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Removal of the insecticide diazinon from liquid media by free and immobilized *Streptomyces* sp. isolated from agricultural soil

Gabriela Briceño[1,2,4], María S. Fuentes[3], Olga Rubilar[1,2], Milko Jorquera[2,4], Gonzalo Tortella[1,2], Graciela Palma[2,4], María J. Amoroso[3,5] and Maria Cristina Diez[1,2]

[1] Department of Chemical Engineering, University of La Frontera, Temuco, Chile
[2] Scientific and Technological Bioresource Nucleus, University of La Frontera, Temuco, Chile
[3] Planta Piloto de Procesos Industriales Microbiológicos (Proimi-Conicet), Avenida Belgrano y Pasaje Caseros, Tucumán, Argentina
[4] Department of Chemical Science and Natural Resources, University of La Frontera, Temuco, Chile
[5] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

From an agricultural soil that had received continuous applications of organophosphorus pesticides, 30 actinobacteria strains were isolated. Two strains, identified as *Streptomyces* sp. AC1-6 and *Streptomyces* sp. ISP4, were selected because of their tolerance to diazinon and based on the relationship between diazinon removal and microbial growth. In liquid medium with diazinon at concentrations of 25 and 50 mg L^{-1}, both strains were able to remove approximately 40–50% and 70–90% of the initial diazinon after 24 and 96 h of incubation, respectively. This diazinon removal was accompanied by microbial growth of the strains, an initial pH decrease, and glucose consumption in the liquid medium. Evaluation of the diazinon removal achieved by the free actinobacteria and *Streptomyces* sp. AC1-6 immobilized on alginate beads revealed that the immobilized cells exhibited a 60% higher diazinon removal compared with the free cells. The reusability of the encapsulated biomass was confirmed, and a diazinon removal rate of more than 50% was obtained after the second batch. This work constitutes one of the few reports that describe *Streptomyces* strains as diazinon degraders. Given the high diazinon removal found, the streptomycetes exhibit suitable potential as diazinon-degrading actinobacteria for elimination of diazinon from liquid residues.

Abbreviations: OP – organophosphorus pesticide; **MM** – minimal medium; **IMHP** – 2-isopropyl-4-methyl-6-hydroxypyrimidine; **IMP** – 2-isopropyl-6-methyl-4-pyrimidinol

Keywords: *Streptomyces* / Diazinon / Free cells / Immobilized cells / Ca-alginate beads

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Introduction

Organophosphorus pesticides (OPs) have been the most widely used group of pesticides in the agricultural industry for more than 40 years. Among OPs, diazinon is an insecticide and acaricide used for the control of insects in crops, ornamentals, lawns, fruits, and vegetables. Moreover, diazinon has been used as an ectoparasiticide formulation for sheep and cattle [1]. In Chile, diazinon is extensively used in the fruit farming and the livestock industry. Because both of these activities are constantly increasing, the use of diazinon has also been increasing, which most likely increases the risk of environmental contamination and health hazards [2].

The route to soil or water for diazinon includes the handling of the pesticide in the farmyard, the run-off from treated animals, spillages, or accidental releases, the rinsing of containers, and disposal [3]. Diazinon presents a solubility of 60 mg L^{-1} and a half-life in the range of approximately 70 h to 12 weeks in surface water

Correspondence: Dr. Gabriela Briceño, Scientific and Technological Bioresource Nucleus, University of La Frontera, Av. Francisco Salazar N° 01145, P.O. Box 54-D, Temuco, Chile
E-mail: gabriela.briceno@ufrontera.cl
Phone: +56 45 592126
Fax: +56 45 734193

and of 10–200 days in soil. Diazinon released in surface water or soil can be degraded by a combination of photolysis, hydrolysis, and biodegradation. The main route of diazinon biodegradation in soil is through cleavage of the P–O pyrimidine group to produce 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP), which is a persistent and less toxic product [4].

Studies on microbial degradation are useful for the development of bioremediation strategies for the decontamination of water or soils contaminated with pesticides. A review by Karpouzas and Singh [5] explained the first reports of diazinon-degrading microorganisms. In this context, bacterial strains such as from the genera *Arthrobacter* and *Streptomyces* were able to use diazinon as a sole source of carbon; the *Flavobacterium* strain ATCC 27551 was able to decompose 95% of the diazinon within 24 h, producing large amounts of metabolites. Recently, *Serratia marcescens* DI101 isolated from agricultural soil by using an enrichment technique was able to completely degrade diazinon at 50 mg L^{-1} in mineral salt medium within 11 days [6].

