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Use of pure and mixed culture of diazinon-degrading *Streptomyces* to remove other organophosphorus pesticides

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ABSTRACT

Chlorpyrifos (CP) is an organophosphorus pesticide (OP) widely used in agriculture. Evidence of CP residues in soil, water and food has generated an urgent need to develop treatment systems that avoid possible damage to human health and the environment. In this study, eight diazinon-degrading *Streptomyces* strains were evaluated for their ability to remove CP as the only carbon source from a liquid medium. Single cultures removed 32–74% of CP, and most of the strains incremented their biomass. Additionally, changes in the protein profile were found. Protein extract analyses demonstrated that four organisms exhibited the enzyme organophosphorus hydrolase (OPH); therefore, the *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains were used as a mixed culture to study the removal of CP and their main metabolite 3,5,6-trichloro-2-pyridinol (TCP). Our results showed a CP removal rate of 0.019–0.034 h^{-1} a half-life of 20–36 h and a maximum TCP concentration of 0.32 mg L^{-1}. The mixed culture used TCP to grow, and up to 58% was removed from the medium. Glucose addition decreased biomass duplication time of the culture and also accelerated pesticide depletion from the medium. Viability analysis showed that after CP and TCP exposure, over 78% of the cells survived. Finally, the mixed culture of *Streptomyces* spp. can simultaneously remove four OPs, reaching a maximum removal of 69.3% and 33.6% for thiophosphotriester and phosphorothiolester compounds respectively.

1. Introduction

Pesticide wastewater characterized by high compound loads and high toxicity are generated by industrial agriculture and by industries related to pesticide manufacturing and packaging. Given the lack of suitable methods of pesticide treatment and disposal, large amounts of these products are improperly eliminated or stockpiled, thus putting human health and the environment at risk (Karas et al., 2011; Yañez-Ocampo et al., 2011). Organophosphorus pesticides (OPs) represent the most important pesticides in the global market, and contamination of soil, water systems, atmosphere and foods have been reported worldwide due to large-scale use of these compounds (Chishti et al., 2013; Fosu-Mensah et al., 2016; Pozo et al., 2016). OPs are highly toxic compounds capable of poisoning insects and other animals, including birds, amphibians and mammals, primarily by phosphorylation of the acetylcholinesterase enzyme at nerve endings (Roberts and Reigart, 2013). Moreover, these compounds are considered to be the major food chain contaminants (Rezg et al., 2010) and therefore related to a wide variety of metabolic human health problems, such as type 2 diabetes (Lasram et al., 2014).

Chile is classified as one of the world's top ten pesticide consumers on agricultural land (Verma et al., 2014), with OPs commonly used against pests and diseases present in agricultural and fruit crops, as well as livestock. Among OPs, the insecticide chlorpyrifos (CP) (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is extensively used in the fruit farming industry,

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especially in blueberry crops, characterized by high productivity in planted areas that have increased from 1000 ha in 2000 to 13,016 ha in 2012 (Oficina de Estudios y Políticas Agrarias, 2013). This has led to an increase in the use of CP, most likely increasing waste generation and the risks of environmental contamination and health hazards.

CP has short to moderate persistence in the environment as a result of several dissipation pathways. According to Solomon et al. (2014), CP presents a half-life for hydrolysis of 16–73 d, aerobic soil metabolisms of 2–1576 d, aerobic aquatic metabolisms of 22–51 d and terrestrial field dissipation between 2 and 120 d. The primary degradation pathway of CP involves hydrolysis to 3,5,6-trichloro-2-pyridinol (TCP), which is more toxic than the parent compound because it has antimicrobial properties that inhibit microbial growth. Biotic degradation is one of the most viable options for detoxification of CP in soil and water (Chishti et al., 2013). Several studies have focused on microbial degradation due to the need to develop technologies that guarantee safe, efficient and economical detoxification and elimination of this compound.

Numerous microorganisms have been described as CP and TCP-degraders (Chishti et al., 2013; Verma et al., 2014), including bacteria such as *Ochrobactrum* sp. (Abraham and Silambarasan, 2016), *Stenotrophomonas* sp. (Deng et al., 2015), *Mesorhizobium* sp. (Jabeen et al., 2015a) and many others. *Streptomyces* spp. isolated from contaminated agricultural soils and marine water samples are recognized as OP-degraders. Specific studies with pure cultures of *Streptomyces* strains have been performed for CP (Briceño et al., 2012; Naveena et al., 2013) and diazinon (DZ) (Briceño et al., 2015). Apart from single *Streptomyces* sp. few mixed cultures with the ability to remove OPs have also been reported. The use of microbial mixed cultures is recognized as a biotechnological tool that can more easily increase pesticide removal, avoid the generation and accumulation of toxic metabolites derived from microbial degradation, and facilitate the degradation of complex mixtures of pesticides (Pino and Peñuela, 2011; Jabeen et al., 2015b). A study performed by Fuentes et al. (2013) demonstrated that in a pesticide mixture consisting of pentachlorophenol and CP, the latter was removed preferentially by a single culture but not by a mixed culture of *Streptomyces* spp. In our recent study, we founded that diazinon removal was increased when mixed cultures of *Streptomyces* spp. were used (Briceño et al., 2016). Even though, this was the first report on the diazinon removal by single and mixed cultures of *Streptomyces* spp., our results indicated that this could be an useful option to obtain an efficient OPs removal from liquid medium.

There have been no studies of specific CP removal, let alone of the removal of a complex mixture of OPs by a mixed culture of *Streptomyces* spp. Moreover the removal of pesticides like azinphos methyl (AZM) and methidathion (MTD) has been poorly studied, although there are some studies performed with fungi species (Jauregui et al., 2003) and *Serratia* sp. (Li et al., 2013). However, there are no reports on the simultaneous removal of OPs and specifically of CP, DZ, AZM and MTD by a mixed culture of *Streptomyces* spp.

The aim of the present investigation was to assess pure cultures of diazinon-degrader *Streptomyces* spp. in order to define a mixed culture able to remove CP and its metabolite TCP from liquid media. The ability of the selected mixed culture to simultaneously remove a complex mixture of OPs formed by the compounds chlorpyrifos, diazinon, azinphos methyl and methidathion was also studied.

2. Materials and methods

2.1. Chemicals, bacterial strains and culture media

Analytical grade CP, TCP, MTD, AZM and DZ were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents used during the study were purchased from standard sources.

Eight diazinon-degrading *Streptomyces* strains were assayed. The strains were *Streptomyces* sp. AC5 (JQ289350), *Streptomyces* sp. AC6 (JQ289351), *Streptomyces* sp. AC7 (JQ289352), *Streptomyces* sp. AC9 (JQ289353), *Streptomyces* sp. GA3 (KT271898), *Streptomyces* sp. GA11 (KT271897), *Streptomyces* sp. ISP4 (JQ289354) and *Streptomyces* sp. ISP13 (JQ289355) (Briceño et al., 2016).

To prepare the *Streptomyces* cultures, ISP-2 medium (malt extract, 10.0 g; yeast extract, 4.0 g; glucose, 4.0 g; and distilled water, 1000 mL) was used. Liquid minimal medium (MM) (L-asparagine, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $FeSO_4 \cdot 7H_2O$, 0.01 g and distilled water, 1000 mL) was used for the removal assays. The initial pH of the media was adjusted to 7.0 prior to sterilization by autoclaving at 121 °C for 20 min.

2.2. Inoculum preparation

Starter cultures of the spores and mycelia of the strains maintained in slants were grown in 100-mL flasks that contained 30 mL of ISP-2 medium. The inoculated flasks were incubated for 96 h at 28 °C and 120 rpm in a rotary shaker. The cultures were centrifuged (8500 × g for 10 min at 4 °C), and the cell pellets were then washed with sterile 0.85% NaCl solution. For the experiments, biomass was used at a concentration of 1% (*w*/*v*) wet weight.

