., 2006; Bempah and Donkor, 2010; Lal et al., 2010; Jia et al., 2010). There is great concern about the adverse effects on the ecosystem. It is not only important to monitor sites where chlordane was used and where pesticide stockpiles were managed but it is also crucial to assess the risk of chlordane remaining in the environment.

To date, little fundamental research on chlordane biodegradation has been carried out, there exist only very few studies on the degradation pathways or degradation products of this compound (Yamada et al., 2008).

Actinobacteria have a great potential for biodegradation of organic and inorganic toxic compounds (Ravel et al., 1998). There

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exist studies demonstrating that these microorganisms have been able to oxidize and partially dechlorinate and dealkylate organochlorine pesticides such as aldrin, DDT, metolachlor and atrazine (Liu et al., 1990, 1991; Radosevich et al., 1995). In our laboratory, Benimeli et al. (2003, 2006, 2007) and Fuentes et al. (2010, 2011) isolated and selected wild type actinobacteria strains, which were tolerant to lindane and able to remove it from culture media and soil. Cuozzo et al. (2009) detected dechlorinase activity and lindane catabolism products as a result of microbial lindane degradation by *Streptomyces* sp. M7, isolated in Tucumán, Argentina. However, to our knowledge, there exists only one report about an actinobacteria strain (*Nocardiopsis* sp.) isolated from soil that has been able to metabolize pure cis- and trans-chlordane in a pure culture (Beeman and Matsumura, 1981).

The aim of this work was to evaluate if indigenous actinobacteria strains isolated from contaminated environments in Argentina would be able to remove and degrade technical-grade chlordane from culture medium and soil samples under aerobic conditions.

2. Materials and methods

2.1. Chemicals

Technical-grade chlordane was purchased from Sigma–Aldrich, MO, USA. All other chemicals used throughout the study were of analytical grade unless stated otherwise and were purchased from recognized manufacturers.

2.2. Microorganisms and media

Four strains of actinobacteria (A2, A5, A6 and A13) had been previously isolated from a contaminated environment in Santiago del Estero, Argentina, where in 1994 about 30 t of organochlorine pesticides were found. The isolates were selected for their ability to grow in the presence of chlordane and identified as *Streptomyces* sp. (Fuentes et al., 2010).

Streptomyces coelicolor A3 (2) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and *Streptomyces* sp. M7 was isolated at our laboratory from waste-water sediment of a copper filter plant, a pesticide-contaminated site (Benimeli et al., 2003).

In order to prepare *Streptomyces* spore suspensions, strains were cultured on Starch Casein (SC) agar containing in g L^{-1}: starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; agar, 15.0 (Hopwood, 1967). The pH was adjusted to 7.0 prior to sterilization by autoclaving at 121 °C for 20 min. The plates were incubated at 30 °C for seven days.

All *Streptomyces* strains were cultured in liquid Minimal Medium (MM), containing in g L^{-1}: $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7H_2O$, 0.01 (Hopwood, 1967).

The pH was adjusted to 7.0 and then the medium was sterilized by autoclaving at 121 °C for 20 min.

Chlordane was dissolved in dimethyl sulfoxide (DMSO, pesticide grade, Merck, Argentina), and then added aseptically to the autoclaved MM at final concentrations of 1.66, 8.3 and 16.6 mg L^{-1}.

2.3. Batch cultures of Streptomyces strains in MM supplemented with chlordane

Spore suspensions (150 μL) of *Streptomyces* strains were inoculated in Erlenmeyer flasks containing 30 mL of MM supplemented with chlordane (1.66 mg L^{-1}) as carbon source. The cultures were incubated at 30 °C for 72 h under constant agitation at 200 rpm and then centrifuged at 8500×*g* for 10 min at 4 °C. Supernatants were discarded and pellets were used as precultured inocula for next cultures.

Flasks containing 30 mL of MM supplemented with 1.66 mg L^{-1} chlordane as sole carbon source were inoculated with precultured *Streptomyces* strains as described previously at a final concentration of 2 g L^{-1}. The cultures were incubated at 30 °C for 7 days on a rotary shaker (200 rpm) and then centrifuged (8500×*g* for 10 min at 4 °C). 15 mL and 5 mL of supernatants were used for residual lindane determination by GC-μECD and release of chloride ions, respectively. Biomass was estimated by washing the pellets with 25 mM Tris–EDTA buffer (pH 8.0) and drying to constant weight at 105 °C.

