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Increased diazinon hydrolysis to 2-isopropyl-6-methyl-4-pyrimidinol in liquid medium by a specific *Streptomyces* mixed culture

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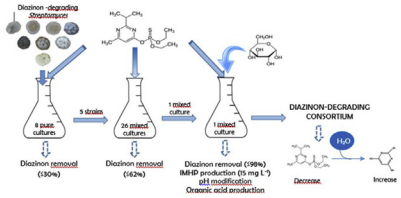
HIGHLIGHTS

- Diazinon is used as an only carbon source for growth by studied *Streptomyces*.
- Mixed cultures of *Streptomyces* spp. increase the diazinon removal.
- Formation of the diazinon metabolite 2-isopropyl-6-methyl-4-pyrimidinol was observed.
- Glucose metabolism stimulate changes in the culture increasing diazinon hydrolysis.
- Selected mixed culture of *Streptomyces* spp. remove diazinon and its metabolite.

GRAPHICAL ABSTRACT



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ABSTRACT

Actinobacteria identified as *Streptomyces* spp. were evaluated for their ability to remove diazinon as the only carbon source from a liquid medium. Single cultures of *Streptomyces* strains were exposed to diazinon at a concentration of 50 mg L^{-1}. After 96 h incubation, six of the eight cultures grew and five strains showed an increase in their total protein concentrations and changes in their protein profile. Up to 32% of the diazinon was removed by the single *Streptomyces* cultures. A compatibility assay showed that the different *Streptomyces* species were not antagonistic. Twenty-six mixed cultures were then prepared. Diazinon removal was increased when mixed cultures were used, and maximum diazinon removal of 62% was observed when the *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 were mixed; this was defined as the selected mixed culture (SMC). Diazinon removal was positively influenced by the addition of glucose into the liquid medium. Our study showed a diazinon degradation rate of 0.025 h^{-1}, half-life of 28 h^{-1} and 2-isopropyl-6-methyl-4-pyrimidinol (IMHP) production of 0.143 mg L h^{-1}. Rapid diazinon hydrolysis to IMHP was associated with a decrease in the pH of the medium as a consequence of microbial glucose metabolism and organic acid exudation. Moreover, the SMC of *Streptomyces* was able to remove IMHP. This work constitutes a new, if not the only, report on diazinon degradation by mixed

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cultures of *Streptomyces* spp. Given the high levels of diazinon removal, the SMC formed by four *Streptomyces* strains has the potential to be used to treat the diazinon present in environmental matrices.

1. Introduction

Organophosphate pesticides (OPs) represent the majority of the pesticide global market, and contamination of soil and water systems has been reported around the world due to the large-scale use of these compounds (Karpouzas and Singh, 2006). Although OPs are biodegradable, they exhibit moderate to extremely acute toxicity because they irreversibly inhibit the acetylcholinesterase in the central nervous system synapses of the target insects as well as non-target organisms, including lower vertebrates and humans, resulting in a subsequent loss of nerve function and eventual death (Zhang et al., 2006; Cao et al., 2013). Moreover, chronic exposure to low doses of OPs may result in the development of neuromuscular diseases and cancer (Yair and Amir, 2008). The World Health Organization has estimated that there are three million cases of pesticide poisoning and approximately 200,000 deaths each year, most of which are related to OPs (Theriot and Grunden, 2011; Odukkathil and Vasudevan, 2013). Therefore early detection and subsequent decontamination and detoxification of polluted pesticide wastes are essential. The accumulation of pesticide wastewaters and obsolete compounds is another problem associated with pesticide use and management. The FAO estimates that there are half a million tons of obsolete pesticides in storage worldwide, two-hundred thousand tons of which correspond to OPs (Singh, 2009). According to the National Council for Clean Production in Chile, approximately 23 tons of obsolete pesticides were eliminated and four million dollars were invested to improve the infrastructure for the accumulation of obsolete pesticides.

Among the OPs, diazinon (*O,O*-diethyl-*O*-[2-isopropyl-4-methyl -6-pyrimidinyl] phosphorothioate) is widely used throughout the world as an insecticide, acaricide and nematicide. Chile is one of the top ten global consumers of pesticides due to the amount of land devoted to agriculture (Verma et al., 2014); diazinon is used extensively as an insecticide in the fruit farming industry and as an ectoparasiticide in the livestock industry. Considering that both these activities are increasing constantly, the use of diazinon has increased. According to the Chilean Ministry of Agriculture, diazinon sales have increased by 7%, with annual sales greater than 180,000 kg or liters of this compound (SAG, 2010).

The routes by which diazinon enters the soil or water can include direct application, spray drift, run-off from treated animals, spills, accidental releases, rinsing of containers and disposal (Fenlon et al., 2011; Cycoń et al., 2013). In the environment, diazinon appears to be mobile and persistent, with a water solubility of 60 mg L^{-1} and a half-life in the range of approximately 12–138 days in water and 21–103 days in soil. Diazinon degradation can occur by a combination of processes, including chemical hydrolysis, photolysis and biodegradation (Mahiudddin et al., 2014), with 2-isopropyl-6-methyl-4-pyrimidinol (IMHP) as the main degradation by-product in water and soil (Kouloumbos et al., 2003; Karpouzas and Singh, 2006).

The methods for disposing of pesticides such as diazinon include land cultivation, disposal pits, evaporation ponds and landfills, while pesticide treatment methods include thermal treatment, chemical treatment, physical treatment and biological treatment (Al Hattab and Ghaly, 2012). Currently, the most promising methods for pesticide detoxification, which could also be complemented with other methods, are biological treatments (Yoder et al., 2001; Pino and Peñuela, 2011; Theriot and Grunden, 2011).

