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Study of the removal of a pesticides mixture by a *Streptomyces* strain and their effect on the cytotoxicity of treated systems

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ABSTRACT

Currently, organochlorine pesticides (OPs) are restricted or banned from agricultural and public health areas. However, many of these compounds have been found in high concentrations in contaminated sites, and therefore continue to pose environmental and health concerns. Thus, it is imperative to identify efficient microorganisms to remove them and to develop methods to remediate environments contaminated with OPs. In this regard, the aim of this work was to evaluate the individual and simultaneous removal of three OPs; specifically lindane (LIN), γ-chlordane (CLD) and methoxychlor (MTX) (2 mg L^{-1} of each pesticide alone or as constituent of a mixture), from liquid contaminated systems, employing a native and non-GMO *Streptomyces* strain. Toxicity test of the treated systems against the cell line Caco-2, and the detection in the genome of the used microorganism of sequences related with the pesticides catabolism are reported. The results demonstrated that, *Streptomyces* sp. A5 was able to remove individually 57.4%, 100.0% and 6.5% of LIN, CLD and MTX, respectively. From the pesticide mixture, LIN and CLD were efficiently removed on 62.2% and 68.6%, respectively by the studied strain. On the other hand, we demonstrated that the liquid systems contaminated with the OPs mixture and bioremediated with *Streptomyces* sp. A5 showed lower cytotoxicity than their respective controls without inoculate. Moreover, molecular studies revealed the presence in *Streptomyces* sp. A5 of putative *lin* genes with potential dehydrochlorinase, haloalkane dehalogenase and NAD-dependent dehydrogenase activities. Therefore, the potential of *Streptomyces* sp. A5 to efficiently remediate OPs alone or in mixtures from liquid contaminated system, was demonstrated.

1. Introduction

Industrial worldwide development has led to an increase in environmental pollution, resulting in anthropogenic activities. These effects produce an alteration of environmental equilibrium [1]. Pesticide production is one of the most important economic activities of the world. Since 1950, the use of pesticides for pest control has increased dramatically and has resulted in an increased productivity of various crops. However, deleterious effects including the detection of pesticide residues in river ecosystems as sediment and aquatic biota [2], as well as in groundwater courses, which generally flow into rivers [3], has also been detected. Additionally, it is necessary to consider that pesticides are usually applied simultaneously or one after another, and a combined contamination of these compound residues could be detected in the environment [4]. The presence of these toxic compounds is not only a result of their use but also improper disposal. In this sense, many officially recognized pesticide contaminated sites have been reported in Brazil, Argentina, Chile [5,6], Poland [7], Spain [8,9], China [10], Canada, United States [11] and India [12], amongst other countries. However, these reports may underestimate the actual situation, due to the presence of illegal, non-reported, contaminated storage sites. Of particular concern is the past use of pesticides, such as organochlorines

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(OPs), due their high toxicity and persistence [13]. Among them, lindane, chlordane and methoxychlor were previously used world-wide for crop protection against insects and other pests [14–16]. Thus, these kinds of xenobiotic compounds may be simultaneously present in the environment, constituting an important problem due their high toxicity and persistence [13]. Despite the fact that most countries have restricted or banned their use, currently they are detected in sites with and without a history of pesticide application, in environmental samples and diverse organisms [17–19].

Removing pesticide residues by microbial degradation plays an important role, and it has received a great deal of attention due to its low cost, high efficiency and environmentally friendly characteristics [20]. Different microorganisms, including actinobacteria, have the ability to degrade pesticides, but only a few reports focus on the biodegradation pathways and intermediate compounds detection of hexachlorocyclohexane isomers, gamma-chlordane and methoxychlor [15,21–24]. The complete mineralization of pesticides is difficult to achieve, and this is associated with their persistence, which can be due to the presence of a diversity of structural groups that require several catabolic enzymes for degradation. One promising approach may be the use of unspecific oxidases, followed by hydrolases or ring cleavage enzymes, able to generate products that could be mineralized by catabolic pathways present in a single bacterium [25]. It is this possibility that motivated our search for an actinobacteria with the putative genes and capacity to degrade OPs involved in the pesticide metabolism. Based on the exposure, the aim of this work was the study of the ability of *Streptomyces* sp. A5 to remove organochlorine pesticides, specifically a mixture of lindane (LIN), gamma-chlordane (CLD) and methoxychlor (MTX) from liquid systems, and to evaluate the toxicity of these treated systems against the cell line Caco-2. Also, the genomic analysis of this actinobacteria strain was realized to identify sequences related with genes involved in the encoding of enzymes with pesticide catabolic activities.

2. Materials and methods

2.1. Chemicals

The standards of pesticides used in this study were lindane (LIN) (99% purity), methoxychlor (MTX) (99.8% purity) and γ-chlordane (CLD) (98.4% pure), which were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade.

2.2. Microorganism and culture media

Streptomyces sp. A5 previously isolated from OPs-contaminated soil [26], was used in this study. Minimal medium (MM) formulated with ($g\,L^{-1}$): L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$, 0.01; glucose, 1.0, was used for pesticide removal assays. The pH was adjusted to 7.0 prior to sterilization (Hopwood, 1967). Tryptone Soy Broth (TSB) constituted by ($g\,L^{-1}$): tryptone, 17.0; soy peptone, 3.0; NaCl, 5.0; K_2HPO_4, 2.5; glucose, 2.5, was used for the inoculum formulation. The pH was adjusted to 7.3 prior to sterilization. All media were sterilized by autoclaving at 121 °C for 15 min.