The use of microorganisms isolated from soil contaminated with pesticides could increase the degradation of these toxic compounds because these microorganisms have adapted to this contaminated habitat. Actinobacteria constitute a significant fraction of the microbial population in soils [7]. These microorganisms have been recognized as having great potential for the degradation of pesticides. In particular, actinobacteria of the genus *Streptomyces* have the ability to degrade diuron [8], lindane, chlordane, methoxychlor [9, 10], cypermethrin [11], and chlorpyrifos [12].

A very promising technique that has been gaining increasing attention during the last year is cell immobilization. Immobilized cells present several advantages over freely suspended cells, such as a higher cellular content in the support, enhanced cellular viability, greater tolerance to high concentrations of pollutants, the reuse of the cells, easier solid–liquid separation, and reduced competition with the indigenous microflora [13]. One of the most suitable methods for cell immobilization is the entrapment of cells in calcium alginate, because this technique is simple and inexpensive and does not exert toxic effects on the cells [14]. A review by Briceño *et al.* [15] reports that immobilized actinobacteria have been assayed using pesticides such as 2,4,6-trichlorophenol, *p*-nitrophenol, atrazine, and lindane. Recently, the removal of chlorpyrifos and a pentachlorophenol mixture by a mixed culture of *Streptomyces* spp. immobilized in Ca-alginate was reported by Fuentes *et al.* [16]. However, no information is available on the use of immobilized actinobacteria for diazinon removal from liquid waste. Therefore, the aim of this work was to isolate and characterize diazinon-degrading actinobacteria that were isolated from agricultural soil with a history of OP applications, and to compare the removal of diazinon using free actinobacteria and actinobacteria immobilized onto alginate beads as a model support. The reusability of the immobilized cells for diazinon removal was also examined.

Materials and methods

Isolation and screening of diazinon-tolerant actinobacteria

Actinobacteria were isolated from soil exposed to continuous applications of chlorpyrifos for a considerable period of time by the serial dilution technique and inoculation in petri dishes according to Briceño *et al.* [12]. The microbial selection of actinobacteria was based on Bergey's manual [17]. The actinobacteria strains were maintained on agar medium slants at 4 °C, and they were filed at the Laboratory of Environmental Biotechnology from La Frontera University.

For the selection of tolerant actinobacteria, both qualitative and quantitative assays were performed. The qualitative screening consisted of evaluating the negative or positive growth of isolated strains on plates containing solid-defined minimal medium (MM) [18] supplemented with diazinon dissolved in methanol (Sigma–Aldrich, 99% pure) at concentrations of 1, 10, 25, and 50 mg L^{-1}. For quantitative assays, the removal of diazinon at 25 mg L^{-1} in liquid MM with 4 g L^{-1} glucose [12] by the strains selected in the previous screening was evaluated. We selected a concentration of 25 mg L^{-1} to perform the assays because, at this concentration, diazinon-tolerant strains exhibited a higher growth compared with solid medium with 50 mg L^{-1} diazinon. After 96 h of incubation, the diazinon removal (%) and microbial growth (%) results were used to select the strains according to the index proposed by Benimeli *et al.* [9] and modified by Briceño *et al.* [12], with the criterion of obtaining the highest degradation with the least amount of biomass.

Characterization of diazinon-tolerant strains

DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio, Inc., USA). For genetic affiliation analysis, the 16S rDNA gene fragments were amplified by PCR according to Peace *et al.* [19]. The amplicons obtained were purified and sequenced by Macrogen, Inc. (Korea). The sequences obtained in this study were deposited in and compared with those present

in the GenBank database from the National Center for Biotechnology Information (NCBI), using the BLAST tool for alignment and identification. Assessment of the phylogenetic affiliation in relation to representative OP-degrading bacteria in GenBank was performed using MEGA5 (http://www.megasoftware.net/).

After the genetic characterization, the selected strains were subjected to biochemical and enzymatic characterization using API 20E and API CORYNE test strips (bioMerieux®). Their tolerance to pH, to NaCl in the concentration range of 0–10%, and to lysozyme in the concentration range of 0–5 mg L^{-1} was evaluated.

Removal of diazinon from liquid medium

A small amount of mycelium was picked from the purified slants, and the strains were grown in 100-ml flasks containing 30 ml ISP-2 medium (20.0 g; malt extract, 10.0 g; yeast extract, 4.0 g; glucose, 4.0 g; distilled water, 1000 ml). After incubation (28 °C, 100 rpm for 96 h) in a rotary shaker, the cultures were centrifuged (8000*g*, 10 min, 4 °C), and the cell pellets were washed with sterile saline solution and used as inocula.

The degradation experiments in liquid media were conducted in 100-ml flasks containing 30 ml liquid MM with 4 g L^{-1} glucose. Diazinon dissolved in methanol was added to the flasks at concentrations of 25 and 50 mg L^{-1}. Then, samples of the cell pellets of the studied strains were added at inoculum concentrations of 4% w/v (wet weight), and non-inoculated flasks were used as control. The flasks were then incubated, and samples were collected at 0, 24, 48, 72, and 96 h. The parameters analyzed were pH, glucose consumption, biomass amount (expressed as dry weight), and residual diazinon.