2.3. Removal of CP by single cultures

An inoculum of each *Streptomyces* strain was incubated in a flask containing 30 mL of liquid MM supplemented with CP (50 mg L^{-1}) as the only carbon source. This concentration was chosen because a previous qualitative screen showed that CP-tolerant strains had higher growth and formed more colonies on solid media at these concentrations compared with those in the presence of higher CP concentrations (Briceño et al., 2012). In this study, the cultures were incubated in an orbital shaker at 120 rpm, 28 °C for 96 h. This incubation time was considered pertinent given that complete depletion of CP has been observed for *Streptomyces* strains in liquid medium supplemented with glucose (Briceño et al., 2012). After centrifugation at 8500 × g for 10 min at 4 °C, 10 mL of the supernatant was aseptically removed to determine residual CP and TCP concentrations by HPLC. Non-inoculated flasks and inoculated flasks without CP were used as abiotic and biotic controls respectively. Pellets were used to estimate both the dry weight of microbial growth at 105 °C and the total protein concentrations of cell-free extracts. The results of residual concentration of CP and biomass of the *Streptomyces* strains in this experiment were used to examine the relationship between the two parameters and thus to identify the strains that showed the most efficient removal and high biomass (Benimeli et al., 2007; Fuentes et al., 2011).

2.4. Protein profiles and organophosphorus hydrolase activity determination

To determine the total protein concentrations of the cell-free extracts, the pellets obtained in Section 2.3 were washed and suspended in 0.05 M phosphate buffer (pH 7.0). Cells were disrupted by ultrasonication on ice and centrifuged at 8500*g* for 10 min at 4 °C. The supernatants were then obtained as cell-free extracts, and protein concentrations were determined using the Bradford method and a BioRad Protein Assay, with bovine serum albumin as the standard. After that, protein concentrations were adjusted to 10 μg, and the protein profiles of the cultures were analyzed using 0.1% sodium dodecyl sulfate 12% polyacrylamide gel

electrophoresis (SDS-PAGE), as described by Laemmli (1970) with Coomassie blue staining.

Organophosphorus hydrolase (OPH) activity was assayed with paraoxon by monitoring the increase in absorbance at 405 nm ($\varepsilon 405 = 17{,}700\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol) for 10 min at 37 °C using a Synergy H1 multi-mode microplate reader (BioTek). For each assay, 40 μL of cell-free extracts were added to 150 μL of 50 mM citrate-phosphate buffer (pH 8.0) and 10 μL of 40 mM paraoxon (Pestanal®) in 10% methanol. Activities were expressed as units (micromoles of paraoxon hydrolyzed per minute) per mg of protein (Cao et al., 2013).

2.5. Removal of CP and TCP by the selected mixed culture

A defined mixed culture was used to study CP and TCP removal at different time intervals. To assess the influence of carbon source on CP and TCP removal, the liquid MM (G0) was enriched with 4 g L^{-1} of glucose (G4). The microorganisms obtained in Section 2.2 were added at a final concentration of 1% wet weight in triplicate flasks containing 30 mL of medium with CP (50 mg L^{-1}) or TCP (12 mg L^{-1}). Inoculated flask without the contaminants and a non-inoculated flask with addition of CP or TCP were used as biotic and abiotic controls respectively. In this study, the cultures were incubated at 28 °C, 120 rpm and 168 h. A longer incubation time was evaluated in order to monitor and learn in more detail the growth kinetic of the mixed culture and the removal of CP and TCP, the latter known for its antimicrobial properties. After 24, 48, 96 and 168 h, samples were collected and centrifuged using the pellet for measuring dry weight microbial growth at 105 °C. Moreover, 10 mL of supernatant were removed to determine residual CP and TCP concentrations via HPLC. CP and TCP removal were estimated by comparing the concentrations in the samples and controls without inoculum over time.

2.6. Survival of the mixed culture

At the end of the assay outlined in Section 2.5, culture samples were collected and processed for live/dead cells using flow cytometry and confocal microscopy. Briefly, cell concentrations were adjusted to 0.1 OD (1×10^6 cell mL^{-1}) and then a suspension of 500 μL was stained with 10 μM of SYTO9 for live cells and 66 μM of propidium iodide for dead cells. After incubation for 15 min at room temperature, 10 μL of stained cells were analyzed using 10,000 eV flow cytometry (Becton Dickinson FACSCanto II) and the software FACs DIV 6.0 (USA). Images were obtained using an Olympus FV1000 confocal laser microscope and the software FV10 (ver. 02.0c, Olympus-Japan).

2.7. Removal of an organophosphorus pesticides mixture

Removal experiments were performed using four OPs: CP, DZ, AZM and MTD, in order to evaluate the ability of the *Streptomyces* mixed culture to remove a complex pesticide mixture. A liquid medium supplemented with 4 g L^{-1} of glucose was contaminated with each pesticide at 25 mg L^{-1} giving a final concentration of 100 mg L^{-1}. The flasks were inoculated with the *Streptomyces* mixed culture added at a final concentration of 1% wet weight. An inoculated flask without the contaminants and a non-inoculated flask with addition of CP, DZ, AZM and MTD were used as biotic and abiotic controls respectively. Incubation was carried out at 28 °C, 120 rpm and 96 h of incubation; this incubation time was established considering that it takes 20 h for 50% of 50 mg L^{-1} CP to disappear. After 24, 48, 72 and 96 h samples were collected and centrifuged, using the pellets to determine the dry weight microbial growth at 105 °C; the supernatant was used at the end of the study to determine pesticide removal by comparing the concentrations in the samples with the abiotic controls.

2.8. Pesticide analysis

Samples of the supernatants from the centrifuged cultures were filtered through a 0.22-μm filter and used to determine pesticide concentrations. Pesticides were extracted with dichloromethane and ethyl acetate. For extractions, 2.5 mL of each sample was mixed with 5 mL of dichloromethene, shaken on a rotary shaker for 60 min at 300 rpm and then vortexed for 10 s. This procedure was repeated twice, replacing dichloromethane with ethyl acetate. Then the organic solvents were combined, concentrated in a SPD121P SpeedVac® Concentrator (Thermo Scientific Savant®), and the sample was re-suspended in 2.5 mL of acetonitrile before storage at −20 °C for chromatographic analyses. Approximately 85% of pesticide residues were recovered using this method.

Analyses were performed using a Shimadzu LC-20AT liquid chromatograph with a diode array equipped with a Purospher Star RP-18e column (Merck®, film thickness 5 μm, 150 × 4.6 mm). The oven temperature was 35 °C and the mobile phase was 25% of a 0.1% phosphoric acid-75% acetonitrile solution injected at a flow rate of 1 mL min^{-1}. Under these chromatographic conditions, the retention times of CP and TCP were 8.26 (λ = 289) and 2.49 (λ = 298) min respectively. Calibration was performed using a standard for each compound, with a linear curve ranging from 0.05 to 10 mg L^{-1}. Analyses of samples containing the pesticide mixture were performed using the same conditions, except that the column was replaced with a Prontosil® RP-18e (5 μm), 250 × 4.6 mm column. Under these conditions the retention time and the wavelength for MTD, AZM, DZ and CP were 4.51 (λ = 225), 4.51 (λ = 298), 7.71 (λ = 246) and 12.24 (λ = 229) min respectively.

2.9. Kinetics and statistics analysis

The data obtained in assay 2.5 were used to determine the specific growth rate (μ) by plotting $\ln(B_t/B_0)$ against time, where B_0 is the amount of biomass in liquid medium at time zero and B_t is the amount of biomass at time *t*. The biomass duplication time was determined as ln(2)/μ. The removal of CP and TCP was described using the first-order kinetic model $\ln C_t/C_0 = e^{-kt}$, where C_0 is the amount of contaminant in the liquid medium at time zero and C_t is the amount of contaminant at time *t*, and *k* and *t* are the rate constant and degradation time in hours respectively. The time at which the CP and TCP concentrations in the liquid medium were reduced by 50% ($T_{1/2}$) was calculated using the equation $T_{1/2} = \ln(2)/k$.