2.3. Biodegradation of pesticides in liquid medium

In order to obtain an appropriate inoculum of *Streptomyces* sp. A5, the strain was pre-cultured in glass flasks containing 30 mL of TSB at 30 °C on a rotary shaker (200 rpm) for 72 h. Then, the cultures were centrifuged at 8500 × *g*, for 10 min at 4 °C, the supernatants were removed and bacterial pellets were resuspended in sterile distilled water and used as inoculum. The inoculum was added ($2.0\,g\,L^{-1}$) into glass flasks containing 30 mL of MM, individually or simultaneously contaminated with $2.0\,mg\,L^{-1}$ of each pesticide (LIN, CLD or MTX). Triplicate cultures were incubated at 30 °C under constant agitation (200 rpm) for 144 h. Both biotic (MM inoculated without pesticide) and abiotic (MM contaminated without inoculum) controls were included. Samples were taken for determinations of microbial growth (dry weigh), residual pesticide concentrations by electron microcapture detector-gas chromatography (μECD-GC), metabolite identification by gas chromatography coupled to mass spectrometry (GC-MS) and cytotoxicity test, as appropriate.

2.4. Analytical procedures

Microbial growth: this parameter was measured by dry weight technique. First the cultures were centrifuged, the precipitate biomass was washed with 25 mM Tris–EDTA buffer (pH 8), and dried to constant weight at 105 °C.

Residual OPs concentrations: from cell free supernatants of liquid cultures the residual pesticide concentrations were determined. First, a solid phase extraction (SPE) was realized using a C18 column (Agilent Technologies Inc., USA). The extracts obtained were injected in a gas chromatograph (Agilent Technologies 7890A) equipped with an HP5 capillary column (30 m × 0.53 mm × 0.35 m), a ^{63}Ni-μECD detector, a split/splitless injector (Agilent 7693B), and Agilent ChemStation software. The chromatographic conditions were set as described by Saez et al. [27]. Quantitative analyses were performed using appropriate calibration standards (AccuStandard, New Haven, CT, USA).

Metabolites identification: in order to identify intermediate metabolites of LIN, CLD and MTX, extractions were performed from supernatant cultures and cell-free extracts using SPE with C18 columns. The extracts obtained were analyzed in a gas chromatograph (Agilent Technologies 6890) equipped with a Network Mass Selective Detector (Agilent 5973) (GC-MS), a HP-5MS capillary column (30 m × 0.25 mm × 0.25 m) and a split/splitless injector (Agilent 6850). The chromatographic conditions were as follows: inlet temperature 280 °C, carrier gas (helium) with constant flow rate at $1.0\,mL\,min^{-1}$; oven temperature was programmed to increase from 50 to 125 °C at $3\,°C\,min^{-1}$, and then at $17.5\,°C\,min^{-1}$ to 300 °C, remaining at this temperature for 10 min. The mass detector was used in single ion monitoring mode. Tentative identification of metabolites was done by comparing their MS spectra with those in the NIST mass spectra library.

2.5. Cytotoxicity test

The human colon cancer cell line Caco-2, was grown in High Glucose Dulbecco's Modified Eagle's Minimum Medium (DMEM, Gibco, Life Technologies, Grand Island, NY, USA), containing 10% foetal bovine serum (Natocor, Córdoba, Argentina), $100\,\mu g\,mL^{-1}$ penicillin and $100\,\mu g\,mL^{-1}$ streptomycin (Gibco), at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were grown to 80–90% of confluence in 25-cm^2 vented tissue culture flasks, and then transferred to 96-well plates. The inhibition of Caco-2 cell viability by the samples (samples from assays added with individual pesticides or with the pesticides mixture, treated or untreated with *Streptomyces* sp. A5), was determined by two different methodologies: trypan blue exclusion and MTT [(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide] assay.

The trypan blue exclusion method is based on the principle that dead cells do not possess intact cell membranes and cannot exclude certain dyes, such as trypan blue. Cells (1×10^5 cells/well) were seeded on 24-well tissue culture plate on a final volume of 1000 μL/well (900 μL of DMEM and 100 μL of the sample to be evaluated), for 24 h. Following treatment, non-attached and attached (trypsinized) cells were collected and 0.4% trypan blue dye and cell suspension were combined in equal parts. Cells were counted using a Neubauer chamber. Each assay was performed in triplicate, and the results expressed as the average percentage of dead cells ± standard deviation.

The MTT assay is based on the conversion of water soluble MTT to an insoluble purple formazan, through mitochondrial dehydrogenases

in viable cells. Cells (1×10^4 cells/well) were seeded on 96-well tissue culture plates for 24 h to allow attachment. The different samples under study were added at a dilution 1/10 in the culture medium (10 µL of the sample for a final volume of 100 µL of medium), and incubated for 24 h. MTT reagent (2.5 mg mL^{-1}) was added (10 µL) to each well and then incubated at 37 °C for 3 h. Medium was removed and replaced with 100 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals (with purple colour). Plates were shaken for 15 min, and read in a microplate reader at 570 nm. Data were expressed as a percentage absorbance of that of control cells incubated with DMEM.