Diazinon removal by free and immobilized *Streptomyces* sp. AC1-6

Pre-inocula were prepared by inoculating small amounts of mycelium of the strain in 100-ml flasks containing 30 ml ISP-2 medium. The culture was incubated at 28 °C, 100 rpm for 96 h. After centrifugation, the biomass obtained was mixed in a sterile solution of 3% w/v sodium alginate to a final concentration of 7% w/v (wet weight) [20]. The alginate-cell suspensions were added dropwise to a well-stirred and sterilized 0.1 M $CaCl_2$ solution using a syringe. The resulting beads with a radius of 1.5–2.0 mm were maintained in the $CaCl_2$ solution with gentle stirring for 2 h and then overnight at 4 °C to allow them to harden. After that, the beads were washed with distilled water and stored at 4 °C in a solution of 0.85% NaCl until use. To determine the size and surface structure of the pellets, microphotographs of free and encapsulated biomass pellets were obtained using a scanning electron microscope model JEOL JSM-6380 LV 314.

The removal experiments were conducted in 100-ml flasks containing 30 ml liquid-defined MM with 0.25 g L^{-1} $CaCl_2$ to allow hardening of the beads [14]. Diazinon dissolved in methanol was added to the flasks at a concentration of 25 mg L^{-1}. Then, 20 beads were added to the flask; assuming that all of the biomass was encapsulated in the beads, an inoculum concentration of 1.2% w/w was used for encapsulated and free biomass. Beads without biomass were also used as a control. The flasks were incubated (28 °C and 100 rpm), and samples were collected at 24 and 96 h for analysis of the diazinon concentration. The reusability of the immobilized bacteria for diazinon removal was then tested in two additional 96-h cycles.

Chemical analyses

The residual diazinon was determined by HPLC on a Chromolith RP-18e column (100 mm × 4.6 mm; Merck®) with a Merck-Hitachi LaChrom Elite instrument equipped with a diode array detector (L-2455) set at 246 nm and an autosampler (L-2200). The mobile phase was 70% acetonitrile and 30% 1 mM ammonium acetate, injected at a flow rate of 1 ml min^{-1}. Under these chromatographic conditions, the retention time, recovery, detection limit, and quantification limit were 3 min, 90%, 0.01 and 0.03 mg L^{-1}, respectively.

Data analysis

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 separated using Tukey's minimum significant difference test ($p \leq 0.05$).

Results

Isolation and screening of diazinon-tolerant strains

A total of 30 actinobacteria were isolated from agricultural soil that was characterized by the repeated application of OPs. The strains were initially screened for microbiological tolerance in solid culture medium with increasing concentrations of diazinon ranging from 1 to 50 mg L^{-1}. These assays showed that 23, 17, 16, and 4 of the 30 tested strains grew in diazinon concentrations of 1, 10, 25, and 50 mg L^{-1}, respectively. The four strains that showed tolerance for higher diazinon concentrations were named AC5, AC1-6, ISP4, and ISP13.

Table 1. Phylogenetic assignment of isolated actinobacteria strains tolerant to 50 mg L^{-1} diazinon in agar medium.

Strain	Closest relative sequence (accession number)[a]	Similarity (%)	Accession no.
AC5	*Streptomyces chattanoogensis* strain CGMCC 4.1415 (JN566019)	99	JQ289350
AC1-6	*Streptomyces* sp. HBUD30310 from root nodules (JF439426)	99	JQ289356
ISP4	*Streptomyces purpeofuscus* strain YSSPG (JN558347)	99	JQ289354
ISP13	*Streptomyces* sp. RU-75 from soil (DQ846831)	99	JQ289355

[a]Based on partial sequencing of the 16S rRNA genes and comparison with those present in the NCBI GenBank database by using BLAST.

The quantitative assay showed that three of the four strains degraded over 80% of the initial diazinon. Strain AC5 demonstrated the least degradation (65.5%) and the highest increase in biomass. Based on these results and its low index value (0.72), obtained from the pesticide removal/biomass relationship, strain AC5 was not used in the subsequent assays. After 96 h of incubation, the results of removal and biomass revealed index values of 1.45, 1.10, and 1.08 for strains ISP4, AC1-6, and ISP13, respectively. As a result, the diazinon-tolerant strains ISP4 and AC1-6 were selected for the subsequent assays.