Several microorganisms have been described as pesticide degraders, including actinobacteria, a special group of Gram-positive bacteria. Specific *Streptomyces* strains may be well suited as inocula for pesticide treatment because these bacteria are characterized as having a mycelial growth habit, relatively rapid growth rates, colonization of semi-selective substrates, susceptibility to genetic manipulation (Alvarez et al., 2012) and an ability to produce surfactant, which may facilitate the bioavailability of the toxic compounds (Larkin et al., 2005). In addition, *Streptomyces* strains have exhibited high metabolic diversity and the ability to degrade pyrethroid, carbamate, organochlorine, chloroacetanilide and OPs (Briceño et al., 2013). OP degradation by *Streptomyces* has primarily been demonstrated with single isolates (Briceño et al., 2012; Naveena et al., 2013), which in some cases produce degradation by-products which can be more toxic than the original compounds. Thus the use of treatment systems with mixed microbial populations could be more effective at removing the toxic compounds, alone and in combination, than pure cultures (Fuentes et al., 2013). Microbial consortia have a greater ability to adapt to stress conditions and therefore increase microbial survival. In addition, they can increase the number of catabolic pathways available for pesticide biodegradation and can more easily avoid accumulation of the toxic compounds derived from microbial degradation (Fuentes et al., 2011; Abraham et al., 2014).

Little information is available on the ability of *Streptomyces* species to biotransform OPs (Naveena et al., 2013). Moreover, reports on the treatment of liquid samples contaminated with diazinon are scarce, as are studies performed using mixed cultures of microorganisms. The objectives of this work were therefore (1) to study diazinon removal in a liquid medium by pure and mixed cultures of *Streptomyces* spp.; and (2) to select an efficient *Streptomyces* mixed culture to enhance diazinon removal. The influence of glucose on diazinon removal was also studied.

2. Materials and methods

2.1. Chemicals, bacterial strains and culture media

Analytical grade diazinon and its metabolite, IMHP, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents used during the study were purchased from standard sources. Methanol stock solution of diazinon and IMHP were sterilized by filtration through 0.22 μm-pore size Biofil ® membranes and used for contamination of the media.

Eight *Streptomyces* strains that had been previously isolated from a soil sample exposed to OPs were assayed. These isolates were characterized using a genetic affiliation analysis of 16S rDNA gene fragments from the *Streptomyces* genus and for their ability to tolerate and degrade organophosphorus insecticides. The GenBank accession numbers of the 16S rRNA gene sequences of the *Streptomyces* spp. strains AC5, AC6, AC7, AC9, ISP4 and ISP13 are JQ289350, JQ289351, JQ289352, JQ289353, JQ289354 and JQ289355 respectively (Briceño et al., 2012; Briceño et al., 2015). Strains GA3 and GA11 were included in this study. They were identified using

the F341/R534 primer pair and the 16S rRNA sequences were deposited in the GenBank database under accession numbers KT271898 and KT271897, respectively.

ISP-2 medium (malt extract, 10.0 g; yeast extract, 4.0 g; glucose, 4.0 g; and distilled water, 1000 ml) was used to prepare the *Streptomyces* cultures. Liquid minimal medium (MM) (L-asparagine, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4*7H_2O$, 0.20 g; $FeSO_4*7H_2O$, 0.01 g, and distilled water, 1000 ml) was used for the degradation assays, and minimal medium agar (15 g L^{-1}) was used for the tolerance assay. 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 slant 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 at 8500 × g for 10 min at 4 °C. The supernatant was eliminated and then the cell pellets were washed with sterile 0.85% NaCl solution. The resulting biomass was used as the inoculum for the pure and mixed cultures.

2.3. Diazinon removal by pure cultures of the Streptomyces spp.

Biomass of the *Streptomyces* strains obtained in section 2.2 was added at a final concentration of 1% (*w/v*) wet weight in a flask containing 30 ml of liquid MM supplemented with diazinon (50 mg L^{-1}) as the only carbon source. The concentration of 50 mg L^{-1} was chosen because a previous qualitative screen showed that the diazinon-tolerant strains had higher growth and formed more colonies on solid medium in the presence of higher diazinon concentrations. The cultures were incubated at 28 °C for 96 h in an orbital shaker at 120 rpm. After centrifugation at 8500 × g for 10 min at 4 °C, 10 ml of the supernatant were aseptically removed to determine the residual diazinon and IMHP concentrations by HPLC. The non-inoculated flasks were used as a control. The pellets were used to estimate both the dry weight microbial growth at 105 °C and the total protein concentrations of the cell-free extracts. To determine the total protein concentrations of the cell-free extracts, the pellets were washed and re-suspended in 5 ml of phosphate buffer (0.05 M, pH 7.0). The cells were then disrupted by ultrasonication on ice and centrifuged (8500 × g, 10 min, 4 °C); the supernatants were obtained as the cell-free extract. The protein concentrations of the cell-free extracts were determined by the Bradford method using a Bio-Rad Protein Assay, with bovine serum albumin as the standard. The 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), and Coomassie blue staining.

2.4. Compatibility assay between the Streptomyces spp.

To determine growth compatibility among the strains studied, the technique described by Fuentes et al. (2011) was used. Petri dishes with solid MM were sown as follows: one *Streptomyces* spp. strain was spread in the center of the plate and was faced transversely with the other strain to be assayed. A strain was considered to be antagonistic to the other assayed strain if growth inhibition was observed. The presence of antagonism among the strains studied was assessed by considering all possible combinations.

2.5. Diazinon degradation by mixed cultures of Streptomyces spp.

The microorganisms obtained in section 2.2 were inoculated in 100-ml flasks containing 30 ml of MM with diazinon (50 mg L^{-1}) as the only carbon source, with all possible combinations of two, three, four and five strains; the final inoculum concentration of the mixed cultures was 1% (*w/v*) wet weight. The assays were performed in triplicate, and the cultures were incubated at 28 °C and 120 rpm for 96 h. Next, the cultures were centrifuged at 8500 × g for 10 min at 4 °C. Finally 10 ml of the supernatants from each mixed culture were aseptically removed to determine the residual diazinon and IMHP concentrations by HPLC. Diazinon removal at 96 h was calculated according to the initial pesticide concentration. The mixed culture that exhibited the highest diazinon degradation was selected for the subsequent assays.