2.6. Searching of genes involved in organochlorine pesticides degradation

Genome sequencing of *Streptomyces* sp. A5 was made using the 600 Cycles V3 Reagent kit (Illumina) in MiSeq (Illumina). The RAST (Rapid Annotation using Subsystem Technology) platform and the KEGG (Kyoto Encyclopedia of Genes and Genomes) database were used for the annotation and catabolic genes search. The alignments of the protein sequences were performed using the BLOSUM62 identity matrix.

2.7. Plasmid detection

Streptomyces sp. A5 was grown, mycelia collected, plugs prepared, and pulsed field gel electrophoresis (PFGE) performed as described by Ravel et al. [28]. PFGE gels were stained with ethidium bromide prior to photography with 302-nm UV light illumination. The gels were digitized with a FluorImager 573 (Molecular Dynamics, Sunnyvale, California).

2.8. Statistical analyses

All assays were conducted in triplicate and the results averaged. One-way analysis of variance (ANOVA) was used to test for significant differences between treatments in liquid systems. When significant differences were found, Tukey post-test was used to separate the effects among treatments. Tests were considered significantly different at $P < 0.05$. These statistical analyses were performed using professional versions of Infostat and Statistica 6.0 software.

3. Results and discussion

3.1. Biodegradation of pesticides in liquid medium

3.1.1. Growth of Streptomyces sp. A5, in presence of LIN, CLD and MTX

The growth of *Streptomyces* sp. A5 was observed when it was inoculated in both MM individually supplemented with LIN, MTX or CLD, or in MM simultaneously contaminated with the three pesticides (Fig. 1). These findings indicate that OPs are not toxic for *Streptomyces* sp. A5 at the assayed concentration. No lag growth phase was detected and a marked increase in biomass during the first 48 h of incubation was observed (Fig. 1). The growth without an initial period of adaptation was previously described for other microorganisms including actinobacteria belonging to the genus *Arthrobacter*, which were able to grow in the presence of α, β and γ-HCH without a lag phase [29]. These findings are consistent with the behaviour previously observed for *Streptomyces* sp. A5, isolated by Fuentes et al. [26] from a site contaminated with different OPs (including LIN, CLD and MTX); whereby it may have adapted to the presence of these pesticides, with the ability to tolerate and degrade them, without showing a lag phase. Additionally, it is important to highlight that the microbial growth of *Streptomyces* sp. A5 did not show a stationary phase in presence of the pesticide mixture, in which it reached the highest biomass values at the end of the assay. These observations could be due to the ability of this microorganism to use the pesticides for growth. In this sense, Fuentes et al. [30] demonstrated that a mixture of LIN, CLD and MTX was not toxic for an actinobacteria consortium, and that the pesticides were used to sustain microbial growth since another carbon source was not added to MM.

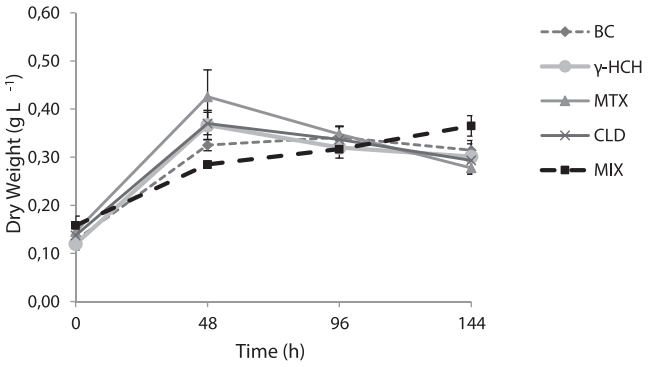

Fig. 1. *Streptomyces* sp. A5 growth in minimal medium supplemented with lindane (LIN), methoxychlor (MTX), chlordane (CLD), a mixture of these pesticides (MIX), and minimal medium uncontaminated (Biotic Control: BC).

3.2. Ability of Streptomyces sp. A5 to remove pesticides

In the test in which LIN was added as the only contaminant, a significant reduction of the pesticide concentration ($P < 0.05$) by *Streptomyces* sp. A5 was detected, compared to the corresponding uninoculated control, at the final of the incubation time. The concentration of LIN at 48 h was 0.97 ± 0.16 mg L^{-1}, corresponding to a 34.5% removal of the pesticide, and 0.63 ± 0.01 mg L^{-1} at 144 h of incubation, corresponding to a 57.4% removal at the end of the assay (Fig. 2a). In a previous study, different strains including *Streptomyces* sp. A5 were evaluated, in MM contaminated with LIN (1.66 mg L^{-1}) as the only carbon source [31], and a removal of 30% of the pesticide was obtained