Characterization of diazinon-tolerant actinobacteria

The four strains that were selected for their tolerance to diazinon (50 mg L^{-1}) in agar medium were identified as bacteria belonging to the phylum Actinobacteria, the order Actinomycetales, the family Streptomycetaceae, and the genus *Streptomyces* (Table 1). A dendrogram illustrating the relationship between the *Streptomyces* strains and other selected bacteria is presented in Fig. 1. The phylogenetic analysis indicates that the isolates have higher similarity to the 16S rDNA genes from actinobacteria strains belonging to the genus *Streptomyces* reported as degraders of OPs.

Biochemical and enzymatic characterization of the diazinon-tolerant *Streptomyces* strains AC1-6 and ISP4 showed that both streptomycetes were positive for pyrazinamidase and catalase. *Streptomyces* sp. AC1-6 was positive for alkaline phosphatase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucosidase, gelatinase, arginine hydrolase, ornithine decarboxylase, and citrate utilization. Unlike *Streptomyces* sp. AC1-6, *Streptomyces* sp. ISP4 was positive for urease activity. Moreover, it was observed that *Streptomyces* sp. AC1-6 was tolerant to a pH range of 5.0–10.0 and to concentrations of up to 10% NaCl and 5 mg ml^{-1} lysozyme. In contrast, *Streptomyces* sp. ISP4 was tolerant to pH values in the

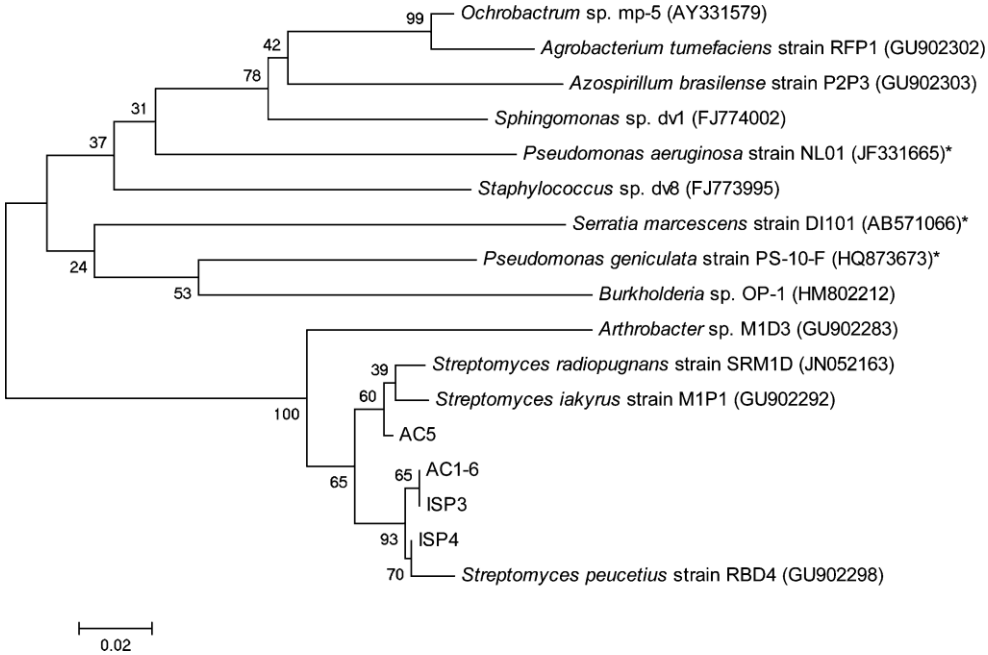

Figure 1. Phylogenetic tree showing the affiliation of the isolated diazinon-degrading actinobacteria (strains AC5, AC1-6, ISP4, and ISP13) in relation to representative bacteria with the ability to degrade OPs. Asterisks indicate sequences from bacteria reported as diazinon degraders.

range of 4.0–10.0 and concentrations of up to 5% of NaCl and 0.5 mg ml^{-1} lysozyme.

Degradation of diazinon in liquid medium by *Streptomyces* sp. AC1-6 and ISP4

The results of diazinon removal and microbial growth by *Streptomyces* sp. AC1-6 and ISP4 are shown in Fig. 2. In general, the statistical analyses between strains showed that the diazinon removal values were significantly different ($p \leq 0.05$) when different time points and different diazinon concentrations were evaluated. The main difference in the extent of diazinon removal was observed after 24 h, with the disappearance of more than 61 and 38% of the initial concentration when *Streptomyces* sp. AC1-6 and ISP4 were inoculated, respectively. However, specifically for both strains, the diazinon concentration had no influence on the removal of the compound, and this tendency was maintained at 48, 72, and 96 h of incubation. After 48 h of incubation, removal of more than 52% was observed for both strains. Finally, the difference in the residual diazinon concentrations after 96 h of incubation was not significant, with the residual concentrations in the liquid incubation medium ranging between 6.25 and 7.25 mg L^{-1} for *Streptomyces* sp. AC1-6 and between 3.25 and 6.25 mg L^{-1} for *Streptomyces* sp. ISP4.