All of the experiments were performed in triplicate. The data were statistically analyzed by analysis of variance (ANOVA). When significant differences were observed, the means were separated using Tukey's minimum significant differences test ($p \leq 0.05$). In the study with single cultures, Pearson correlation was done to determine correlation between variables.

3. Results

3.1. Removal of CP and microbial growth of single cultures

Biomass growth and CP removal by each of the eight individual *Streptomyces* strains was studied. The results, presented in Table 1, indicated that after 96 h the final biomass in the strains was significantly different ($p \leq 0.05$). The initial biomass for each strain was 0.30 mg mL^{-1}; accordingly, six of the eight strains grew in liquid MM without any other added carbon source except for CP. The strains were not able to grow in the control without CP. The highest

Table 1
Dry weight biomass and removal of chlorpyrifos (CP) by single cultures of the *Streptomyces* spp. strains AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13 cultured for 96 h in minimal medium with 50 mg L^{-1} CP.

Strain	Dry weight biomass (mg mL^{-1})	CP removal (%)	RCP/B
Streptomyces sp. AC5	0.415 ± 0.012 abc	66.7 ± 4.8 a	0.04
Streptomyces sp. AC6	0.220 ± 0.023 cd	32.1 ± 2.9 b	0.15
Streptomyces sp. AC7	0.435 ± 0.018 abc	50.5 ± 4.5 ab	0.06
Streptomyces sp. AC9	0.440 ± 0.020 ab	75.8 ± 0.8 a	0.03
Streptomyces sp. GA3	0.355 ± 0.022 bcd	32.0 ± 3.0 b	0.10
Streptomyces sp. GA11	0.328 ± 0.025 bcd	67.1 ± 4.2 a	0.05
Streptomyces sp. ISP4	0.216 ± 0.049 d	40.5 ± 2.5 b	0.14
Streptomyces sp. ISP13	0.602 ± 0.055 a	74.4 ± 4.4 a	0.02

The average values and the standard error are presented (n = 3). The analyses were done in the same column between strains. The values with different letters indicate significant differences ($p \leq 0.05$, Tuckey test). RCP = Residual chlorpyrifos (mg L^{-1}) and B = Biomass (dry weight, mg L^{-1}).

biomass was observed for the *Streptomyces* spp. strain ISP13, while a depletion of biomass at the evaluation time was observed for strains AC6 and ISP4. Similarly these strains, together with the GA3 strain, presented the lowest CP removal (32–40%); the AC7 strain showed 50% removal, while the *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 showed the highest CP removal (66–76%), and therefore a residual CP concentration between 12 and 28 mg L^{-1}. The CP metabolite, TCP was detected in all samples, but not in concentrations higher than those observed in the abiotic control.

The relation between the residual concentration of CP and microbial growth was examined in order to estimate and select the best strains to form the mixed culture. In this study, the microorganisms with the lowest ratio (low CP concentration and high biomass production) were strains AC5, AC7, AC9, GA11 and ISP13 (Table 1); these could therefore be considered as the most efficient strains for CP removal. However, all strains were subjected to protein and OPH analysis before the final selection was made.

3.2. Effect on protein profile and OPH activity of single cultures

Analyses performed on cell-free extracts from the eight strains cultivated in liquid MM with and without CP as the only carbon source showed that protein concentrations in some strains was increased significantly ($p \leq 0.05$) after CP exposure. This increment ranged from 0.048 to 0.067 mg mL^{-1} compared with controls for *Streptomyces* spp. strains AC5 and ISP4, and from 0.366 to 0.786 mg mL^{-1} for the other strains, with the exception for *Streptomyces* spp. AC6 and AC7 for which protein concentration was not increased (Table 2).

The same cell-free extracts with adjusted protein concentrations were observed using SDS-PAGE (Fig. 1). The results showed an increased intensity and more protein bands in the lane that represent the strains cultured in the presence of CP. *Streptomyces* spp. strains AC5, AC6, AC9, GA3, GA11 and ISP13 presented the most noticeable changes, while *Streptomyces* spp. strains AC7 and ISP4 showed visually similar profiles to those observed in the MM without CP.

Finally, cell-free extracts from the eight strains exposed to CP were used to evaluate the enzyme OPH using paraoxon, which is hydrolyzed to colored *p*-nitrophenol. Enzymatic activity was detected for four of the eight strains with values between 0.0046 and 0.0629 U mg^{-1} proteins. Strains exhibiting OPH were *Streptomyces* spp. AC9, GA11, ISP13 and especially *Streptomyces* sp. AC5, for which the highest activity was observed (Table 2).

Pearson analysis of the results for CP removal, microbial growth, total protein and OPH activity showed that CP removal was significantly correlated with microbial biomass ($r = 0.624$, $p < 0.01$), total proteins ($r = 0.524$, $p < 0.01$) and OPH activity ($r = 0.413$, $p < 0.05$). Based on this information, the four *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 that showed an increase in biomass and total proteins, and showed positive for OPH activity, were selected for mixed culture formation and for use in the following assays.

3.3. Microbial growth and CP removal by the selected mixed culture

Fig. 2 shows the microbial growth and CP removal in treatment G0 and G4 contaminated with 50 mg L^{-1} CP and inoculated with the *Streptomyces* spp. mixed culture formed by the AC5, AC9, GA11 and ISP13 strains. These results show that the addition of glucose significantly increased ($p \leq 0.05$) biomass growth after 24 h; the specific growth rates were 0.004 and 0.026. It is seen that biomass duplication time was reduced from 161 h in the absence of glucose to 27 h when glucose was added to the liquid medium (Table 3). At 48 h, the biomass in G4 reached a maximum value of 1.41 mg mL^{-1} dry weight, after which it began to decline. In the case of treatment G0, the mixed culture was not able to grow in MM containing L-asparagine as the sole carbon and nitrogen source. However, in the medium with CP, the biomass began to increase after 24 h, with the largest values of biomass (1.07 mg mL^{-1}) observed at 96 h; this trend was maintained until the end of the study (Fig. 2a).

CP removal increased significantly ($p \leq 0.05$) in the presence of glucose. The results showed disappearance of nearly 40% in G0 and 85% in G4 at 24 h. The kinetic data showed that CP removal by the G0-treated culture was described by a rate constant of 0.019 h^{-1} and $T_{1/2}$ of 36 h, while a rate constant of 0.034 h^{-1} and $T_{1/2}$ of 20 h was observed in G4-treated culture (Table 3). After 96 h and 168 h, CP removal in the treatments did not differ significantly. A CP

Table 2
Total protein content from cell-free extracts and organophosphorus hydrolase (OPH) activity observed for the single cultures of the *Streptomyces* spp. strains AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13, cultured for 96 h in minimal medium with 50 mg L^{-1} CP.

Strain	Increment of the total protein concentration* (mg mL^{-1})	OPH activity (U mg^{-1} protein)
Streptomyces sp. AC5	0.048 ± 0.014 d	0.0629 ± 0.0010 a
Streptomyces sp. AC6	n.i	n.d
Streptomyces sp. AC7	n.i	n.d
Streptomyces sp. AC9	0.366 ± 0.015 b	0.0046 ± 0.0002 c
Streptomyces sp. GA3	0.473 ± 0.086 c	n.d
Streptomyces sp. GA11	0.778 ± 0.009 a	0.0066 ± 0.0023 c
Streptomyces sp. ISP4	0.067 ± 0.006 d	n.d
Streptomyces sp. ISP13	0.786 ± 0.006 a	0.0193 ± 0.0076 b

The average values and the standard error are presented (n = 3). The analyses were done in the same column between strains. The values with different letters indicate significant differences ($p \leq 0.05$, Tuckey test). * The increment of protein content was respect the observed in the control treatment without CP. OPH= Organophosphorus hydrolase; n.i = no increment; n.d. = no detection.