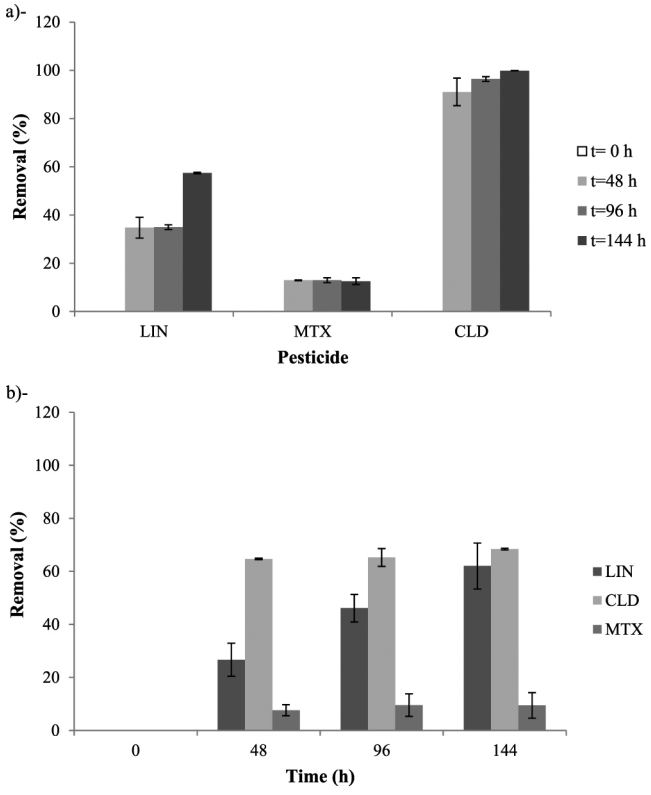

Fig. 2. (a) Lindane (LIN), methoxychlor (MTX) and chlordane (CLD) removal (%) by *Streptomyces* sp. A5, inoculated in minimal medium individually contaminated with each pesticide. (b) Removal of the mixture of the three pesticides (LIN, MTX and CLD) by *Streptomyces* sp. A5.

after 4 incubation days (96 h). In the present work similar results were obtained at the same incubation time; however, a greater LIN removal (57.43%) was detected at the end of the assay (144 h). This increase in the removal obtained could be due to the inoculation of *Streptomyces* sp. A5 into MM supplemented with 1 g L^{-1} of glucose and contaminated with a higher pesticide concentration (2 mg L^{-1}), or probably by the major incubation time assayed. In relation to the glucose added, it is known that this carbon source could exert a favourable effect on the degradation of the pesticide. In this sense, Benimeli et al. [32] demonstrated the feasibility of LIN removal, through the inoculation of *Streptomyces* sp. M7 into a MM amended with low glucose concentration, and contaminated with LIN. These findings demonstrate the ability of *Streptomyces* strains to remove LIN both in the presence and in the absence of glucose. In this study, in the abiotic control (MM supplemented with pesticide but uninoculated) the residual LIN concentration remained constant throughout the experiment, indicating that the pesticide removal is associated with the microbial activity and not to physical–chemical transformations.

When analyzing the MM contaminated with MTX and inoculated with *Streptomyces* sp. A5, a reduction of the pesticide concentration was detected at the end of the assay. After 144 h of incubation MTX concentration was 1.75 ± 0.03 mg L^{-1} in the inoculated samples, corresponding to a 12.5% of pesticide removal. In this case, a slight decrease in the MTX concentration (1.88 ± 0.02 mg L^{-1}), equivalent to a 6.0% of abiotic removal, was detected in the control (Fig. 2a). As a result, only 6.5% of the MTX removed could be attributed to the biotic degradation. The abiotic removal could be due to physical–chemical transformations of the pesticide by processes such as photolysis, hydrolysis, adsorption, oxidation or reduction [33]. Similar results have been reported by other authors; for example, Sánchez Díaz Granados et al. [34] found degradation of aldrin in a control without inoculate, and attributed this phenomenon to possible abiotic effects such as photochemical processes and loss by volatilization. In respect to MTX, Bourguignon et al. [35] evaluated their removal by different streptomycetes (*Streptomyces* sp. A3, A6, A12 and A14) in MM contaminated with this pesticide as the sole carbon source (without glucose supplementation), and removal percentages ranging from 97% to 100% were detected. Similarly, Fuentes et al. [26] detected almost 100% of MTX removal by using different streptomycetes strains, including *Streptomyces* sp. A5, in MM without the addition of glucose. Based on these results, it could be inferred that the presence of glucose adversely affects the removal of MTX in particular.

Moreover, a total removal of the CLD was detected in MM contaminated and inoculated with *Streptomyces* sp. A5 at the end of the incubation time (144 h). In this case there was a marked decrease in the pesticide concentration during the first 48 h of incubation to 0.07 ± 0.00 mg L^{-1}, a value corresponding to 96.5% removal, and a removal of 100% was reached at the end of the test (Fig. 2a). No abiotic removal was detected in the corresponding controls. In previous work referring to the anaerobic biodegradation of γ-CLD (trans), α-CLD (cis) and hexachlorobenzenes from river sediments, Hirano et al. [36] found degradation percentages that ranged between 0% and 33% for γ-CLD. In studies on aerobic degradation of CLD after 20 weeks of incubation at 30 °C in the dark, Murray et al. [37] detected only 9% removal of the pesticide after 21 days of incubation when using bacteria native to a banana farm. Meanwhile, Cuozzo et al. [38] and Fuentes et al. [39] detected CLD aerobic removals with values corresponding to 99.8% and 91.3%, respectively, by *Streptomyces* sp. A5. These results show the ability of *Streptomyces* sp. A5 to remove CLD effectively.