Inoculated sterile flasks without chlordane and non-inoculated sterile flasks with chlordane were used as biotic and abiotic controls, respectively. All experiments were carried out in duplicate.

2.4. Gas chromatography analysis

Residual chlordane (α-chlordane and γ-chlordane) was extracted from cell-free supernatants (9000×*g*, 30 min, 4 °C) by solid phase extraction (SPE) using C18 columns (Varian, Lake Forest, USA).

Extracts were then injected into an Agilent 7890A Gas Chromatograph equipped with an HP5 capillary column (30 m × 0.53 mm × 0.35 μm) and ^{63}Ni μECD detector, split/splitless injector (Agilent 7693B) and Agilent ChemStation software. Quantitative sample analysis was performed using appropriate calibration standards (AccuStandard, New Haven, CT, USA). The detection limit for each pesticide was 0.007 μg L^{-1}.

2.5. Colorimetric assay for dechlorination activity

Cell-free supernatants were immediately used for indirect determination of the release of chloride ions using modified procedures by Phillips et al. (2001), in which Phenol Red Sodium Salt was added to 1 mL of supernatant at a ratio of 1:10 as a pH indicator. A color change in the supernatant from red through orange to yellow in the presence of chloride was indicative of dechlorination of chlordane, and, therefore, a positive result. Culture medium without substrate but with pH indicator was used as a blank. Chloride concentrations were determined colorimetrically at 540 nm and compared with standard HCl solutions using a Beckman spectrophotometer. A decrease in optical density at 540 nm (ΔA_{540}) was indicative of microbial dechlorination activity.

2.6. Soil assays: conditioning and inoculation

Surface soil samples (5–15 cm depth) were taken from an experimental site in the northwest of Tucumán, Argentina. Samples were kept at 10–15 °C in the dark and used within the next days. Glass pots were filled with 200 g of soil (20% moisture on dry weight base) and kept at room temperature for 36 h, so that water equilibrated in the soil. One part of the soil samples was sterilized (three successive sterilizations every 24 h, at 100 °C for 1 h each time) and the soil humidity was adjusted with sterile water or a chlordane solution to 20%. A final pesticide concentration of 16.6 mg kg^{-1} (wet weight) of soil was used. The sterility of the soil was checked for each set of pots by enumeration of bacteria (CFU g^{-1}).

For soil inoculation, strains were precultured in Erlenmeyer flasks containing 50 mL of Trypticase Soy Broth (TSB) at 30 °C on a rotary shaker (100 rpm) for approximately 3–4 days. Soil pots were inoculated with precultured strains at a final microbial concentration of 2.0 g kg^{-1} (wet weight) of soil. Soil, inoculum and

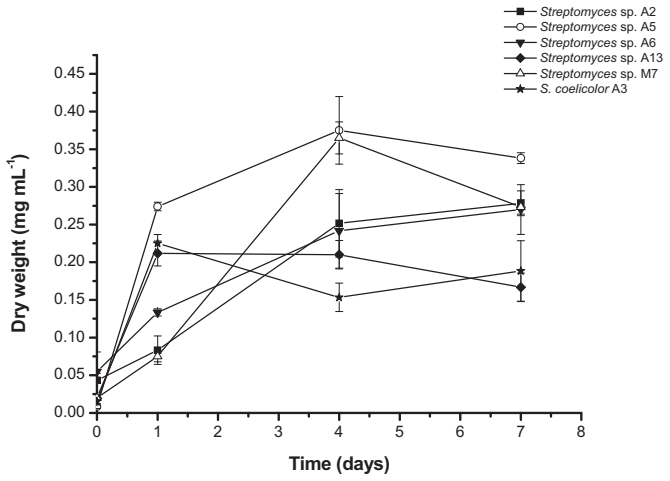

Fig. 1. Time-course of bacterial growth at 30 °C, initial pH 7.0 and at 200 rpm of the six *Streptomyces* strains assayed in the presence of 1.66 mg L^{-1} technical-grade chlordane. Error bars represent standard deviation.