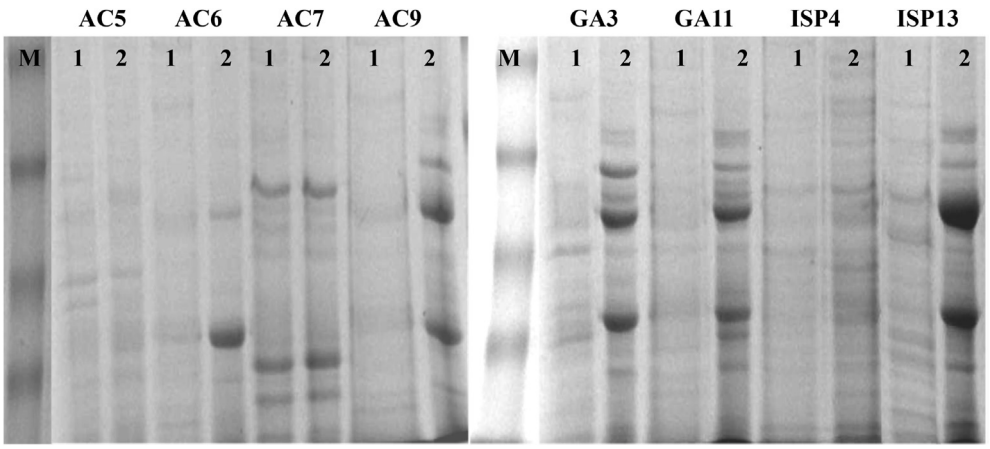

Fig. 1. Denaturing PAGE analysis of proteins from the *Streptomyces* spp. strains AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13. Cells were cultured in minimal medium with 50 mg L^{-1} chlorpyrifos (CP). Lane M: Molecular weight marker; lane 1: without CP; lane 2: with CP.

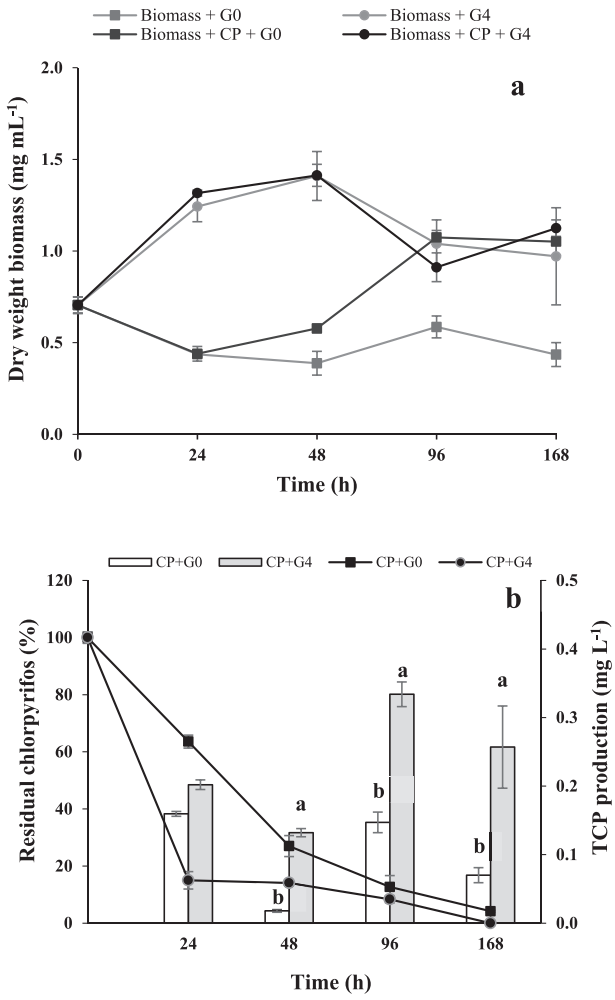

Fig. 2. Growth of the *Streptomyces* spp. mixed culture (a), chlorpyrifos removal (CP) and 3,5,6-trichlo-2-pyridinol (TCP) production (b) in minimal medium (G0) contaminated with 50 mg L^{-1} CP and supplemented with 4 g L^{-1} glucose (G4). The error bars represent the standard error of the means of three replicates. The bars with different letters indicate significant differences ($p \leq 0.05$, Tuckey test). The analyzes for TCP production were done for each different incubation time.

concentration of 2 mg L^{-1} was found in treatment G0, while no CP residues were detected in treatment G4 at the end of the study (Fig. 2b). The mixed culture of *Streptomyces* spp. increased CP removal by about 15–31%. However, this increase in CP removal was not significant when compared to that observed for strains AC5, AC9, GA11 and ISP13 in MM at 96 h.

In addition to CP removal, the production of TCP, its principal metabolite, was evaluated. The results showed that TCP production was significantly different ($p \leq 0.05$) among the treatments, which was evident after 48 h of incubation. The general trend observed was of higher TCP production in the treatments performed in the presence of glucose, especially after 96 h of incubation in which TCP production was over 0.3 mg L^{-1} (Fig. 2b).

3.4. Microbial growth and TCP removal by the selected mixed culture

The results of microbial growth and TCP removal are shown in Fig. 3. Similar to previous observations, the mixed culture of *Streptomyces* spp. was not able to grow in the absence of TCP or glucose as a carbon source. The biomass increased significantly in both treatments after 48 h. The biomass in G0 presented a maximum value of 0.79 mg mL^{-1}, after which it decreased to values similar to those observed in absence of TCP. In treatment G4 similar microbial growth was observed during the first 24 h in both the absence and presence of TCP. However, this trend changed after 48 h of incubation, when the biomass decreased in the liquid medium with TCP (Fig. 3a). In accordance with these results, a specific growth rate of 0.019 and a biomass duplication time of 36 h were observed for treatment G0 with TCP addition, while this time was reduced to 14 h in G4 where a specific growth rate of 0.051 was observed (Table 3).

TCP removal by the mixed culture of *Streptomyces* was significantly different ($p \leq 0.05$) when the liquid medium was supplemented with glucose. After 24 h, 9% was removed in treatment G0 and 21% in G4. TCP removal increased slightly in G0, reaching 30% disappearance by the end of the study. The treatment that favored TCP removal most was G4, where 50% TCP was removed after 96 h and close to 58% at the end of the study (Fig. 3b). The kinetic data showed that TCP removal by the G0 culture was characterized by a rate constant of 0.002 h^{-1} and $T_{1/2}$ of 250 h, while in the G4 culture the rate constant was 0.005 h^{-1} and the $T_{1/2}$ was reduced to 139 h (Table 3).

3.5. Survival of the mixed culture exposed to CP and TCP

In addition to studying the microbial growth of the *Streptomyces*

Table 3
First order kinetics data for chlorpyrifos (CP) and 3,5,6-trichlo-2-pyridinol (TCP) removal, specific growth rate (μ) and biomass duplication time of the mixed culture of *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains in liquid medium without glucose (G0) and supplemented with 4 g L^{-1} glucose (G4).

Parameters	CP		TCP	
	G0	G4	G0	G4
Regression equation	0.071 + 0.019x	0.447 + 0.034x	0.016 + 0.002x	0.066 + 0.005x
k (h^{-1})	0.019 ± 0.000	0.034 ± 0.000	0.002 ± 0.000	0.005 ± 0.000
$T_{1/2}$ (h^{-1})	36 ± 1 a	20 ± 0 b	250 ± 6 a	139 ± 10 b
R^2	0.992	0.973	0.960	0.971
Specific growth rate (μ)	0.004 ± 0.000	0.026 ± 0.000	0.019 ± 0.000	0.051 ± 0.001
Biomass duplication time (h^{-1})	161 ± 2 a	27 ± 0 b	36 ± 0 a	14 ± 3 b

The error bars represent the standard error of the means of three replicates. The values with different letters indicate significant differences ($p \leq 0.05$, Tuckey test) considering the concentration of glucose for each compound.