Finally, taking into account the high toxicity and persistence of the OPs and the fact that these pesticides could be simultaneously present in the environment constituting mixtures that contaminate soils and sediments [13], the capacity of *Streptomyces* sp. A5 to simultaneously remove the three OPs under study was evaluated. Two of the three constituents of the mixture were removed by this *Streptomyces* strain (Fig. 2b). The CLD removal was the most effective, with a marked decrease in its concentration during the first 48 h of incubation and with a residual concentration of 0.72 ± 0.01 mg L^{-1} corresponding to 64.7% of removal. This percentage remained practically constant until the end of the assay (144 h) when the concentration was 0.64 ± 0.01 mg L^{-1}, corresponding to 68.6% of removal (Fig. 2b). In the case of LIN, there was a progressive decrease of its residual concentration over the incubation time, reaching a LIN concentration of 0.82 ± 0.18 mg L^{-1}, corresponding to a 62.2% pesticide removal at 144 h. No MTX removal was detected from the mixture in MM inoculated with *Streptomyces* sp. A5, neither in the abiotic controls (Fig. 2b).

An increase in the removal of LIN from the mixture was detected in comparison to removal in the individually contaminated assay. It is known that in a mixture of organic pollutants, the presence of more than one carbon source can increase their degradation [40]. With regard to this, Fuentes et al. [26] showed the ability of different actinobacteria, including *Streptomyces* sp. A5, to grow in MM with LIN, CLD or MTX without traditional carbon sources, thus these pesticides could act as carbon sources for *Streptomyces* sp. A5. In the present work, their simultaneous presence in the culture medium may increase their degradations. In contrast to the observed for LIN, for the CLD and MTX cases, the removal observed from the mixture was less than that detected from the assays tested individually for each pesticide. Similarly, Rama Krishna and Philip [41] found that the degradation efficiency of a mixture of carbofuran, LIN and methyl parathion in submerged soil systems was less than that detected in individually contaminated systems.

The obtained results have shown the ability of *Streptomyces* sp. A5 to remove different OPs from individual or simultaneously contaminated systems.

3.3. Intermediate metabolites detection in the pesticides biodegradation assays

It is important to consider that in order to facilitate the study of the catabolism of a mixture of compounds by a particular microorganism, the evaluation of their ability to catabolize the individual compounds, and the determination of the resultant intermediates is important. In this way it could have an antecedent on the behaviour of the microorganism with each particular compound and a subsequent comparison when treated with the mixture. Therefore, initially samples of supernatants and cell-free extracts belonging to different individually contaminated assays were evaluated at 144 h of incubation by GC-MS to determine the presence of intermediate metabolites. The supernatant of the culture grown in the LIN presence showed two peaks, one of which was detected at a retention time (Rt) of 30.82 min, and was associated to a potential chlorinated compound (Table 1). It is important to note that this peak was not identified as a specific chlorinated metabolite by comparison to those reported in the NIST mass spectrum library. However, based on the charge/mass ratio and the relative intensity of the signals in the mass spectrum, typical of chlorinated compounds, this peak was related to a chlorinated compound. The second peak was detected at Rt of 31.12 min and corresponded to LIN with an identity of 99% (Table 1). These results confirm the ability of *Streptomyces* sp. A5 to degrade LIN, and metabolize this pesticide producing a chlorinated intermediate compound. In the same way, Cuozzo et al. [21] previously demonstrated the ability of a *Streptomyces* strain (*Streptomyces* sp. M7) to degrade LIN on plates inoculated with the microorganism and sprayed with this pesticide. The formation of a clear halo around the colonies was observed, and this was indicative of the ability of the microorganism to degrade the toxicant. This was corroborated by the detection of different metabolic intermediates through GC-MS such as γ-pentachlorocyclohexene and 1,3,4,6-tetrachloro-1,4-cyclohexadiene. In other work, Manickam et al. [42] studied the aerobic degradation of the γ-HCH isomer (LIN) by *Sphingomonas* sp. NM05, and determined the presence of 1,2,4-trichlorobenzene as metabolite. Similarly, Datta et al.

Table 1
Metabolites identified during the biodegradation of lindane, gamma chlordane and methoxychlor by *Streptomyces* sp. A5, using a GC–MS.

Carbon source	Suggested compound[a]	Retention time (min)	Identity (%)	Monitored ion in SIM mode
Lindane				
Culture supernatant	Chlorinated compound	30.82	–	N/D
	Lindane	31.12	99	181
Cell-free extract	N/D	N/D	N/D	N/D
Methoxychlor				
Culture supernatant	N/D	N/D	N/D	N/D
Cell-free extract	1,1-Dichloro-2,2-bis(4-methoxyphenyl) ethylene	34.36	96	310, 308 (bp, mp), 273, 238, 223, 195, 166, 152, 119
	1,1-Dichloro-2,2-bis(4-methoxyphenyl) ethane	34.66	90	312, 310 (mp), 275, 240, 227 (bp), 212, 169, 153, 114
	Methoxychlor (1,1,1-trichloro-2,2-bis (4-methoxyphenyl) ethane)	35.12	98	346, 344 (mp), 311, 309, 276, 274, 228, 227 (bp)
γ-Chlordane				
Culture supernatant	N/D	N/D	N/D	N/D
Cell-free extract	γ-Chlordane	33.30	99	N/D

Nomenclature of compounds is according to IUPAC (International Union of Pure and Applied Chemistry). bp, base peak; mp, molecular ion peak; SIM, selected ion monitoring.