2.6. Monitoring diazinon degradation by the selected mixed culture (SMC) of Streptomyces spp. in liquid medium in the presence of glucose

The SMC from section 2.5 was used to study diazinon degradation and IMHP production at different time intervals. To assess the influence of the carbon source on diazinon degradation, the MM was enriched with 2 g L^{-1} and 4 g L^{-1} of glucose, henceforth called treatments (SMC + DZ + G0), (SMC + DZ + G2) and (SMC + DZ + G4) respectively. Samples were collected at 24, 48, 96 and 168 h, and, after centrifugation, 10 ml of the supernatants were removed to determine the residual diazinon and IMHP concentrations by HPLC. In addition, the supernatant was used to determine pH and organic acid concentrations, which were included as complementary analyses to provide a better understanding of the results. Diazinon degradation and IMHP production were estimated by comparing the concentrations in the samples and controls over time.

2.7. IMHP removal by the SMC of Streptomyces spp.

IMHP removal was performed in 30 ml of MM containing 10 mg L^{-1} IMHP in the presence of 2 g L^{-1} and 4 g L^{-1} glucose, henceforth called treatments (SMC + IMHP + G0), (SMC + IMHP + G2) and (SMC + IMHP + G4) respectively. After the cultures were incubated in an orbital shaker at 28 °C for 96 h, the microbial growth and residual IMPH levels were evaluated.

2.8. Chemical analysis

Samples of the supernatants from the centrifuged cultures were filtered through a 0.22-μm filter and used to determine the pesticide and metabolite concentrations. Diazinon and IMHP were extracted with dichloromethane and ethyl acetate. For extraction, 2.5 ml of each sample was mixed with 5 ml of dichloromethane, shaken in 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 the chromatographic analyses. The efficiency of diazinon and IMHP recovery from the liquid samples was 100% and 90% respectively. The analyses were performed using a Shimadzu LC-20AT liquid chromatograph equipped with a Purospher Star RP-18e column (Merck®, film thickness 5 μm, 150 × 4.6 mm). The oven temperature was 35 °C. 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 IMHP and

diazinon were 1.59 and 4.94 min respectively. Calibration was performed using a standard for each compound, with a linear curve ranging from 0.05 to 10 mg L^{-1}.

The organic acids were analyzed by HPLC in a Symmetry C18 column (water 5 μm, 250 mm × 4.6 mm) with a Merck-Hitachi instrument, model 3400, equipped with a UV detector at 210 nm. The mobile phase was 200 mM phosphoric acid pH 2.1 at a flow of 1.0 ml min^{-1}. The organic acids analyzed in this study were gluconic, oxalic, malic, pyruvate, lactic, acetic, maleic, citric and succinic acid. The presence of the acids was confirmed by the addition of a standard into the sample.

2.9. Data analysis

To analyze diazinon degradation by the SMC of *Streptomyces* spp. a kinetic model was quantified by plotting $\ln(C_t/C_0)$ against time, where C_0 is the amount of diazinon in the liquid medium at time zero and C_t is the amount of diazinon at time *t*. A straight line was obtained for the treatments with the different glucose concentrations and the reaction followed the first-order kinetic model $\ln C_t/C_0 = e^{-kt}$, where *k* and *t* are the rate constant and degradation time in hours respectively. The time at which the pesticide concentration in the liquid medium was reduced by 50% ($T_{1/2}$) in hours was calculated using the equation $T_{1/2} = \ln(2)/k$. The IMHP production (Q_P) was determined by calculating the slope of the respective plots versus time (h). The specific growth rate (μ) was determined by plotting the $\ln(B_t/B_0)$ 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.

All of the experiments were performed in triplicate. The data were statistically analyzed using one-way analysis of variance (ANOVA). When significant differences were observed, the means were analyzed by Tukey's minimum significant differences test ($p \leq 0.05$).

3. Results

3.1. Diazinon removal by pure cultures of Streptomyces spp.

The results for microbial growth, total protein content of the biomass and diazinon removal by pure cultures of *Streptomyces* spp. are shown in Table 1. The highest biomasses and protein concentrations were observed for strains AC5, AC7, AC9, GA11 and ISP13. While strain ISP4 showed a slight increase, strains AC6 and GA3 were the only strains that were affected by the presence of 50 mg L^{-1} of diazinon, and their biomasses and total protein contents were reduced after 96 h incubation.

The protein analysis performed using SDS-PAGE (Fig. 1) showed increased intensities and more protein bands in the lane that represented the strains cultured in the presence of diazinon. The AC9, GA11 and ISP13 strains exhibited marked effects in their protein profiles, while a close inspection of the images indicated that when strains AC5, AC6 and GA3 were exposed to diazinon, new bands appeared that were not present in the profile without the contaminant. Finally, *Streptomyces* spp. strains AC7 and ISP4 showed a similar profile to that observed in the MM without diazinon.

Diazinon removal after 96 h incubation was significantly different ($p \leq 0.05$) when pure cultures of *Streptomyces* spp. strains AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13 were studied (Table 1). At this time point, the highest diazinon removal was exhibited by the ISP13, AC7, AC9 and GA11 strains, which reached values of 30%; the ISP4 and AC5 strains showed 21% and 24% removal respectively, while the lowest degradation (11–16%) was exhibited by the AC6 and GA3 strains.

According to these results, *Streptomyces* spp. AC7, AC9, GA11 and ISP13 fulfilled many of the requirements for selection and use in the subsequent assays. However, we wanted to include the AC5 strain due to its capacity to degrade other OPs, such chlorpyrifos (Briceño et al., 2012).

3.2. Compatibility assay between the Streptomyces spp.

The compatibility assay performed between the *Streptomyces* spp. selected in section 3.1 showed that the *Streptomyces* sp. AC7 strain was the only one that presented a slight decrease in growth when faced with the AC5 strain (Fig. 2). However, there was no effect on the individual growth of the other strains, which suggested that the strains could be cultured together as a mixed culture. In our case, despite the fact that the AC7 strain was difficult to grow, we did not want to exclude any previously selected strain. The next step was to grow the strains together to determine their combined effect on diazinon removal.