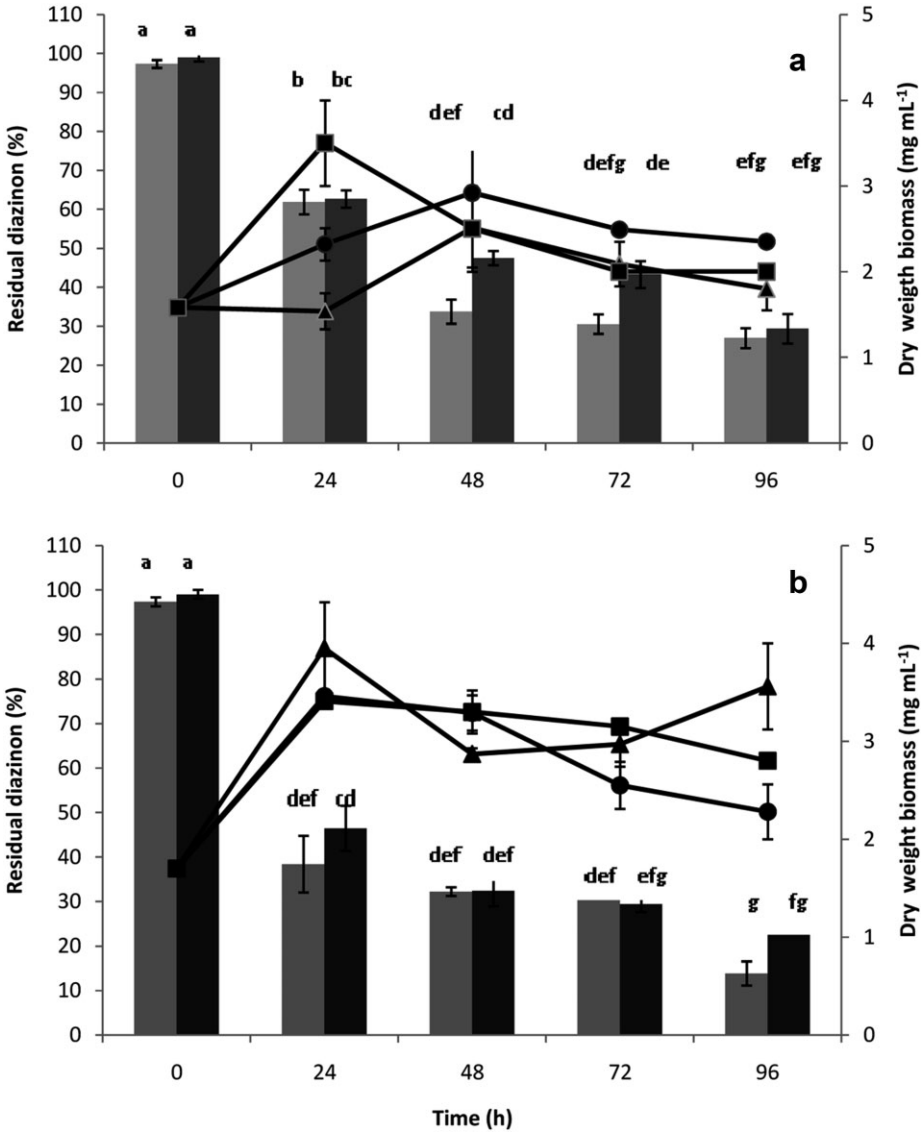

Figure 2. Microbial growth of *Streptomyces* sp. AC1-6 (a) and *Streptomyces* sp. ISP4 (b), and removal of diazinon. The curves represent microbial growth in medium with diazinon at concentrations of 0 (■), 25 (▲), and 50 mg L^{-1} (●). The columns represent residual diazinon (%) detected at initial concentrations of 25 (▬) and 50 mg L^{-1} (▬).

The results obtained for *Streptomyces* sp. AC1-6 showed different biomass concentrations ($p \leq 0.05$) during incubation with diazinon. After 24 h of incubation, the biomass increased from an initial biomass value of 1.58–2.32 mg ml^{-1} in the presence of 50 mg L^{-1} diazinon and to 3.50 mg ml^{-1} in the absence of diazinon. After 48 h of incubation, the amount of biomass decreased in the absence of diazinon and increased in the presence of diazinon to a level higher than that obtained after 24 h of incubation. After 96 h of incubation, a decrease in the biomass amount was observed in all of the treatments evaluated. The microbial growth, evaluated at different times of incubation for *Streptomyces* sp. ISP4, was significantly different ($p \leq 0.05$). This strain doubled its biomass after 24 h of incubation, from an initial biomass of 1.70 to 3.41–3.95 mg ml^{-1} in media with and without the pesticide. In this evaluation, a larger biomass production was observed after 24 h when 25 mg L^{-1} of diazinon was applied. However, after this time, a sharp drop was observed. At 48 h of incubation, the biomass amount began to decrease in the treatment without diazinon and with 50 mg L^{-1}, while in the media with 25 mg L^{-1} diazinon applied a new increment was observed.