[a] Identification is based on mass spectrum compared with the WILEY7 NIST library.

[43] described the degradation of γ-HCH by the actinobacteria *Arthrobacter citreus* BI-100, and identified metabolites such as pentachlorocyclohexane, tetrachlorocyclohexane, trichlorocyclohexadiene, 2-chlorophenol, phenol and catechol. In the present study, a single metabolite was detected. Nevertheless, the LIN removal was evident from the low percentage of residual LIN detected by GC-μECD from culture supernatants of *Streptomyces* sp. A5, at 144 h of incubation. It is important to highlight that the removal process may involve not only biodegradation, but also a combination of mechanisms such as adsorption, absorption and desorption [44]. On the other hand, it is important to note the absence of phenolic compounds in the supernatants of the culture of *Streptomyces* sp. A5. This result was also observed when α- and β-HCH degradation by *Streptomyces* sp. M7 was studied [24]. However, other authors reported phenolic compounds as products of aerobic degradation of LIN by using other microorganisms such as *Arthrobacter citreus* BI-100 [43]. Phenolic compounds, due to their physical–chemical nature, are more persistent and toxic than the parental compounds [45]. Thus, this represents an important advantage in the removal of LIN by *Streptomyces* sp. A5, since it does not lead to the production of phenols.

Three peaks were detected in chromatograms corresponding to cell-free extract samples from cultures of *Streptomyces* sp. A5, in the presence of MTX. These peaks with Rt of 34.36 min, 34.66 min and 35.12 min corresponded to 1,1-dichloro-2,2-bis(4-methoxyphenyl) ethylene, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane and MTX, and showed identity values of 96%, 90% and 98%, respectively (Table 1). These metabolites have been described in previous studies. For example, Bourguignon et al. [35] identified four intermediates resulting from the degradation of MTX by the strain *Streptomyces* sp. A14, including the metabolites of the present work. They detected that this pesticide was gradually converted to 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane, 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethylene, 1-chloro-2,2-bis(4-methoxyphenyl)ethane and 2,2-bis(4-hydroxyphenyl) acetonitrile, indicating that MTX is predominantly degraded by dechlorination, dehydrogenation, demethylation and CN substitution. Formation of 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane is consistent with the removal of a chloride ion from the MTX molecule, whereas the 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethylene is a product of the dehydrogenation process, according to the MTX degradation pathway by the catabolic action of the white rot fungus *Stereum hirsutum*, proposed by Lee et al. [46]. In other studies, 1,1-dichloro-2,2-bis (4-methoxyphenyl)ethane was detected as a result of the detoxification reaction present in the MTX degradation pathways proposed for *Corbicula japonica* [23] and *Bradyrhizobium* sp. [47].

The results obtained in the present work are consistent with previous studies by Fuentes et al. [26,31,39], who determined that cell-free extracts from various *Streptomyces* sp. have specific dechlorinase activity and the ability to degrade OPs. Despite the low percentage of MTX removal obtained in the present study, the detection of MTX metabolic intermediates confirms the ability of *Streptomyces* sp. A5 to partially degrade this pesticide.

The chromatogram corresponding to the cell-free extract from CLD assays, showed a signal with Rt of 33.30 min, with 99% identity with CLD (Table 1). This is indicative of the pesticide presence inside the cells of *Streptomyces* sp. A5. These findings, and the absence of residual CLD in the culture medium after 144 h of incubation, are consistent with the removal of the pesticide through a mechanism of intracellular accumulation by this strain. In relation to this, Fuentes et al. [39] detected 94.3% of CLD removal, in a defined mixed culture consisting of six *Streptomyces* strains, without detecting specific dechlorinase activity, and concluded that these strains could have a capacity to accumulate the pesticide inside cells. Similarly, Nakast and Litchfield [48], when studying the effects of CLD on the marine bacterium *Aeromonas proteolytica*, determined that the pesticide was located on the plasma membrane of the microorganism.

With the background of the behaviour of the *Streptomyces* studied strain, on individually contaminated assays, supernatants and cell free extracts obtained from the assays contaminated with the pesticides mixture were analyzed. In this case, intermediate metabolites by MS-GC were not detected; however, the decrease in the LIN and CLD concentration detected by μECD-GC, showed the removal pesticide capability of *Streptomyces* sp. A5. At this point the null MTX removal detected from the mixture, and the absence of intermediaries when *Streptomyces* sp. A5 eliminates CLD in the individually contaminated assays, are important results to consider. For this the expectation is to find the presence of the chlorinate intermediate on the bioremediate mixture detected in the assay individually contaminated with LIN and remediated by *Streptomyces* sp. A5. Therefore, new analyses to clarify the removal of these pesticides from the complex mixture are being made.

3.4. Cytotoxicity test

It is known that the aim of bioremediation is to reach the mineralization of a toxic compound or at least to obtain intermediate products less toxic than the parental compound. On this basis, and considering the highest pesticide concentration, the highest potential toxicity of the mixture, and the greater proximity to a contaminated

Table 2
Cytotoxic assays on pesticides contaminated samples, bioremediated or without remediate by *Streptomyces* sp.A5.