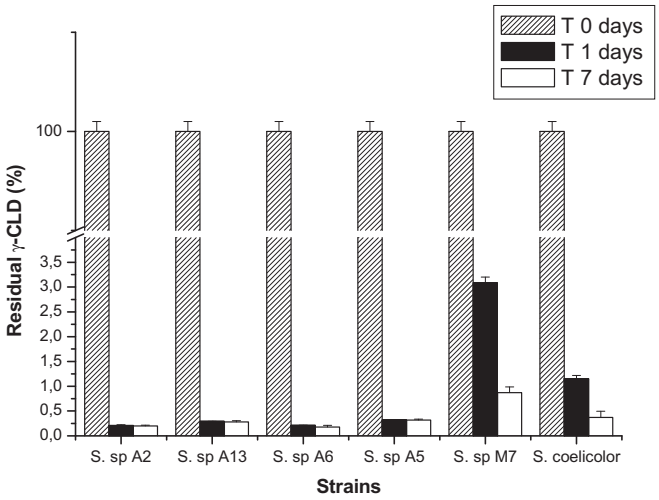

Fig. 2. γ-Chlordane removal in minimal medium by the six *Streptomyces* strains assayed. Initial CLD concentration was 1.66 mg L^{-1}. Error bars represent standard deviation.

technical-grade chlordane were mixed thoroughly to ensure uniform distribution. Inoculated soil samples without chlordane and non-inoculated soil pots were used as controls. Pots were incubated at 30 °C for 4 weeks, and the soil humidity was controlled twice a week. Samples were taken once a week for residual chlordane concentration and microbial growth determination.

3. Results

3.1. Bacterial growth and technical-grade chlordane removal by Streptomyces strains in minimal medium

Fig. 1 shows the growth profiles of the six *Streptomyces* strains cultured in MM in the presence of 1.66 mg L^{-1} technical-grade chlordane (CLD) as the sole carbon source. The microorganisms showed dissimilar behaviors; different biomass values were obtained under the same culture conditions. After one day of incubation, it was observed that the highest biomass values were achieved by *Streptomyces* sp. A5, A13 and *S. coelicolor* A3. However, *Streptomyces* sp. A13 and *S. coelicolor* A3 showed a decrease in the biomass production after 7 days of incubation, which could be due to the presence of toxic metabolites produced during microbial growth.

Analysis of the growth kinetics of the six microorganisms exhibited duplication times (DT) from 6.77 h to 18.78 h, with *Streptomyces* sp. A5 showing the lowest value (Table 1).

Technical-grade CLD added to culture medium is a mixture of over 140 different but related compounds, and the three most common components are α-chlordane (15%), γ-chlordane (15%) and trans-nonachlor (9.7%) (Dearth and Hites, 1990). Residual γ-CLD in the culture media was determined by GC/μECD (Fig. 2). All *Streptomyces* strains showed high average rates of γ-CLD removal, from 97 to 99.8% after 24 h of incubation, and *Streptomyces* sp. A5 was the most efficient strain (99.8%). From then until 7 days of incubation, no significant change in γ-CLD concentration was observed indicating that the metabolism of the strains was highly adaptable and previous adaptation to the pesticide was not necessary.

The six selected *Streptomyces* strains showed release of chloride ions into supernatants. Maximum values were reached within 24 h of incubation, indicating microbial dechlorination activity in MM and the use of CLD as sole carbon source (Fig. 3). The strains with highest Cl^- release were *Streptomyces* sp. M7 (Cuozzo et al., 2009) and *Streptomyces* sp. A6, reaching ΔA_{540} values of 0.12 approximately.

3.2. Selection of the most efficient chlordane-degrading Streptomyces strain

Based on previous results, three criteria were established for selection of the most efficient strain regarding chlordane biodegradation: (i): biomass production and duplication time during microbial growth; (ii): residual chlordane concentration and release of chloride ions (measured as ΔA_{540}) in culture supernatants; (iii): comparison of growth kinetics in the presence of glucose (1 g L^{-1}) or chlordane (1.66 mg L^{-1}).