3.3. Selection of a mixed culture of Streptomyces spp. for diazinon removal

In this study, 26 different combinations were tested to obtain and select a mixed culture with the highest diazinon removal capacity. Diazinon removal by the mixed cultures of *Streptomyces* spp. was significantly different ($p \leq 0.05$) (Table 2), ranging between 13 and 37% (average 30%), 31–46% (average 39%) and 43–62% (average 49%) for the mixed cultures formed by two, three and four strains respectively, after 96 h incubation. The minimum diazinon removal was observed for the mixed cultures of AC5-AC7, AC7-AC9-ISP13 and AC5-AC7-AC9-GA11, while the highest value of diazinon degradation (62%) was observed for the mixed culture formed by the *Streptomyces* spp. strains AC5-AC9-GA11-ISP13. In this study, the combination of five strains removed 25% of the

Table 1
Biomass growth, total protein content from cell-free extracts and diazinon removal by pure cultures of *Streptomyces* spp. AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13.

Strain	Biomass growth* (mg mL^{-1})	Total protein* (mg mL^{-1})	Diazinon removal (%)
Streptomyces sp. AC5	0.17 ± 0.01 abc	0.13 ± 0.01 c	23.7 ± 0.4 ab
Streptomyces sp. AC6	n.g	n.i	10.8 ± 0.8 c
Streptomyces sp. AC7	0.23 ± 0.07 ab	n.i	31.7 ± 0.7 a
Streptomyces sp. AC9	0.16 ± 0.01 abc	0.65 ± 0.04 b	30.9 ± 3.7 a
Streptomyces sp. GA3	n.g	n.i	16.2 ± 0.5 bc
Streptomyces sp. GA11	0.04 ± 0.02 c	0.77 ± 0.08 a	29.8 ± 1.2 a
Streptomyces sp. ISP4	0.01 ± 0.00 c	0.02 ± 0.00 c	20.7 ± 2.6 abc
Streptomyces sp. ISP13	0.32 ± 0.07 a	0.78 ± 0.01 ab	31.7 ± 0.7 a

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 biomass growth or increment of protein content was respect the observed in the control treatment (without diazinon). No growth = n.d and no increment = n.i.

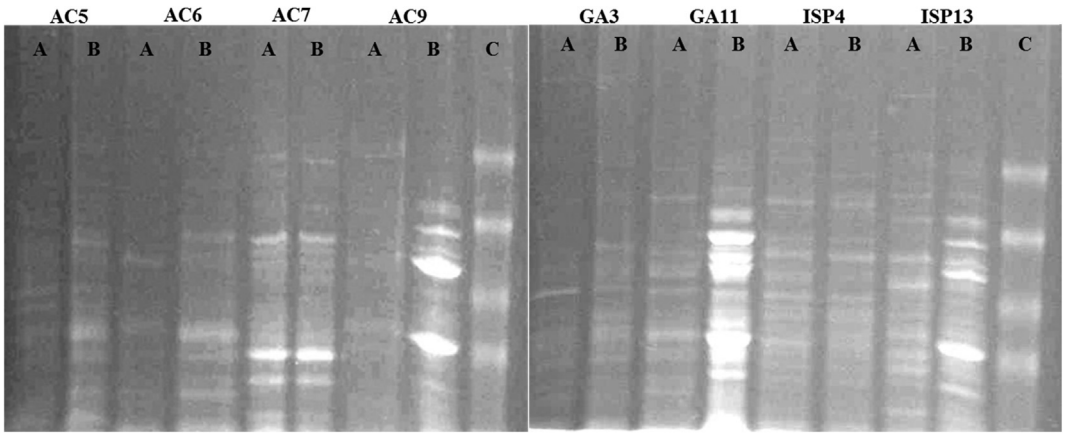

Fig. 1. Denaturing PAGE analysis of protein from *Streptomyces* spp. AC5, AC6, AC7, AC9, GA3, GA11, ISP4 and ISP13. The cells were cultured in MM medium with 50 mg L^{-1} diazinon. Lane A: without diazinon, lane B: with diazinon, and lane c: molecular weight marker.

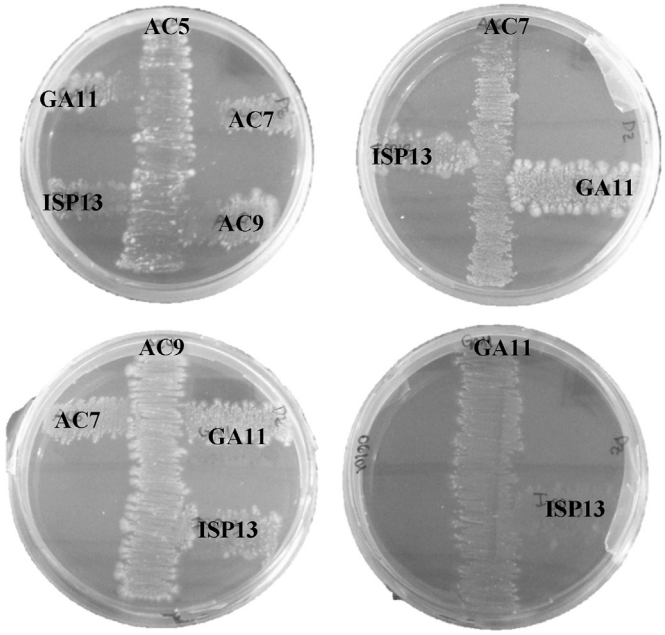

Fig. 2. Compatibility assay among *Streptomyces* spp. strains AC5, AC7, AC9, GA11 and ISP13.

diazinon; this mixed culture was therefore discarded in the subsequent assays.

In addition to diazinon degradation, we evaluated the production of its metabolite, IMHP. The results showed that after 96 h, IMHP production was significantly different ($p \leq 0.05$) in the different mixed cultures of *Streptomyces* (Table 2). In general, the concentrations of IMHP were between 0.060 and 0.950 mg L^{-1}.

Considering the above results, and that we were interested in obtaining the mixed culture that exhibited the highest diazinon removal, the mixed culture formed by the *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains was defined as the selected mixed culture (SMC) and used in the subsequent assays.