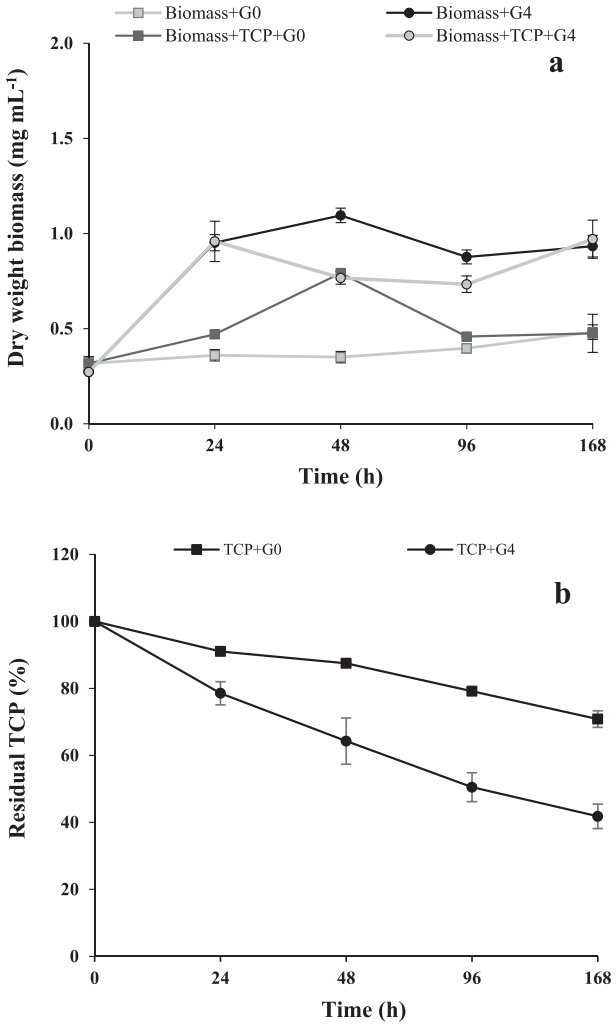

Fig. 3. Growth of the *Streptomyces* spp. mixed culture (a) and 3,5,6-trichlo-2-pyridinol (TCP) removal (b) in minimal medium (G0) contaminated with 12 mg L^{-1} TCP and supplemented with 4 g L^{-1} glucose (G4). The error bars represent the standard error of the means of three replicates.

mixed culture exposed to 50 mg L^{-1} CP or 12 mg L^{-1} TCP, culture survival after pesticide exposure was analyzed by live/dead cell analysis. Fig. 4 shows the confocal images of samples obtained from liquid culture contaminated with CP (Fig. 4a) and TCP (Fig. 4b). As may be seen in the figure, most cells were viable (green) at the end of the study, with very few dead cells (red). These results were confirmed by flow cytometry analysis, which obtained 78% live cells and 22% dead cells after exposure of *Streptomyces* mixed culture to CP, and approximately 79% live and 21% dead cells after exposure to TCP. This was similar to the results obtained for the control treatment without pesticide, where 78% live and 22% dead cells were found.

3.6. Microbial growth and removal of an OP mixture by the mixed culture

The capability of the mixed culture of *Streptomyces* spp. to remove other OPs (DZ, MTD and AZM) in addition to CP was also investigated in liquid medium supplemented with 4 g L^{-1} glucose and containing each pesticide at a concentration of 25 mg L^{-1}. Fig. 5 shows microbial growth during 96 h of incubation and pesticide removal at the end of the assay. The results show that the mixed culture was able to grow in the presence of the contaminant mixture with a biomass duplication time of 40 h, while in the control without pesticides, the culture grew rapidly with a biomass duplication time of 16 h. Despite initial differences in growth, after 72 h the maximum biomass (1.2 mg mL^{-1}) was observed in both the liquid cultures with the pesticide mixture and the control (Fig. 5a). With regard to pesticide removal, the *Streptomyces* mixed culture showed the highest removal of CP at 69.3% after 96 h of incubation, followed by 58.9% for DZ, while 27.9% and 33.6% of MTD and AZM were removed, respectively (Fig. 5b).

4. Discussion

The insecticide CP has become one of the most studied OP pesticides for bioremediation in both soil and water. Several bacteria strains of the genera *Bacillus* sp., *Sphingomonas* sp., *Mesorhizobium* sp. and *Cupriavidus* sp. have been characterized for their ability to degrade these compounds (Li et al., 2007; Anwar et al., 2009; Lu et al., 2013; Jabeen et al., 2015a). *Streptomyces* sp. is a genus of Gram-positive bacteria that grows in various environments and is characterized by the presence of a specialized and coordinated metabolism (de Lima et al., 2012). In bioremediation, *Streptomyces* sp. has not been extensively studied compared with other genera. However it has been reported in various studies that these microorganisms possess the ability to degrade pesticides of such different chemical classes as organochlorines (Fuentes et al., 2011), pyrethroids (Lin et al., 2011), carbamates (Rahmansyah et al., 2012), ureas (Castillo et al., 2006), chloroacetanilides (Sette et al., 2005) and OPs (Briceño et al., 2012; Naveena et al., 2013).

We used eight *Streptomyces* spp. strains tolerant to and able to remove the insecticide DZ (Briceño et al., 2016). In this study, we report that six and five strains exhibit appropriate microbial growth and CP removal (over 50%) respectively when this pesticide was the only carbon source. The strains AC6 and ISP4 showed a CP removal of 32 and 40% respectively, in spite of a decrease in biomass which could be because at 96 h these strains were in the death phase of

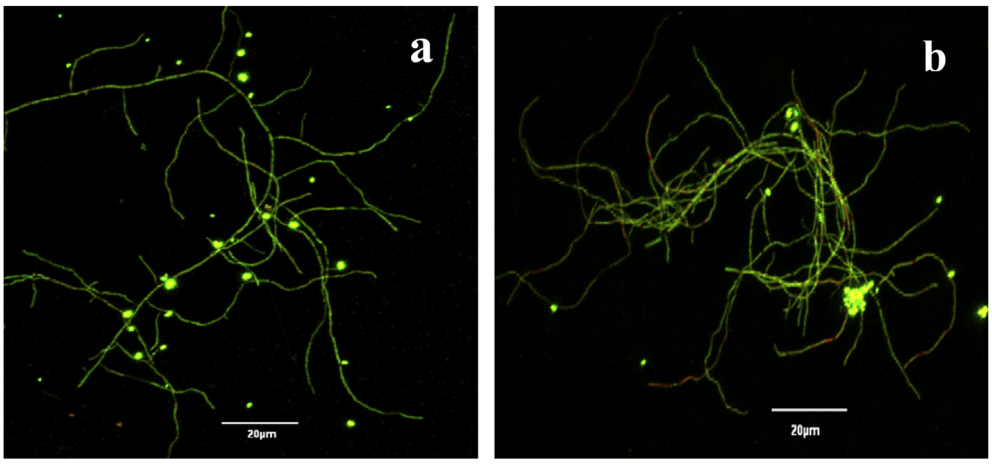

Fig. 4. Confocal laser-scanning fluorescence microscopy analysis of the *Streptomyces* spp. mixed culture exposed to 50 mg L^{-1} chlorpyrifos (CP) (a) and 12 mg L^{-1} 3,5,6-trichlo-2-pyridinol (TCP) (b). Samples were stained with SYTO 9 and propidium iodide. The red and the green colors indicate dead and living mycelium or spores, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the bacterial growth curve. Considering that no direct relationship between biomass of *Streptomyces* strains and removal of organochlorine pesticides has been reported (Benimeli et al., 2007; Fuentes et al., 2011), in our study, the ratio between the residual CP removal and biomass was calculated in order to recognize the strains with the highest ability to remove CP.