Streptomyces sp. A5 was selected according to the first two criteria, as it showed the highest growth potential, reaching the highest biomass of the strains assayed after 1 and 7 days of incubation: 0.23 and 0.29 mg mL^{-1}, respectively (Fig. 1). The strain also exhibited the lowest duplication time (6.44 h) in MM supplemented with 1.66 mg L^{-1} technical-grade chlordane (Table 1) and it removed 99.8% of γ-CLD from the medium (Fig. 2) with a high release of chloride ions ($\Delta A_{540} = 0.11$) (Fig. 3).

3.3. Influence of pH, temperature and concentration of chlordane on its degradation by Streptomyces sp. A5

Appropriate conditions for chlordane degradation by *Streptomyces* sp. A5 were examined by assaying pH, temperature and CLD

Table 1
Duplication times (DT) of *Streptomyces* in MM supplemented with 1.66 mg L^{-1} technical-grade chlordane.

Strains	*Streptomyces* sp. A2	*Streptomyces* sp. A5	*Streptomyces* sp. A6	*Streptomyces* sp. A13	*Streptomyces* sp. M7	*S. coelicolor* A3
DT (h)	13.06	6.77	18.78	6.98	12.58	8.71

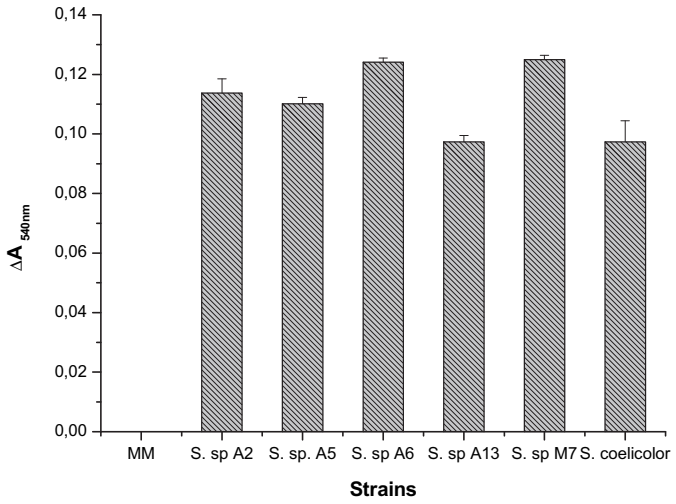

Fig. 3. Chloride ion released from chlordane after 24 h of incubation by the six *Streptomyces* strains cultured in minimal medium. Error bars represent standard deviation.

concentration. At a fixed agitation speed (200 rpm), initial pH of 7.0 and a CLD concentration of 1.66 mg L^{-1}, the biomass production of *Streptomyces* sp. A5 varied according to the temperature (Fig. 4b). Duplication times of the strain at 25, 30 and 35 °C were 7.17, 6.77 and 6.55 h, respectively, and 35 °C was considered the optimum temperature for microbial growth.

However, no considerable difference was observed in chlordane removal at different temperatures; highest CLD removal was observed at 30 and 35 °C with 98.8 and 99.8%, respectively. Highest ΔA_{540} value (0.11) was detected in the culture supernatant after seven days of incubation at 30 °C, indicating a release of chloride ions.

Chlordane degradation by *Streptomyces* sp. A5 was also monitored in MM at different initial pH values (5.0, 7.0 and 9.0) at 200 rpm and at 30 °C (Fig. 4c). Highest microbial growth (0.34 mg L^{-1}) was found at pH 7.0 and lowest duplication time (6.36 h) was observed at pH 9.0. However, no significant difference ($p < 0.05$) was observed for γ-CLD removal at different pH values: at pH 5.0, 7.0 and 9.0 removal was 99.2, 99.8 and 99.5%, respectively. Because of these results, pH 7.0 was used for further studies on chlordane degradation by *Streptomyces* sp. A5 in MM at 200 rpm and 30 °C. It should be emphasized that a pH of 7.0 is generally considered optimal for metabolic activities of the genus