3.4. Diazinon removal at different times and in the presence of different concentrations of glucose by the SMC of Streptomyces spp.

Residual diazinon and IMHP production were studied at 24, 48, 96 and 168 h after the addition of 50 mg L^{-1} diazinon and different glucose concentrations to the liquid medium (Fig. 3a). The results revealed no difference in diazinon removal by the SMC + DZ + G0- and SMC + DZ + G2-treated cultures. In both of the treated cultures, approximately 10, 20, 50 and 55% of the diazinon was removed after 24, 48, 96 and 168 h incubation respectively. Diazinon removal increased significantly ($p \leq 0.05$) in the SMC + DZ + G4-treated cultures; approximately 70% and 98% of the diazinon was removed after 48 h and 168 h of incubation respectively. The kinetic data showed that diazinon removal by the SMC + DZ + G0- and SMC + DZ + G2-treated cultures was characterized by rate constants of 0.005 h^{-1} and $T_{1/2}$ of 134 h^{-1} respectively, while a rate constant of 0.025 h^{-1} and $T_{1/2}$ of 28 h^{-1} was observed in the SMC + DZ + G4-treated culture (Table 3).

In parallel to diazinon removal from the liquid medium, the IMHP concentration increased over time (Fig. 3a). When diazinon was removed as the only carbon source (SMC + DZ + G0), IMHP increased from 0.09 mg L^{-1} to 0.29 mg L^{-1}. Similarly, the IMHP concentrations increased from 0.25 mg L^{-1} to 0.61 mg L^{-1} in the SMC + DZ + G2-treated cultures, while a significant increase ($p \leq 0.05$) in IMHP production was observed in the SMC + DZ + G4-treated cultures. In this culture, the rate of IMHP production was 0.143 mg L h^{-1} (Table 3); therefore, an IMHP concentration of approximately 15 mg L^{-1} was produced after 96 h incubation. At this time point, approximately 34% of the IMHP had disappeared from the liquid media.

Microbial growth increased significantly ($p \leq 0.05$) in the presence of glucose addition after 24 h incubation, but this was not the case for diazinon (Fig. 3b). For the SMC + DZ + G2- and SMC + DZ + G4-treated cultures, the specific growth rates were 0.037 and 0.042 and required 19 h and 17 h respectively for the mixed cultures to double their biomass. Approximately 168 h were required for the mixed culture to double its biomass when diazinon was the only carbon source (Table 3).

The addition of glucose significantly ($p \leq 0.05$) modified the pH values of the liquid media. The results showed (Fig. 3c) that the pH increased to over 8.20 after 24 h of incubation in SMC + DZ + G0. The pH value dropped after 24 h of incubation in SMC + DZ + G2 and then increased to approximately 7.70. The pH decreased after incubation in SMC + DZ + G4 and reached its lowest value of approximately 4.0 after 168 h of incubation. These results were observed in the absence and presence of diazinon.

Finally, analysis of the organic acids in the supernatant of SMC + DZ + G4 identified that 2.2–37.0 mg L^{-1} malic acid, 16.6–38.2 mg L^{-1} succinic acid and 3.7–101.4 mg L^{-1} oxalic acid were present.

Table 2
Diazinon removal and IMHP production by mixed cultures of *Streptomyces* spp. after incubation for 96 h. The mixed cultures were formed with two, three, four and five strains.

Strains	Mixed culture	Diazinon removal (%)	IMHP production (mg L^{-1})
Two	AC5-AC7	13.0 ± 0.6 e	0.242 ± 0.003 b
	AC5-AC9	16.6 ± 1.1 e	0.361 ± 0.027 b
	AC5-GA11	31.9 ± 3.2 cd	0.170 ± 0.057 b
	AC5-ISP13	35.2 ± 0.2 c	0.176 ± 0.068 b
	AC7-AC9	33.4 ± 0.1 cd	0.258 ± 0.071 b
	AC7-GA11	36.5 ± 2.0 c	0.216 ± 0.006 b
	AC7-ISP13	31.9 ± 1.8 cd	0.341 ± 0.033 b
	AC9-GA11	36.8 ± 1.2 c	0.232 ± 0.023 b
	AC9-ISP13	36.7 ± 1.7 c	0.256 ± 0.034 b
	GA11-ISP13	34.2 ± 0.5 c	0.239 ± 0.018 b
Three	AC5-AC7-AC9	39.9 ± 2.2 c	0.160 ± 0.020 b
	AC5-GA11-ISP13	38.8 ± 1.9 c	0.195 ± 0.021 b
	AC5-AC9-ISP13	43.3 ± 1.7 bc	0.211 ± 0.041b
	AC5-AC9-GA11	44.9 ± 0.5 bc	0.207 ± 0.007b
	AC7-AC9-GA11	46.2 ± 2.8 b	0.172 ± 0.025 b
	AC7-GA11-ISP13	44.0 ± 2.1 bc	0.193 ± 0.021 b
	AC9-GA11-ISP13	35.0 ± 2.7 c	0.760 ± 0.030 ab
	AC7-AC9-ISP13	31.0 ± 3.3 cd	0.060 ± 0.005 c
	AC5-AC7-ISP13	31.7 ± 2.9 cd	0.950 ± 0.040 a
	AC5-AC7-GA11	37.8 ± 4.1 c	0.770 ± 0.030 ab
Four	AC5-AC7-AC9-ISP13	48.9 ± 0.2 b	0.078 ± 0.008 c
	AC5-AC7-AC9-GA11	41.6 ± 0.8 bc	0.253 ± 0.033 b
	AC7-AC9-GA11-ISP13	45.3 ± 2.7 bc	0.196 ± 0.048 b
	AC5-AC9-GA11-ISP13	61.7 ± 3.6 a	0.587 ± 0.063 ab
	AC5-AC7-GA11-ISP13	49.8 ± 4.5 b	0.315 ± 0.066 b
Five	AC5-AC7-AC9-GA11-ISP13	25.1 ± 2.3 d	0.201 ± 0.001 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).