Before selecting potential microorganisms for use in mixed cultures, microbial growth and CP removal were contrasted with the effect on protein concentration and profile. In this context, changes in protein concentrations and protein profiles were observed when the strains were exposed to 50 mg L^{-1} CP. These changes could be attributed to the formation of new proteins that may be helping *Streptomyces* to tolerate CP, as observed in cyanobacteria (Kumar et al., 2011), or by the induction or synthesis of enzymes involved in CP degradation. The most studied potentially OP-degrading enzyme is OPH, which has broad substrate specificity and is able to degrade OP compounds with P–O (present in CP), P–CN, P–F and P–S bonds (Lai et al., 1995; Theriot and Grunden, 2011; Jain and Garg, 2013). The OPH enzyme has been reported and characterized for numerous fungi and bacteria able to hydrolyze CP into TCP and diethylthiophosphate (Singh, 2009; Chishti et al., 2013). According to our results, four *Streptomyces* strains presented OPH activity (0.005–0.063 U mg^{-1} protein), which was confirmed by the release of *p*-nitrophenol after paraoxon transformation using cell-free extracts. These results suggest that OPH was present in *Streptomyces* cells as an intracellular enzyme. Similar to our results, an intracellular methyl parathion hydrolase secreted by *Stenotrophomonas* sp. G1 was reported by Deng et al. (2015), which is able to degrade eight OP pesticides, including CP. Among the actinobacteria, the marine *Streptomyces venezuelae* ACT1 exhibited OPH activity, proving capable of degrading parathion (Naveena et al., 2013). However, our study is the first report of CP removal mediated by an OPH enzyme from *Streptomyces* sp. isolated from agricultural soils. According to these results, OPH activity was demonstrated in the four strains that exhibited an increase of biomass and total protein, beside of the highest CP removal. Therefore, the *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13 were selected for mixed culture formulation. The selection of these strains to form a mixed culture of *Streptomyces* spp. could favor the CP removal in the same way to that observed for diazinon (Briceño et al., 2016).

Several studies report the efficacy of mixed microbial cultures instead of single species in pesticide removal (Pino and Peñuela, 2011; Abraham et al., 2014) because microbial mixed cultures have a higher ability to adapt to stress conditions and therefore show increased microbial survival. In addition, they can increase the number of catabolic pathways available for pesticide biodegradation and can more easily prevent the accumulation of toxic compounds derived from microbial degradation (Pattanasupong et al., 2004). In the present study, CP removal by a mixed culture of *Streptomyces* sp. was studied. The results indicate that the mixed culture utilized CP as the sole carbon source for growth as well as glucose when present. Rapid CP removal from the liquid medium was observed in the presence of glucose, which indicates that CP could also be degraded co-metabolically by the mixed culture. This response is probably due to an environmental adaptation of these bacteria that were isolated previously from a CP-contaminated environment (Xu et al., 2008). On the other hand, the presence of glucose in the culture medium increased the number of microorganisms and therefore its capacity to degrade, which was verified by a lower biomass duplication time and half-life of CP than observed for the culture in the absence of glucose. This suggests that the mixed culture, and therefore its members, have the metabolic capacity to degrade the contaminant (Pino and Peñuela, 2011). Single cultures of *Streptomyces* sp. strains AC5 and AC7 demonstrated efficient CP removal in the presence of glucose (Briceño et al., 2012). In a study carried out with pure and mixed cultures of *Streptomyces* strains, *Streptomyces* sp. M7 was observed to exhibit the best CP removal capability (99%) followed by the mixed culture *Streptomyces* spp. AC5 and AC7 strains with a CP removal of 92% (Fuentes et al., 2013). The mixed culture of *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 increased in about 15–31% the CP removal. However, compared with pure cultures this increase was not significantly different. CP can therefore be effectively removed by both single and mixed cultures of *Streptomyces* spp.

The first hydrolysis intermediate produced by numerous CP-degrading microorganisms is TCP (Abraham et al., 2013; Lu et al., 2013; Jabeen et al., 2015a), recognized for its higher degree of toxicity than CP and for presenting an antimicrobial effect (Singh and Walker, 2006). In the CP removal study, TCP was detected in all treatments, and the trend showed that it was slightly incremented through time, reaching a maximum concentration close to 0.3 mg L^{-1}. Our observations showed that the *Streptomyces* mixed culture was not affected by the presence of TCP in the medium; therefore, the biomass was incremented. The literature suggests

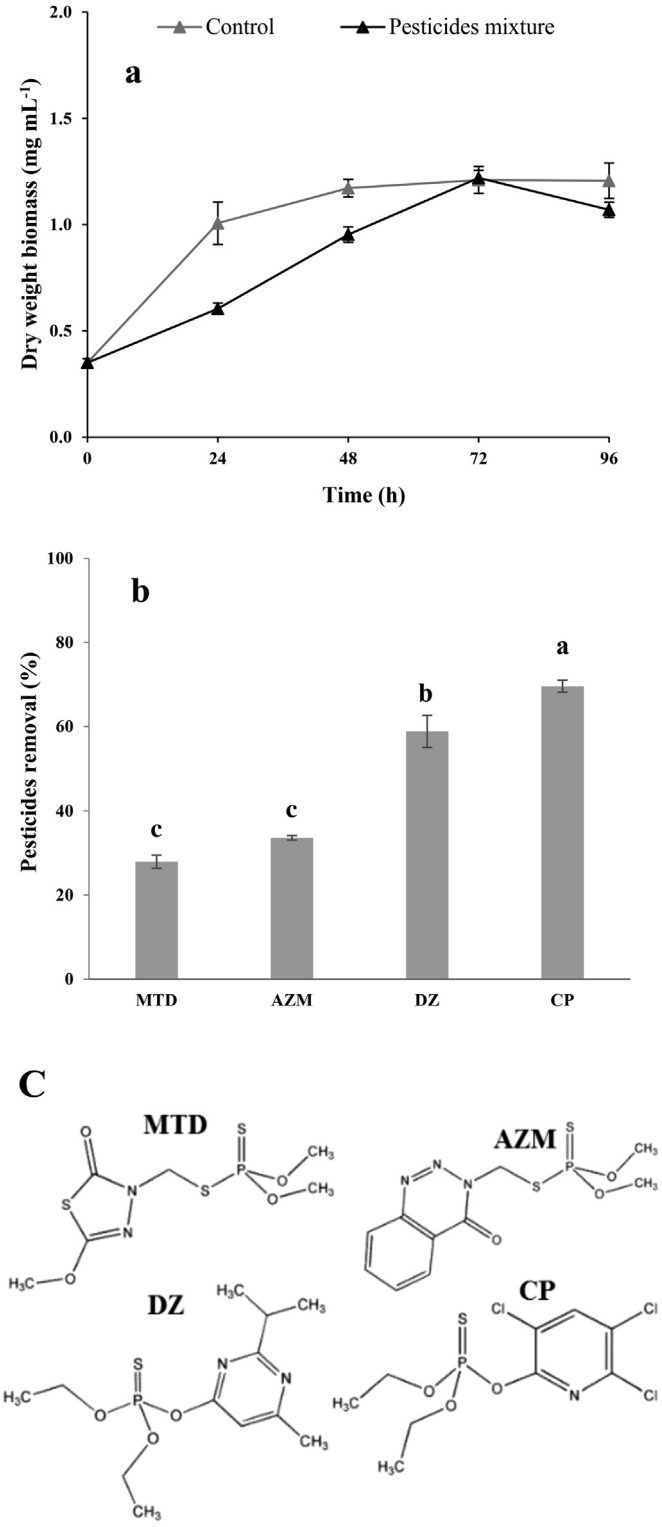

Fig. 5. Growth of the *Streptomyces* spp. mixed culture (a) and removal of methidathion (MTD), azinphos methyl (AZM), diazinon (DZ) and chlorpyrifos (CP) (b) in minimal medium supplemented with 4 g L^{-1} glucose. Chemical structures of MTD, AZM, DZ and CP (c). The error bars represent the standard error of the means of three replicates. The values with different letters indicate significant differences ($p \leq 0.05$, Tuckey test).