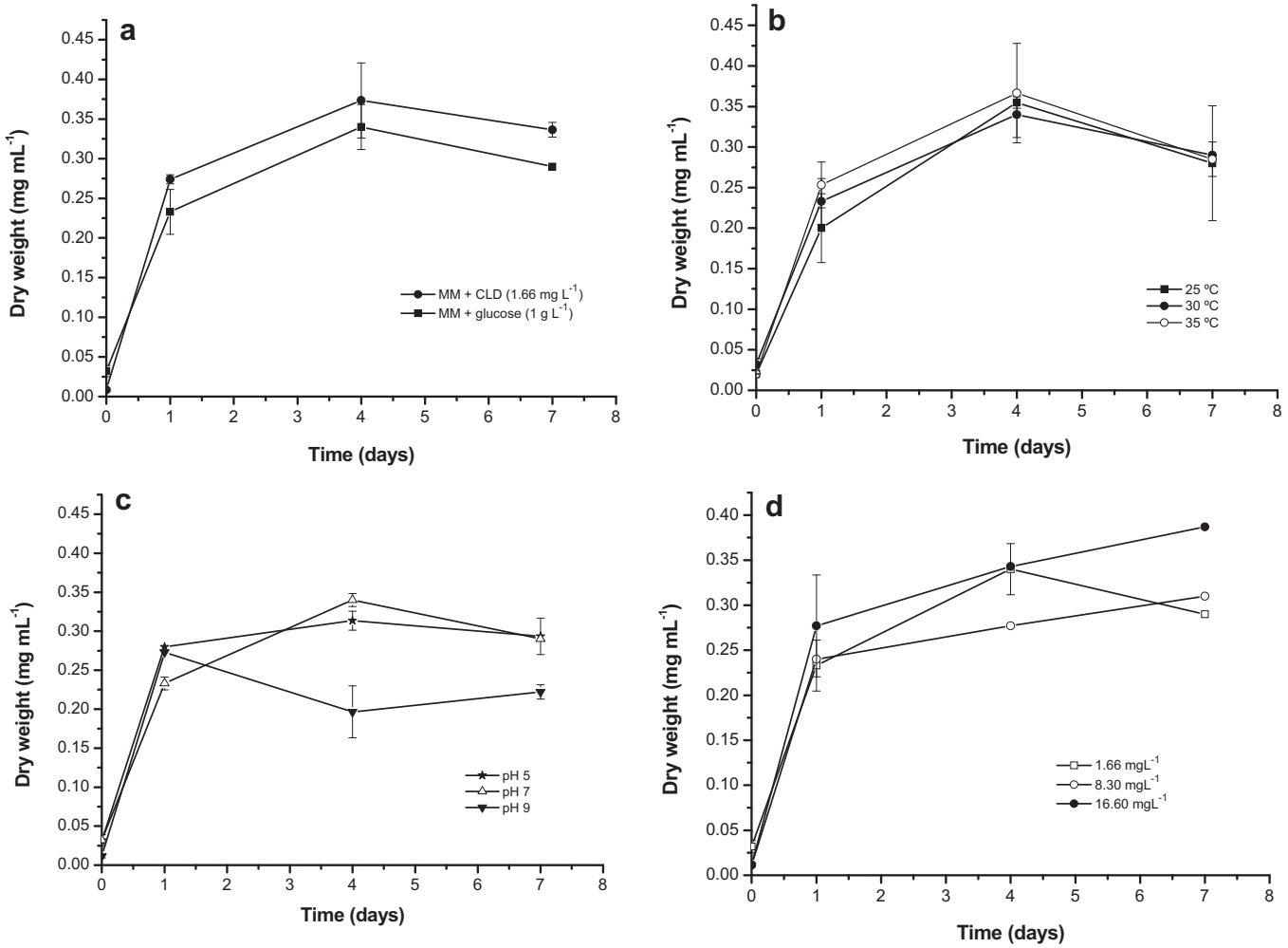

Fig. 4. (a) Growth of *Streptomyces* sp. A5 in the presence of CLD or glucose as sole carbon source. (b) Temperature effect on microbial growth of *Streptomyces* sp. A5 in MM in the presence of 1.66 mg L^{-1} CLD. (c) Effect of pH on microbial growth of *Streptomyces* sp. A5 in MM in the presence of 1.66 mg L^{-1} CLD. (d) Microbial growth of *Streptomyces* sp. A5 in MM supplemented with three different CLD concentrations: 1.66, 8.30 and 16.60 mg L^{-1}. Error bars represent standard deviation.

Streptomyces (Cuozzo et al., 2009). In addition, Robinson et al. (2009) observed that an acidic environment was not favorable for aerobic bacterial dehalogenation.