3.5. IMHP removal

The SMC consisting of the *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains that had been exposed to 10 mg L^{-1} IMHP showed an increase in biomass growth, and this trend was mainly observed in the cultures treated with SMC + IMHP + G2 and SMC + IMHP + G4. Glucose addition and the subsequent increase in biomass did not have any effect on IMHP removal. Approximately 30–50% of the IMHP was removed by the mixed culture, with the highest removal in the liquid medium without an additional carbon source (Fig. 4).

4. Discussion

The present study showed that single cultures of *Streptomyces* spp., with the exception of the AC6 and GA3 strains, used diazinon as a carbon and energy source for growth. The increased microbial growth was confirmed by the increase in the total protein content of the biomass. Changes in the protein profile due to the synthesis of a new set of proteins and increases in the levels of expression of some proteins were observed in the diazinon-treated cultures. This was likely due to the capacity of these bacteria to adapt to the stressful conditions created by diazinon exposure, as has been observed for cyanobacteria exposed to different pesticides (Kumar et al., 2011). Alternatively, the presence of diazinon in the culture medium could induce the expression of some enzymes in the *Streptomyces* strains that could be responsible for degrading this contaminant, such as phosphotriesterases or organophosphorus hydrolases, a group of enzymes that can degrade OP compounds and have been isolated from various microorganisms (Singh, 2009).

In this study, the single cultures of *Streptomyces* that had previously been isolated from agricultural soil with a history of OP application were able to grow and to remove different amounts of diazinon. Previous research by Briceño et al. (2012, 2015) reported that single cultures of *Streptomyces* spp. can degrade chlorpyrifos and diazinon. In general, compared to other contaminants, microbial biodegradation of diazinon by actinobacteria has been poorly studied. Gunner and Zhukerman (1968) studied an *Arthrobacter* spp. and a *Streptomyces* spp. for diazinon degradation in soil. Therefore, this study may be the first report of diazinon removal by *Streptomyces* strains that are able to utilize the contaminant as sole carbon source in liquid samples.

The most recent studies report that bacterial species, such as *Serratia* spp., *Pseudomonas* spp. (Cycoń et al., 2009), *Burkholderia* spp., and *Brevundimonas* spp. (Mahiudddin et al., 2014), epiphytic yeasts, such as *Rhodotorula glutinis* and *Rhodotorula rubra* (Bempelou et al., 2013), and *Stenotrophomonas* spp. G1 isolated from sludge (Deng et al., 2015) have the ability to degrade diazinon. In most cases, these bacteria have been studied as single cultures. However, microbial consortia are recognized to be more efficient in the removal of pollutants than pure cultures because they can increase the number of catabolic pathways available for pesticide biodegradation, more easily prevent the accumulation of toxic compounds derived from microbial degradation, and degrade mixtures of pesticides into non-toxic metabolites (Pattanasupong et al., 2004; Abraham et al., 2014). Because microorganisms living in a common environment are known to compete for space and resources, and because individuals of the same species can compete with each other (Hibbing et al., 2010), we performed an antagonism assay between the *Streptomyces* spp. strains AC5, AC7, AC9, GA11 and ISP13 to determine whether they presented an antagonistic effect. Fuentes et al. (2013) did not report any antagonistic effects between *Streptomyces* spp. isolated from soil and sediment environments in Chile and Argentina. Therefore the formation of different mixed cultures resulted in the simultaneous degradation of an organochlorine and an organophosphorus compound. In our case, the AC7 strain exhibited a slight decrease in growth when was faced with the AC5 strain; therefore this combination, which exhibited minimum diazinon removal, was excluded. To obtain and