that aerobic bacteria tend to transform CP to TCP, which in turn accumulates in the culture medium, as microorganisms that produce the intermediates are unable to metabolize them (Singh and Walker, 2006). In our study, TCP removal by the microorganisms was tested and the *Streptomyces* mixed culture was exposed to a concentration 40 times higher than the highest concentration detected in the culture medium. Although TCP removal was not fully achieved in the evaluated time and a longer experiment is likely required (Lu et al., 2013), TCP removal (29–58%) was probably favored by the presence of the AC5 strain in the *Streptomyces* mixed culture, which has previously been associated with TCP depletion (Briceño et al., 2012). Similar to the observed results for CP removal, the addition of glucose favored TCP removal and microbial growth; therefore, the $T_{1/2}$ was reduced from 250 h to 139 h and the biomass duplication time was reduced from 36 h to 14 h, confirming that TCP at the studied concentration was not toxic and conversely that the *Streptomyces* mixed culture was unable to grow in the absence of TCP when this was supplemented as only source of nutrients. Use of TCP for growth as the only carbon source has been reported for single bacteria strains (Lu et al., 2013; Jabeen et al., 2015a). In the present study we report a DZ-degrading *Streptomyces* mixed culture that is able to remove CP and its primary metabolite, TCP. However, further assays must be performed in order to obtain a viable inoculum that can be used for repeated CP or TCP treatments. In this study, a cell viability of over 78% was observed at the end of the assay. However, it is not known if the remaining 22% represent the death of representative strains of the previously selected mixed culture.

Additionally, it was demonstrated that the *Streptomyces* mixed culture was able to remove different amounts of the four added OPs from the liquid medium. As expected, the highest removal was observed for CP (69.3%), followed by DZ (58.9%). However, based on obtained results and previous studies, the removal efficiency was lower than the efficiency detected in systems contaminated with one pesticide at a time. In this study, the removal of CP was 83% and Briceño et al. (2016) demonstrated a removal of DZ of 90% after 96 h of incubation. These results suggest that there was inhibition of the removal of the pesticides when they are treated in mixture (Pino and Peñuela, 2011). However, the mixed culture also removed MTD (27.9%) and AZM (33.6) but in reduced amounts. Differences in chemical structure of the pesticides studied would be the main cause of the observed response. Substrate specificity in OP degradation by *Stenotrophomonas* sp. G1 demonstrated that strains rapidly degraded phosphotriester compounds but not others (Deng et al., 2015). Similar to results from another microbial consortium (Dong et al., 2015), the mixed culture of *Streptomyces* spp. possessed greater abilities to remove compounds with a similar structure. In our case, CP and DZ are both thiophosphotriesters that are characterized by the presence of a P–O cleavage bond, while in phosphorothiolester compounds, such as MTD and AZM, P–S cleavage bonds are present and removal is slower. The differences in the removal of the different pesticides could be perfectly associated with the capacity of the OPH enzyme present in the members of the mixed culture; it was shown that the OPH enzyme is capable of hydrolyzing various OPs at drastically different catalytic rates (Theriot and Grunden, 2011), generally in the following order: P–O bond > P–F bond > P–S bond (Lai et al., 1995).

In practice, because diverse OPs are used to control insect pests in agricultural and domestic practices, residues are generated in different ways. OP residues are a key concern for the government and environmental science; finding a biological system than can remove one or more OPs simultaneously is therefore a central goal in bioremediation. In our case, both the individual strains of diazinon-degrading *Streptomyces* spp. assayed and the defined mixed culture may offer a promising contribution for the future development of biological treatment systems that efficiently decontaminate CP. We propose the use of a *Streptomyces* mixed culture to remove not only CP but also other OPs from liquids or other matrices in the environment.

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References

Abraham, J., Shanker, A., Silambarasan, S., 2013. Role of *Gordonia* sp. JAAS1 in biodegradation of chlorpyrifos and its hydrolyzing metabolite 3,5,6-trichloro-2-pyridinol. Lett. Appl. Microbiol. 57, 510–516.

Abraham, J., Silambarasan, S., Logeswari, P., 2014. Simultaneous degradation of organophosphorus and organochlorine pesticides by bacterial consortium. J. Taiwan Inst. Chem. E 45, 2590–2596.

Abraham, J., Silambarasan, S., 2016. Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol using a novel bacterium *Ochrobactrum* sp. JAS2: a proposal of its metabolic pathway. Pestic. Biochem. Phys. 126, 13–121.

Anwar, S., Liaquat, F., Khan, Q.M., Khalid, Z.M., Iqbal, S., 2009. Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1. J. Hazard. Mater. 168, 400–405.

Benimeli, C.S., Castro, G.R., Chaile, A.P., Amoroso, M.J., 2007. Lindane uptake and degradation by aquatic *Streptomyces* sp. strain M7. Int. Biodeterior. Biodegrad. 59, 148–155.

Briceño, G., Fuentes, M.S., Palma, G., Jorquera, M.A., Amoroso, M.J., Diez, M.C., 2012. Chlorpyrifos biodegradation and 3,5,6-trichloro-2-pyridinol production by actinobacteria isolated from soil. Int. Biodeterior. Biodegrad. 73, 1–7.

Briceño, G., Fuentes, M.S., Rubilar, O., Jorquera, M., Tortella, G., Palma, G., Amoroso, M.J., Diez, M.C., 2015. Removal of the insecticide diazinon from liquid media by free and immobilized *Streptomyces* sp. isolated from agricultural soil. J. Basic Microbiol. 55, 293–302.

Briceño, G., Schalchli, H., Rubilar, O., Tortella, G.R., Mutis, A., Benimeli, C.S., Palma, G., Diez, M.C., 2016. Increased diazinon hydrolysis to 2-isopropyl-6-methyl-4-pyrimidinol in liquid medium by a specific *Streptomyces* mixed culture. Chemosphere 156, 195–203.

Castillo, M.A., Felis, N., Aragón, P., Cuesta, G., Sabater, C., 2006. Biodegradation of the herbicide diuron by *Streptomycetes* isolated from soil. Int. Biodeterior. Biodegrad. 58, 196–202.

Cao, X., Yang, C., Liu, R., Li, Q., Zhang, W., Liu, J., Song, C., Qiao, C., Mulchandani, A., 2013. Simultaneous degradation of organophosphate and organochlorine pesticides by *Sphingobium japonicum* UT26 with surface-displayed organophosphorus hydrolase. Biodegradation 24, 295–303.

Chishti, Z., Hussain, S., Arshad, K.R., Khalid, A., Arshad, M., 2013. Microbial degradation of chlorpyrifos in liquid media and soil. J. Environ. Manag. 114, 372–380.

Deng, S., Chen, Y., Wang, D., Shi, T., Wu, X., Ma, X., Li, X., Hua, R., Tang, X., Li, Q.X., 2015. Rapid biodegradation of organophosphorus pesticides by *Stenotrophomonas* sp. G1. J. Hazard. Mater. 297, 17–24.

Dong, W., Hou, Y., Xi, X., Wang, F., Li, Z., Ye, X., Huang, Y., Cui, Z., 2015. Biodegradation of fenoxaprop-ethyl by an enriched consortium and its proposed metabolic pathway. Int. Biodeterior. Biodegrad. 97, 159–167.