Group	Trypan blue exclusion‡	MTT assay‡
Control	95 ± 3	98 ± 2
MIX	65 ± 3*	84 ± 1*
MIX + *St* A5	92 ± 1	90 ± 4

Each group corresponds to Caco-2 cells incubated with the mix of the three pesticides (MIX group) treated or untreated with *Streptomyces* sp. A5. Results are expressed as the average ± SD obtained from triplicated.

‡ Results of the assay are expressed as percentage of viable cells. It was considered 100% viability for cells incubated with DMEM alone.

* Means a significant difference ($p < 0.01$) compared to the Control group.

real system, the inhibition of Caco-2 cell viability by samples of the pesticides mixture treated with *Streptomyces* sp. A5 and untreated, were studied. It was observed that the mixture of LIN, MTX and CLD affected the viability of Caco-2 cells when they were evaluated by the trypan blue exclusion assay. The cell viability in the presence of this mixture decreased significantly ($p < 0.01$) (65 ± 3%). In contrast, the cultures contaminated with the mixture on study and treated with *Streptomyces* sp. A5, showed a reversal of the cytotoxic effect and reached a cell viability of 92 ± 1%, with a similar percentage value to the observed in their control without pesticide (95 ± 3%) (Table 2). Similarly, the MTT assay showed a significant decrease in the percentage of viable cells (84 ± 1%) in the pesticides mixture presence, compared to their control without contaminants (98 ± 2%). However, it is important to highlight that the treatment of the contaminated system with *Streptomyces* sp. A5 significantly reversed ($p < 0.01$) the toxic effect of the mixture of pesticides (90 ± 4%). This finding is an important result because in some cases it has been well documented that the mixture of pesticides is devoid of hazard when the concentration of each compound does not reach health concern level. However, there is evidence that demonstrates significant effects of the mixture even when the compounds are present below their individual no observable adverse effect levels [49]. In this case, the toxicity of the mixture on study for the cell line assayed and the ability of *Streptomyces* sp. A5 to remove compounds from the mixture and detoxify the system were demonstrated. In this sense, Aparicio et al. [50] demonstrated the success of the bioremediation of a co-contaminated soil by *Streptomyces* sp. M7 and a quadruple actinobacteria consortium (with *Streptomyces* sp. A5 as constituent), through the use of a different ecotoxicology tests. They demonstrated the increase in the vigour index measured in lettuce seedlings, when the soil contaminated with Cr(VI) and lindane was bioremediated, in comparison to the co-contaminated and non-treated soil.

3.5. Searching of genes involved in organochlorine pesticides degradation and plasmid detection

The pathway for aerobic degradation of LIN by *Sphingobium japonicum* UT26 was previously studied by Nagata et al. [51]. They observed that different enzymes carried out dechlorination reactions. LinA (dehydrochlorinase), LinB (haloalkane dehalogenase) and LinC (NAD dependent dehydrogenase) are enzymes that act in the upper pathways corresponding to the first stage of pesticide degradation. On this basis, and considering the specific dechlorinase activity detected in cell-free extracts of *Streptomyces* sp. A5 by Fuentes et al. [39], sequences with potential dechlorinase activity were identified through a comparative study of the unknown protein sequences of the genome of *Streptomyces* sp. A5, and the existing sequences in the gene databases. In this work it was possible to assign functions to four of the analyzed sequences for the degradation process of LIN.

In the sequence MF346168 present in the genome of *Streptomyces* sp. A5, domains and motifs characteristic of LinA from *Sphingobium*

Table 3
Genes identification of *Streptomyces* sp. A5 with conserved domains and motifs, associated with the degradation pathway of lindane.

GeneBank accession no.	aa no.	Motifs and domains	Function	Identity
MF346168	156	Domains, SnoaL_4(pfam13577) Motif: active site, D24; H72	Dehydrochlorinase, chlorine atoms eliminations from HCH molecule	22% gamma-BHC dehydrochlorinase [*Sphingobium japonicum*] BAA14369.2
MF346169	292	Domains: alpha/beta hydrolase fold Motif: active site: D94; H268; D128	Haloalkane dehalogenase, hydrolytic cleavage of the carbon−chlorine bond	24% LinB [*Sphingobium indicum*]AAR05978.1
MF346170	246	Domains: SDR_c (oxidoreductase, cd05233),adh_short (pfam00106), FabG (COG1028) Motifs: active site S-12X-Y-3X-K; NAD(P) binding site: TG-3X-(AG)-XG	Short chain alcohol dehydrogenase	38% 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase [*Sphingomonas paucimobilis*]AAZ14097.1