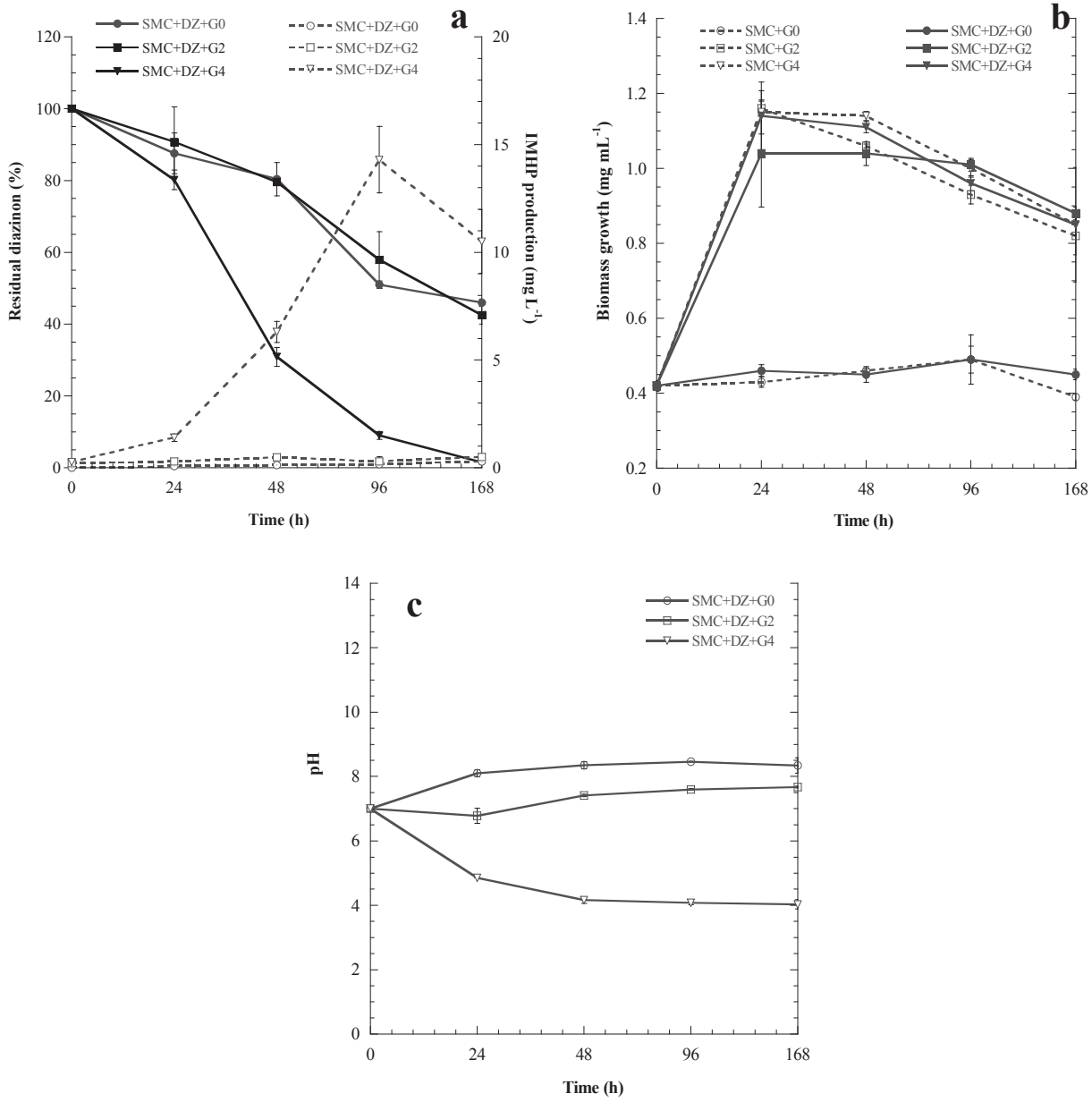

Fig. 3. Diazinon removal (%) and IMHP production (a), biomass growth of SMC formed by the *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains (b) and pH of the liquid medium (c). Diazinon applied at a concentration of 50 mg L^{-1} in the liquid culture supplemented with 0 g L^{-1} (SMC + DZ + G0), 2 g L^{-1} (SMC + DZ + G2) and 4 g L^{-1} glucose (SMC + DZ + G4).

Table 3
First-order kinetics parameter for diazinon degradation, IMHP production rate, specific growth rate (μ) and biomass duplication time of the SMC of *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains in liquid medium supplemented with 0 (SMC + DZ + G0), 2 g L^{-1} (SMC + DZ + G2) and 4 g L^{-1} glucose (SMC + DZ + G4).

Parameters	Treatment		
	SMC + DZ + G0	SMC + DZ + G2	SMC + DZ + G4
Regression equation	0.016 + 0.005x	−0.01 + 0.005x	−0.285 + 0.028x
k (h^{-1})	0.005 ± 0.000	0.005 ± 0.001	0.025 ± 0.002
$T_{1/2}$ (h^{-1})	134.7 ± 5.5	133.5 ± 9.8	27.8 ± 3.1
R^2	0.998	0.985	0.994
IMHP production (mg L h^{-1})	0.002 ± 0.000	0.006 ± 0.001	0.143 ± 0.010
Specific growth rate (μ)	0.004 ± 0.001	0.037 ± 0.006	0.042 ± 0.003
Biomass duplication time (h^{-1})	168.3 ± 17.1	19.0 ± 2.4	16.8 ± 1.1

select a mixed culture with the highest diazinon removal capacity, 26 different combinations of mixed cultures were tested, and this assay did indeed show increased diazinon removal, particularly when mixed cultures of four *Streptomyces* spp. were assayed. In our

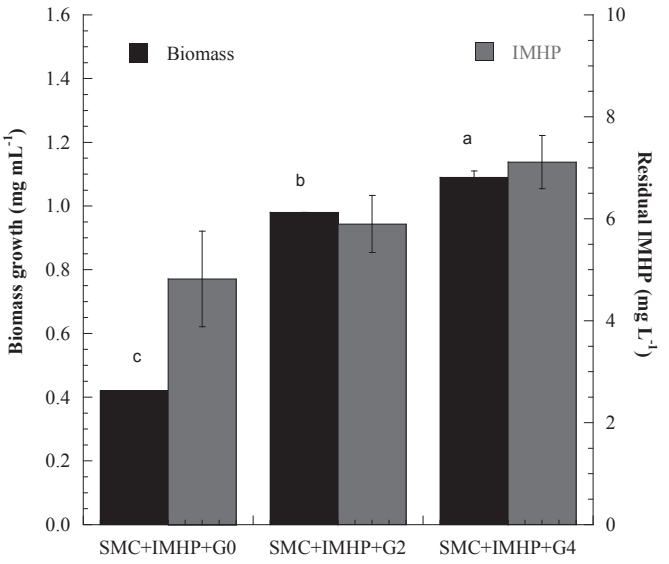

Fig. 4. Residual IMHP and microbial growth of the SMC formed by the *Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains after 48 h incubation. IMHP applied at a concentration of 10 mg L^{-1} in the liquid culture supplemented with 0 g L^{-1} (SMC + IMHP + G0), 2 g L^{-1} (SMC + IMHP + G2) and 4 g L^{-1} glucose (SMC + IMHP + G4).

study, the combination of five strains removed approximately 25% of the diazinon and was therefore discarded for diazinon degradation. Similar to our results, Fuentes et al. (2011) showed that the combination of four *Streptomyces* spp. strains showed the highest lindane removal, while the combinations of five strains and six strains showed a decrease in contaminant removal, likely due to an inhibitory effect between strains. The compatibility test performed in this study is a simplistic method to provide useful information about the behavior of these microorganisms (Fuentes et al., 2011). In mixed cultures, interactions with poorly understood mechanisms may occur which may produce metabolites with antagonistic properties (killing the strain or inhibiting growth) (Abdelmohsen et al., 2015). According to our results, strain AC7 was slightly inhibited by strain AC5 (Fig. 2). The SMC which had the highest diazinon removal therefore did not include the AC7 strain.

In addition to diazinon removal by the mixed cultures, we evaluated the production of IMHP, its principal metabolite. IMHP was produced by *Pseudomonas* spp. and *Bacillus* spp. bacteria isolated from soil after 10, 21 and 30 days of incubation in liquid samples (Thabit and El-Naggar, 2013). In our studies, the presence of IMHP after 96 h indicates the rapid removal of diazinon as the only carbon source by the different *Streptomyces* mixed cultures.