The study was accompanied by an analysis of the pH changes and the glucose uptake (Table 2). The results show that the pH of the liquid media with and without diazinon and inoculated with *Streptomyces* sp. AC1-6 decreased from 7.00 at time zero to 6.09 after 24 h of incubation. After this time, the pH increased again to reach values close to those obtained in the control after 96 h of incubation. When the liquid media without and with diazinon were inoculated with *Streptomyces* sp. ISP4, the pH values decreased from 7.00 at time zero to 6.00–6.74 after 24 h of incubation; thereafter, the pH continued to increase to a value of 8.25 after 96 h of incubation. On the other hand, the analysis of the glucose uptake showed rapid substrate consumption by *Streptomyces* sp. AC1-6 during the first 24 h of incubation (72–87%), and almost all of the glucose was consumed after 96 h of incubation in the media without diazinon (97%) and with diazinon (92%). A similar trend was observed for *Streptomyces* sp. ISP4.

Degradation of diazinon by free and immobilized *Streptomyces* sp. AC1-6

Streptomyces sp. AC1-6 was used to evaluate the removal of diazinon by encapsulated biomass. This strain was selected because of its high viability when encapsulated in calcium alginate under storage conditions (1 year) and the facility to obtain a high amount of biomass (data not shown). The diazinon removal was also evaluated using free biomass that forms pellets (Fig. 3a) of approximately 300 μm in size; they are characterized as stable spheres with an irregular surface composed of an agglomeration of branched filaments (Fig. 3b) formed by the effect of the agitation used during the cultivation. Figure 3c and d shows alginate control beads without cells and immobilized *Streptomyces* sp. AC1-6 inside alginate beads, respectively.

The results obtained from this evaluation showed different removal percentages when free biomass, encapsulated biomass, or reused encapsulated biomass was inoculated into the liquid medium (Table 3). At 24 h

Table 2. Changes in pH and residual glucose levels in the liquid medium.

		pH		Residual glucose (g L^{-1})	
Time (h)	Initial diazinon (mg L^{-1})	Strain AC1-6	Strain ISP4	Strain AC1-6	Strain ISP4
0		7.00 ± 0.07a	7.05 ± 0.07bcd	4.00 ± 0.10a	4.00 ± 0.01a
24	0	6.09 ± 0.20c	6.00 ± 0.17e	0.50 ± 0.18c	1.03 ± 0.10c
	25	6.20 ± 0.10c	6.74 ± 0.02cde	1.00 ± 0.05b	2.20 ± 0.05b
	50	6.19 ± 0.06c	6.18 ± 0.34de	1.11 ± 0.50b	0.66 ± 0.05d
48	0	6.50 ± 0.26bc	6.61 ± 0.62cde	0.33 ± 0.08c	0.34 ± 0.03d
	25	6.28 ± 0.03bc	6.84 ± 0.24cde	0.50 ± 0.07c	0.86 ± 0.04cd
	50	6.55 ± 0.18bc	7.60 ± 0.14abc	0.53 ± 0.10c	0.28 ± 0.02d
72	0	6.68 ± 0.05ab	6.54 ± 0.02de	0.18 ± 0.05d	0.24 ± 0.01d
	25	6.93 ± 0.74ab	7.96 ± 0.48ab	0.44 ± 0.09c	0.25 ± 0.07d
	50	7.40 ± 0.26ab	8.21 ± 0.01a	0.37 ± 0.01c	0.26 ± 0.07d
96	0	7.59 ± 0.09a	6.90 ± 0.09cde	0.10 ± 0.01d	0.10 ± 0.05e
	25	7.07 ± 0.62a	8.25 ± 0.21a	0.40 ± 0.01c	0.18 ± 0.05e
	50	7.30 ± 0.16a	8.25 ± 0.16a	0.30 ± 0.02c	0.20 ± 0.08de

The medium was supplemented with 4 g L^{-1} glucose and diazinon at concentrations of 25 and 50 mg L^{-1}, and inoculated with *Streptomyces* sp. strain AC1-6 or ISP4. The data are presented as average values ± standard error ($n = 3$). Different letters indicate significantly different values, ANOVA with Tukey's test ($p \leq 0.05$). The analyses were done in the same column for each strain separately.