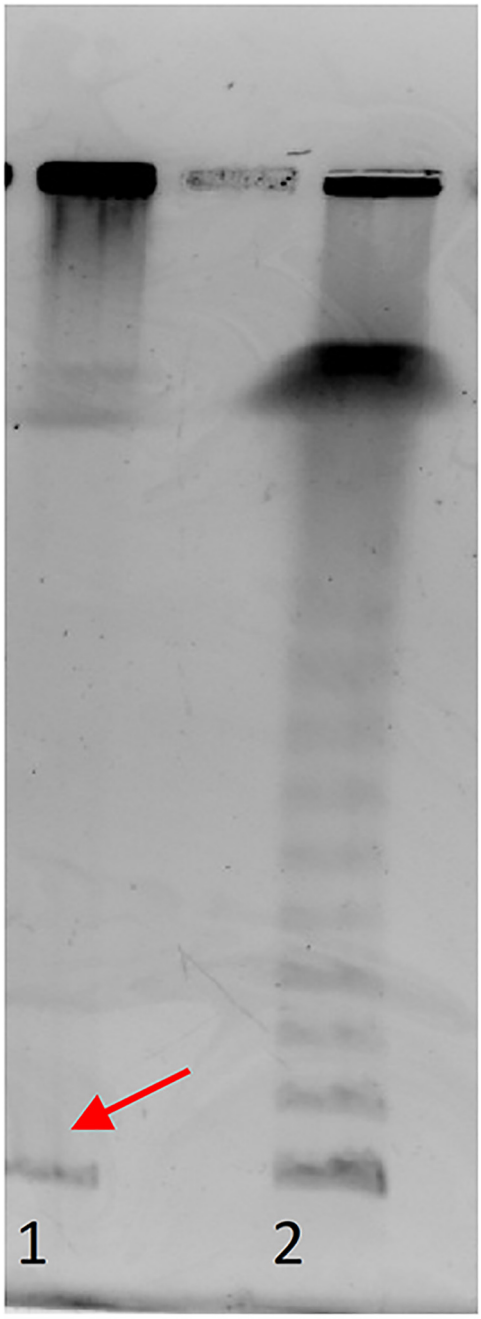

Fig. 3. PFGE analysis of total DNA from *Streptomyces* sp. A5. Lane 1 (indicated by arrow) reveals the presence of a 50 kb plasmid. Lane 2 shows a Lambda ladder. Camping time was 30–50 s for 22 h (6 V/cm at 14 °C).

japonicum were identified (Table 3). The potential active site formed by the conserved motifs D24 and H72 was determined. Comparative studies with the known sequence showed an identity of the order of 22% with respect to the LinA enzyme present in *Sphingobium japonicum* (Table 3).

The sequence MF346169 was identified as putatively encoding the LinB enzyme, where all domains belonging to the alpha/beta hydrolases family were determined, as well as the conserved motifs: D94; H268; D128, corresponding to a catalytic triad of this type of enzyme. The protein sequence of the putative LinB enzyme of *Streptomyces* sp. A5 showed 24% of identity to the LinB sequence present in *Sphingobium indicum* (AAR05978.1) (Table 3).

The sequence MF346170 was identified to have domains and motifs characteristic of the LinC enzyme present in *Sphingobium japonicum*; this protein belongs to the family of short-chain dehydrogenases. Among the conserved motifs present were both, the active site: S-12X-Y-3X-K and the cofactor binding site (NAD +), TGXXX [AG] XG, essential sites in NAD-dependent dehydrogenase. The alignment of MF346170 with LinC from *Sphingomonas paucimobilis* (AAZ14097.1) showed a 38% identity (Table 3).

The results of these comparative studies support the capability of *Streptomyces* sp. A5 to degrade OPs, and generate the possibility that it is degradation produces the formation of less toxic compounds than the starting substrates, probably using these assigned hypothetic proteins. For examples, previous studies detected 1,2,4-trichlorobenzene as a product of the catabolism of the LIN, obtained by dehydrodechlorinase enzyme action [42]. This resultant compound is less toxic than LIN, and is not classified as a human carcinogen.

The PFGE analysis of total DNA revealed a plasmid of 50 kb in *Streptomyces* sp. A5 (Fig. 3), raising the possibility that this plasmid may encode the genes for degradation of LIN, acquired by horizontal transmission [52]. Accordingly, there is information about the presence of plasmids and megaplasmids in different actinobacteria strains such as *Streptomyces reticuli* Tü 45, which has a linear 0.8 Mb megaplasmid, a linear 94 kb plasmid and a circular 76 kb plasmid [53] or *Rhodococcus opacus* strain M213 with two megaplasmids (pNUO1 and pNUO2) estimated to be of 750 kb and 350 kb in size, respectively [54]. Interestingly, in the particular case of *R. opacus* strain M213, several plasmid/genomic islands-encoded genes that likely participate in degrading naphthalene were identified. Currently, the aforementioned possibility regarding the detection of the presence of genes related with LIN degradation in the plasmid of *Streptomyces* A5, is being explored by their sequencing to confirm the *in silico* results.

4. Conclusions

Streptomyces sp. A5 was able to grow in the presence of LIN, CLD and MTX, and remove these pesticides, in a percentage of 57.4%, 100.0% and 6.5%, respectively; when they were added individually to the culture medium. From the mixture of these three pesticides, *Streptomyces* sp. A5 was able to remove 62.2% of LIN and 68.6% of CLD; however, no MTX removal was detected in the assayed conditions. The bioremediation of the mixture of OPs by *Streptomyces* sp. A5 gave rise to a lower cytotoxicity than in the controls without bioremediation. Also, the search for genes involved in OPs degradation in the genome of *Streptomyces* sp. A5, revealed the presence of putative *lin* genes, with potential dehydrochlorinase, haloalkane dehalogenase and NAD-dependent dehydrogenase activities, and the PFGE analysis of total DNA revealed the presence of a plasmid of 50 kb in *Streptomyces* sp. A5.

These results demonstrate the ability of *Streptomyces* sp. A5 to eliminate OPs from contaminated liquid systems, especially from a complex mixture, and therefore this bacterium is proposed as a promising tool for bioremediation processes in environments contaminated with multiple organochlorine pesticides.

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