Once we had selected the mixed culture (*Streptomyces* spp. AC5, AC9, GA11 and ISP13 strains) that favored the highest diazinon removal (68%) in the absence of an additional carbon source, the addition of glucose in the liquid medium at different times was evaluated. The results showed that glucose influenced the specific growth rate, explaining the decrease in the biomass duplication time of the culture that was observed. Moreover, the addition of 4 g L^{-1} glucose significantly increased the diazinon degradation rate as well as IMHP production. Enhanced microbial growth and diazinon removal in a liquid medium supplemented with glucose have been reported for different bacteria (Cycoń et al., 2009; Mahiudddin et al., 2014), including *Streptomyces* spp. isolated from soil (Sethunathan and MacRae, 1969).

Diazinon removal may occur via biological degradation under neutral conditions and via chemical decomposition through hydrolysis under acidic conditions (Cycoń et al., 2009). According to Drufovka et al. (2008), diazinon removal below pH 4.5 predominately occurs through chemistry directly, while above pH 4.5 the microorganisms elicit changes that favor diazinon degradation by a chemical process. In our study, diazinon was removed from the liquid medium by the *Streptomyces* mixed culture under neutral and basic conditions, while glucose addition (4 g L^{-1}) indirectly increased diazinon hydrolysis and thus IMHP production, mainly after 24 h of incubation, when the liquid medium reached a pH value of less than 4.5. The acid pH of the medium at this time point is a consequence of the microbial glucose metabolism and the accumulation of organic acids (Cycoń et al., 2009; Abo-Amer, 2011). In this study, we identified the production or excretion of oxalic acid, succinic acid and malic acid by the *Streptomyces* mixed culture at concentrations favoring medium acidification. Organic acid excretion by pure *Streptomyces* strains was previously observed during chlorpyrifos removal (Briceño et al., 2012).

IMHP is recognized as the main diazinon metabolite that is produced in water, soil and compost by hydrolysis, photolysis and microbiological transformation (Kouloumbos et al., 2003; Drufovka et al., 2008; Thabit and El-Naggar, 2013). Although IMHP is potentially leachable due to its increased polarity, it is less toxic than diazinon (Kouloumbos et al., 2003). In our study, IMHP production by diazinon hydrolysis was clearly confirmed. The selected *Streptomyces* mixed culture favored IMHP production (0.143 mg L h^{-1}) in the liquid medium, with a residual concentration of approximately 15 mg L^{-1} after 96 h. However decreased concentrations of IMHP were observed at later times. This decrease in IMHP was corroborated by the microorganisms ability to grow and remove between 30% and 50% of the IMHP after 48 h incubation. Bacteria with the ability to utilize IMHP as a carbon, nitrogen and energy source for growth were obtained from a chemostat and characterized for their ability to hydrolyze parathion (Sprenger et al., 2003). On the other hand, a member of the SMC, *Streptomyces* spp. strain AC5, possesses the ability to degrade 3,5,6-trichloro-2-pyridinol, which is recognized as the main metabolite of chlorpyrifos (Briceño et al., 2012). Therefore, the incorporation of this strain in the diazinon-degrading mixed culture could be beneficial, as IMHP may be used by one or more members of the consortium.

In general, microbial consortia have been shown to efficiently remove single OP compounds and mixtures of OP compounds. Pino and Peñuela (2011) showed that a bacterial consortium formed by *Acinetobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Citrobacter* spp., *Stenotrophomonas* spp. and others isolated from contaminated soils were able to degrade 150 mg L^{-1} of methyl parathion and chlorpyrifos. A bacterial consortium formed by bacteria such as *Alcaligenes* spp., *Ochrobactrum* spp., *Sphingobacterium* spp. and others isolated from agricultural fields that had previously been exposed to pesticide contamination efficiently removed 300 mg L^{-1} chlorpyrifos and 1000 mg L^{-1} monocrotophos (Abraham et al., 2014). Diazinon degradation by single cultures and a consortium formed by *Serratia liquefaciens*, *Serratia marcescens* and *Pseudomonas* spp. was studied by Cycoń et al. (2009), who reported that 80–92% of the insecticide added as the only carbon source was degraded within 14 days. Little information is available about pesticide degradation by actinobacterial consortia compared to other groups of bacteria. Studies performed by Fuentes et al. (2011, 2013) have demonstrated that organochlorine contaminants, such as lindane and pentachlorophenol, and the organophosphorus insecticide chlorpyrifos, can be degraded by an actinobacterial consortium formed by *Streptomyces* spp. isolated from contaminated soils.

Several researchers have focused on pesticide degradation by microorganisms, which is the most viable option for remediation of agricultural soils. However, efforts are being devoted to treating pesticide-containing wastewater (Yoder et al., 2001; Al Hattab and

Ghaly, 2012). Pesticide removal by bacterial consortia in a bioreactor has been very successful (Yañez-Ocampo et al., 2011). Therefore, we expect that the SMC of *Streptomyces* spp. could be used to efficiently treat liquid samples containing diazinon, as well as other OP compounds.

5. Conclusions

In conclusion, we showed that *Streptomyces* isolated from soil have the ability to degrade the insecticide diazinon as the only carbon source in a liquid medium. The study demonstrated the effectiveness of using a mixed culture instead of single cultures of *Streptomyces* spp. for diazinon removal in liquid medium. The combination of the *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 removed the highest amount of diazinon, 62%, and this combination was selected as the best mixed culture of actinobacteria for diazinon removal. The addition of glucose also increased the specific biomass growth rate of the SMC, the diazinon degradation rate and IMHP production. Increased diazinon hydrolysis was favored by a strong decrease in the pH of the liquid medium, indirectly caused by the organic acids produced by the SMC of *Streptomyces* spp. as part of glucose metabolism. The ability of the SMC to remove diazinon from the liquid medium was complemented by its ability to remove the associated metabolite, IMHP. The results of this study will help to improve current technologies for the biodegradation of this commonly used insecticide by a group of *Streptomyces* which has barely been studied for the removal of toxic OP compounds.

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