Chlordane degradation by *Streptomyces* sp. A5 in MM at pH 7.0 and at 30 °C was assayed after addition of different CLD concentrations (Fig. 4d). Highest microbial biomass (0.39 mg L^{-1}) and lowest duplication time (5.25 h) were obtained with increasing CLD concentration, but no considerable difference in pesticide removal was found at the different CLD concentrations. When the strain was grown in MM supplemented with an initial concentration of 1.66 mg L^{-1} technical-grade CLD (corresponding to 0.26 mg L^{-1} γ-CLD) maximum removal was achieved after 1 day of incubation, but at higher initial CLD concentrations (8.30 and 16.60 mg L^{-1}) maximum removal was observed after 4 days of incubation (data not shown).

3.4. Bioremediation of chlordane-contaminated sterile soil by *Streptomyces sp. A5*

Growth of *Streptomyces* sp. A5 in sterile soil samples contaminated with technical-grade chlordane was studied during 4 weeks. Simultaneously, CLD removal by *Streptomyces* sp. A5 was determined. Identical experiments without CLD were carried out as control. Growth profiles of contaminated and non-contaminated soil samples were similar (data not shown), which strengthens the hypothesis that chlordane present in soil is not toxic to *Streptomyces* sp. A5.

A decline in residual chlordane was observed after 2 weeks of incubation whereas the compound did not disappear from the non-inoculated sterile control (Fig. 5).

4. Discussion

The biodegradation of organic chemicals generally requires acclimatization of the microbial population before detectable bioconversion rates occur. During this period they induce new enzymes, undergo genetic changes and exhaust preferential substrates (Yikmis et al., 2008). This acclimatization period usually appears as a lag period, in which little or no biodegradation is observed. However, in our case no lag period was observed for γ-CLD removal by the *Streptomyces* strains assayed (Fig. 1). This is because these microorganisms were isolated from sites contaminated with organochlorine pesticides, the acclimation period had most likely finished and the strains had probably acquired the ability to biodegrade the chemicals (Elcey and Kunhi, 2010). Comparatively, Hirano et al. (2007) observed that anaerobic degradation of γ-CLD and α−CLD in river sediments occurred after an initial lag period of 4 weeks with residual pesticide concentrations of 67.0 and 88.0%, respectively.

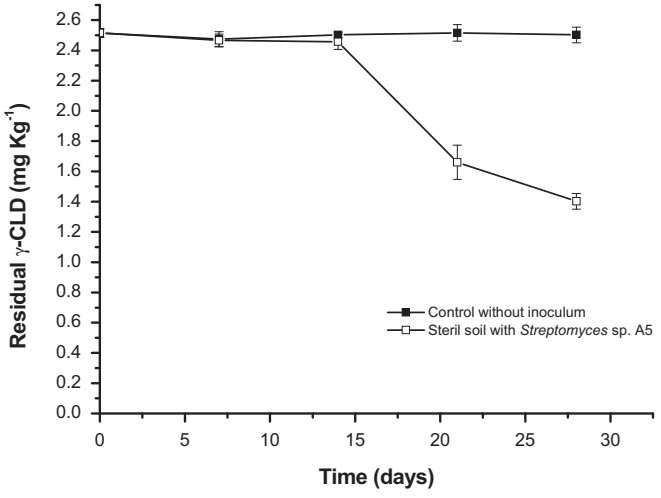

Fig. 5. Removal of γ-chlordane by *Streptomyces* sp. A5 in sterile soil samples after 28 days of incubation. Error bars represent standard deviation.

Aerobic degradation of CLD was reported by Murray et al. (1997), but the authors observed only 9% pesticide removal after 21 days of incubation using indigenous bacteria from a banana farm.

It should emphasized that the *Streptomyces* strains assayed in this study showed removal percentages that are similar or higher compared to those reported in other studies, and pesticide depletion from the medium was achieved in a shorter incubation period (less than 24 h) than those cited in the literature (Hirano et al., 2007; Baczynski et al., 2010).