Fosu-Mensah, B., Dartey, E., Darko, E., Gordon, C., 2016. Organophosphorus pesticide residues in soils and drinking water sources from cocoa producing areas in Ghana. Environ. Syst. Res. 2016, 5–10.

Fuentes, M.S., Saez, J.M., Benimeli, C.S., Amoroso, M.J., 2011. Lindane biodegradation by defined consortia of indigenous *Streptomyces* strains. Water Air Soil Pollut. 222, 217–231.

Fuentes, M.S., Briceño, G.E., Saez, J.M., Benimeli, C.S., Diez, M.C., Amoroso, M.J., 2013. Enhanced removal of a pesticides mixture by single cultures and consortia of free and immobilized *Streptomyces* strains. BioMed Res. Int. 2013, 1–9.

Jabeen, H., Iqbal, S., Anwar, S., 2015a. Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by a novel rhizobial strain *Mesorhizobium* sp. HN3. Water Environ. J. 29, 151–160.

Jabeen, H., Iqbal, S., Anwar, S., Parales, R.E., 2015b. Optimization of profenofos degradation by a novel bacterial consortium PBAC using response surface methodology. Int. Biodeterior. Biodegrad. 100, 89–97.

Jain, R., Garg, V., 2013. Enzymatic degradation of monocrotophos by extracellular fungal OP hydrolases. Appl. Biochem. Biotechnol. 171, 1473–1486.

Jauregui, J., Valderrama, B., Albores, A., Vazquez-Duhalt, R., 2003. Microsomal transformation of organophosphorus pesticides by white rot fungi. Biodegradation 14, 397–406.

Karas, P.A., Perruchon, C., Exarhou, K., Ehaliotis, C., Karpouzas, D.G., 2011. Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. Biodegradation 22, 215–228.

Kumar, N., Kumar, R., Bora, Anubhuti, Kaur, M., 2011. An evaluation of pesticide stress induced proteins in three Cyanobacterial species- *Anabaena fertilissima, Aulosira ferttilissima* and *Westiellopsis prolifica* using SDS-PAGE. Int. J. Biol. Agric. Food Biotechnol. Eng. 5, 139–146.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lai, k., Stolowich, N.J., Wild, J.R., 1995. Characterization of P-S bond hydrolysis in organophosphorothioate pesticides by organophosphorus hydrolase. Arch. Biochem. Biophys. 318, 59–64.

Lasram, M.M., Dhouib, I.B., Annabi, A., El Fazaa, S., Gharbi, N., 2014. A review on the molecular mechanisms involved in insulin resistance induced by organophosphorus pesticides. Toxicology 322, 1–13.

Li, X., He, J., Li, S., 2007. Isolation of a chlorpyrifos-degrading bacterium, *Sphingomonas* sp. strain Dsp-2, and cloning of the *mpd* gene. Res. Microbiol. 158, 146–149.

Li, C., Lan, Y., Zhang, J., Chen, Z., Tang, D., Xu, H., 2013. Biodegradation of methidathion by *Serratia* sp. in pure cultures using an orthogonal experiment design, and its application in detoxification of the insecticide on crops. Ann. Microbiol. 63, 451–459.

de Lima, R.E., da Silva, I.R., Martins, M.K., de Azevedo, J.L., de Araújo, J.M., 2012. Antibiotics produced by *Streptomyces*. Braz. J. Microbiol. 16, 466–471.

Lin, Q.S., Chen, S.H., Hu, M.Y., Haq, M.R., Yang, L., Li, H., 2011. Biodegradation of cypermethrin by a newly isolated actinomycetes HU-S-01 from wastewater sludge. Int. J. Environ. Sci. Technol. 8, 45–56.

Lu, P., Li, Q., Liu, H., Feng, Z., Yan, X., Hong, Q., Li, S., 2013. Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by *Cupriavidus* sp. DT-1. Bioresour. Technol. 127, 337–342.

Naveena, B., Annalakshmi, G., Partha, N., 2013. An efficacious degradation of pesticide by salt tolerant *Streptomyces venezuelae* ACT 1. Bioresour. Technol. 132, 378–382.

Oficina de Estudios y Políticas Agrarias (ODEPA), 2013. Mercado y proyecciones del cultivo de arandanos. Ministerio de Agricultura, Gobierno de Chile. http://www.minagri.gob.cl/wp-content/uploads/2013/08/Mercado-y-proyecciones-del-cultivo-de-arandanos.pdf.

Pattanasupong, A., Nagase, H., Sugimoto, E., Hori, Y., Hirata, K., Tani, K., Nasu, M., Miyamoto, K., 2004. Degradation of carbendazim and 2,4-dichlorophenoxyacetic acid by immobilized consortium on loofa sponge. J. Biosci. Bioeng. 98, 28–33.

Pino, N., Peñuela, G., 2011. Simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by an isolated bacterial consortium from a contaminated site. Int. Biodeterior. Biodegrad. 65, 827–831.

Pozo, K., Llanos, Y., Estellano, V.H., Cortés, S., Jorquera, H., Gerli, L., Pozo, K., Encina, F., Palma, R., Focardi, S., 2016. Occurrence of chlorpyrifos in the atmosphere of the Araucanía Region in Chile using polyurethane foam-based passive air samplers. Atmos. Pollut. Res. 7, 706–710.

Rahmansyah, M., Agustiyani, D., Julistiono, H., Dewi, T.K., 2012. Growth and adaptation of four *Streptomyces* isolates in the media containing propoxur. ARPN J. Agric. Biol. Sci. 7, 773–781.

Rezg, R., Mornagui, B., El-Fazaa, S., Gharbi, N., 2010. Organophosphorus pesticides as food chain contaminants and type 2 diabetes: a review. Trends Food Sci. Tech. 21, 345–357.

Roberts, J.R., Reigart, J.R., 2013. Recogvition and Management of Pesticide Poisoning. EPA735K13001, sixth ed. Washington D.C. www2.epa.gov/pesticide-worker-safety.

Sette, L.D., de Oliveira, V.M., Manfio, G.P., 2005. Isolation and characterization of alachlor-degrading actinomycetes from soil. A. Van Leeuw. 87, 81–89.

Singh, B., Walker, A., 2006. Microbial degradation of organophosphorus compounds. FEMS Microbiol. Rev. 30, 428–471.

Singh, B.K., 2009. Organophosphorus-degrading bacteria: ecology and industrial applications. Nat. Rev. Microbiol. 7, 156–164.

Solomon, K.R., Williams, W.M., Mackay, D., Purdy, J., Giddings, J.M., Giesy, J.P., 2014. Properties and uses of chlorpyrifos in the United States. In: Giesy, J.P., Solomon, K.R. (Eds.), Ecological Risk Assessment for Chlorpyrifos in Terrestrial and Aquatic Systems in the United States, Reviews of Environmental Contamination and Toxicology, vol. 231. http://dx.doi.org/10.1007/978-3-319-03865.

Theriot, T., Grunden, A., 2011. Hydrolysis of organophosphorus compounds by microbial enzymes. Appl. Microbiol. Biotechnol. 89, 35–43.

Verma, J., Kumar, D., Sagar, R., 2014. Pesticide relevance and their microbial degradation: a-state-of-art. Rev. Environ. Sci. Biotechnol. 13, 429–466.

Xu, G., Zheng, W., Li, Y., Wang, S., Zhang, J., Yan, Y., 2008. Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by a newly isolated *Paracoccus* sp. strain TRP. Int. Biodeterior. Biodegrad. 62, 51–56.

Yañez-Ocampo, G., Sánchez-Salinas, E., Ortiz-Hernández, M.L., 2011. Removal of methyl parathion and tetrachlorvinphos by a bacterial consortium immobilized on tezontle-packed up-flow reactor. Biodegradation 22, 1203–1213.