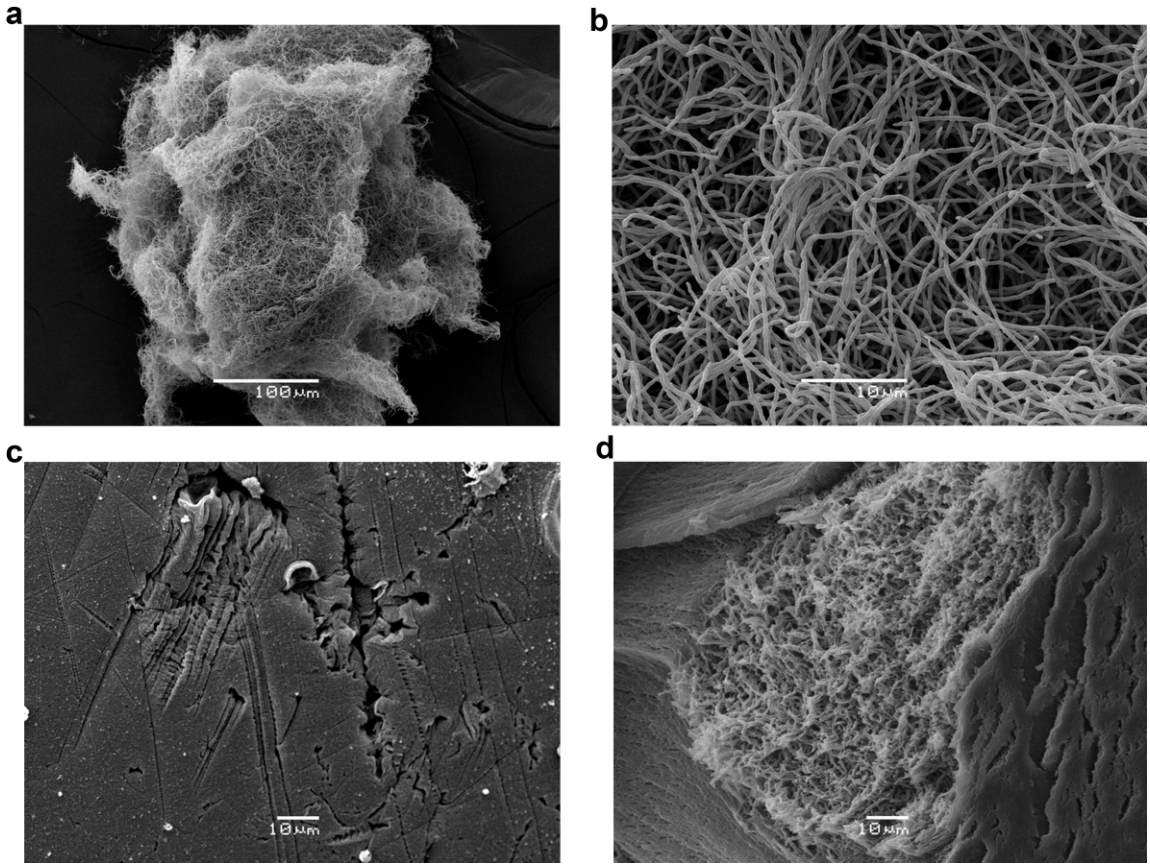

Figure 3. Electron scan micrographs. Pellets of free *Streptomyces* sp. AC1-6 (a) and branched filaments (b). Alginate beads without biomass (c) and with encapsulated biomass of *Streptomyces* sp. AC1-6 (d).

of incubation, the free biomass resulted in a higher removal of diazinon (close to 24%) compared with the encapsulated biomass (close to 15%). However, after 96 h of incubation, this trend was reversed: 53% diazinon removal by the encapsulated biomass compared with 35% diazinon removal by the free biomass.

When evaluating the reusability of encapsulated biomass in the alginate beads, removal of more than 30% of the initial diazinon was observed after 24 h of incubation, and this value increased to 44% after 96 h of incubation. Similarly, when the beads were reused a second time, removal rates close to 27 and 56% were measured after 24 and 96 h of incubation, respectively. It should be noted that, in all studies, a control using beads without biomass was considered, where 16% of the initial diazinon was adsorbed onto the alginate beads after 96 h of incubation.

Table 3. Diazinon removal (%) by free and immobilized *Streptomyces* sp. AC1-6 in alginate beads, during two consecutive cycles of 96 h, each assayed after the first use.

Culture	Time	Removal of diazinon (%)
Free culture	24	23.98 ± 1.15
	96	35.52 ± 1.64
Immobilized culture, first use	24	14.99 ± 2.45
	96	53.76 ± 1.81
Immobilized culture, second use	24	31.80 ± 2.00
	96	44.88 ± 4.00
Immobilized culture, third use	24	27.92 ± 5.00
	96	56.52 ± 4.00

Into the liquid medium, diazinon was added at a concentration of 25 mg L^{-1}. After the first use of the immobilized biomass, the beads containing the biomass were washed and re-inoculated in fresh medium containing diazinon. At 24 and 96 h, samples were taken for analysis. Data are presented as average values $\pm$ standard error ($n = 3$).