It is well known, that the elimination of halogens from halogenated xenobiotics is a key step in their degradation because the carbon–halogen bond is relatively stable (Fetzner and Lingens, 1994). Nagata et al. (2007) determined two different types of dehalogenases, which are involved in the early steps of γ-HCH degradation by *Sphingobium japonicum* UT26: dehydrochlorinase and halidohydrolase. Because dehalogenation plays a central role in biodegradation of many chlorinated compounds, the current study examined the release of chloride ions to assess chlordane degradation by six actinobacteria strains. Manickam et al. (2008) used specific and rapid dechlorinase activity assays to screen bacteria from contaminated soils for HCH-degrading activity. Benimeli et al. (2006) reported on the release of chloride ions from lindane by a streptomycete strain. Cuozzo et al. (2009) demonstrated that synthesis of dechlorinase in *Streptomyces* sp. M7 was induced when the microorganism was grown in the presence of lindane as sole carbon source.

Also, *Streptomyces* sp. A5 was able to grow in the presence of CLD and microbial growth, pesticide removal and degradation were higher when compared with other strains. No significant differences were observed in the growth kinetics of *Streptomyces* sp. A5 in the presence of the two alternative carbon sources, glucose and CLD (Fig. 4a), indicating that the pesticide was not toxic to the cells at the assayed concentration (1.66 mg mL^{-1}) and probably the strain did not accumulate toxic metabolites that could produce growth inhibitory effects. Therefore, this strain could be highly promising for future experiments.

In different degradation conditions, it was observed that the optimum culture temperature was 30 °C (Fig. 4b), which is comparable with previous results obtained by Benimeli et al. (2007), who studied lindane degradation by *Streptomyces* sp. M7 in medium with soil extract. Kennedy et al. (1990) observed 23% removal of γ-CLD under aerobic conditions in liquid medium at 39 °C by a fungus, *Phanerochaete chrysosporium*, after 60 days of incubation. Comparatively, *Streptomyces* sp. A5 showed a greater ability to remove γ-CLD in a much shorter incubation time. As a result, the pesticide concentrations assayed were not toxic enough to inhibit microbial growth and pesticide degradation (Fig. 4d).

In contrast, Arisoy (1998) found that concentrations of heptachlor higher than 50 μM produced a toxic effect on the growth of the fungus *P. chrysosporium*. Wang et al. (2006) observed that degradation of hexazinone by a mixed bacterial culture (*Pseudomonas* sp. and *Enterobacter cloacap*) clearly decreased concomitantly with increasing initial concentration of the herbicide. A hexazinone concentration of 150–200 mg L^{-1} was found to be toxic enough to totally inhibit its degradation.

It was found that the maximum pesticide removal of γ-CLD (44.0%) was observed after 4 weeks of incubation (Fig. 5). Similar

results were found by Benimeli et al. (2008), who demonstrated a depletion of 56% of residual lindane in sterile soil spiked with lindane (100 μg kg^{-1}soil) and inoculated with *Streptomyces* sp. M7. Additional studies are necessary to optimize bioremediation under natural environmental conditions by stimulating bacterial growth and chlordane biodegradation ability. However, survival and activity of the inoculated strain are not always guaranteed, and present another issue to be addressed in bioremediation studies.

5. Conclusions

Five actinobacteria strains isolated from organochlorine pesticide-contaminated soils showed chlordane-degrading ability in MM. Growth of *Streptomyces* sp. A5 in chlordane-supplemented MM and the ability of this microorganism to remove CLD and release chloride ions from the medium was higher than that of the other strains assayed. *Streptomyces* sp. A5 had the shortest duplication time and the highest microbial growth rate. Optimum conditions for the strain to degrade chlordane were pH 7.0, 30 °C and agitation at 200 rpm. The microorganism probably uses the pesticide as carbon and energy source, because an increase in CLD concentration of 10 times still showed similar microbial growth. Besides, the concentrations assayed were not toxic to the bacterium, because it was able to almost completely remove CLD.

A reduction of 56% in γ-chlordane was observed in soil after 28 days, which is much faster than that found in previous studies. Our results also provide the first evidence of aerobic biodegradation of CLD by regional actinobacterias strains. Although *Streptomyces* sp. A5 seems to be a potential agent for bioremediation of environments contaminated with chlordane, many studies have shown that results from laboratory studies can greatly differ from results in field studies due to number of variables. Therefore, mechanisms of uptake, accumulation and biodegradation of chlordane by *Streptomyces* are presently being studied at our laboratory.

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