Discussion

In this study, close to 80% of the isolated actinobacteria exhibited some degree of diazinon tolerance at the concentrations evaluated (1–50 mg L^{-1}). However, only four of the strains exhibited high growth in agar medium with 50 mg L^{-1} diazinon. These strains were identified as

belonging to the genus *Streptomyces*, which is the dominant actinobacteria genus in soil [7]. The 16S rDNA phylogenetic analysis of the isolates showed a closer relation with OP-degrading bacteria of the genus *Streptomyces*, such as *S. radiopugnans*, *S. iakyrus*, and *S. peucetius*.

The quantitative assay performed showed that *Streptomyces* sp. AC5, AC1-6, ISP4, and ISP13 were able to grow and to remove diazinon, but not in a linear relationship. It was therefore necessary to examine the relationship between both of these parameters to select the best strains for future assays [9]. The actinobacteria with the highest values, which corresponded to a high degradation of diazinon and a low biomass production, were selected [12]. As a result, *Streptomyces* sp. AC1-6 and ISP4 were selected due to their efficiency in degrading diazinon with a minor amount of biomass. A similar trend was observed for chlorpyrifos degradation by actinobacteria isolated from Chilean soil [12] and for lindane degradation by *Streptomyces* sp. isolated from contaminated Argentinean soil [9, 21].

To obtain microorganisms that are able to degrade diazinon, the actinobacteria were isolated from agricultural soil with a history of OP application. These soils could lead to the accelerated degradation of this type of pesticides in soil by microorganisms [22, 23]. This assumption could be confirmed through the results obtained, which demonstrated that *Streptomyces* sp. AC1-6 and ISP4 rapidly removed diazinon. Therefore, the use of these actinobacteria for the bioremediation of matrices contaminated by pesticides is an attractive approach since these microorganisms are already adapted to the habitat.

In this study, evaluation of the degradation of diazinon at concentrations of 25 and 50 mg L^{-1} by *Streptomyces* sp. AC1-6 and ISP4 showed similar capacities. Thus, no differences were observed in the evaluated doses of the contaminant at the end of the incubation period. Similar results have also been observed elsewhere. For example, Cycoń *et al*. [24] showed that *Serratia* sp. and *Pseudomonas* sp. in liquid media with glucose and contaminated with 50 mg L^{-1} of diazinon degraded 40–59% of the initial dose of diazinon after 2 days of incubation. According to Drufovka *et al*. [25], an increase in diazinon degradation can be observed in the presence of available organic carbon. In contrast, the addition of an easily biodegradable substrate, such as glucose, increases the growth of bacteria, as was observed with the AC1-6 strain during the first 48 h and with the ISP4 strain during the first 24 h of incubation. According to our results, the *Streptomyces* sp. AC1-6 strain, compared with *Streptomyces* sp. ISP4, required a longer time for the microbial population to acclimate to the contaminant. However, this factor did not influence the removal of diazinon and therefore indicates that the pesticide was not toxic to the cells at the assayed concentrations.

Analyses of the pH and the glucose uptake in the liquid medium were conducted to obtain a better understanding of the degradation of diazinon by the studied streptomycetes. As an effect of the growth of *Streptomyces* sp. AC1-6 and ISP4 and the removal of diazinon, a slight decrease in the pH was observed in the medium with and without diazinon, and this response coincided with the time during which an almost complete glucose uptake was observed. As a consequence of microbial glucose metabolism, an accumulation of organic acids occurs in the liquid medium [24]. The excretion of organic acid metabolites into the extracellular medium has been observed in cultures of *Streptomyces* sp. AC5 and AC7 exposed to chlorpyrifos; the metabolites were apparently re-assimilated by the microorganisms after the depletion of glucose, with a subsequent increase in the biomass amount and in the pH to a value higher than 8.0 [12]. A similar response could explain the tendency observed particularly for the strain ISP4 after 96 h of incubation with diazinon. It has been shown that diazinon dissipation occurs as a result of chemical processes and microbiological degradation. For example, Drufovka *et al*. [25] and Cycoń *et al*. [24] reported that a low pH value (e.g., 4.5) results in the hydrolysis of diazinon and a faster loss of the pesticide. Hence, our results indicate that the microbial removal of diazinon by *Streptomyces* sp. AC1-6 and ISP4 is the main mechanism of diazinon dissipation under the evaluated conditions. According to Cycoń *et al*. [24], biodegradation, primarily under aerobic conditions, is a major fate process for diazinon associated with water and soil. The degradation products of diazinon include 2-isopropyl-6-methyl-4-pyrimidinol (IMP), which is reported as the major metabolite in compost, soil, and water, and as less toxic compared to its parent compound. Other products are diazoxon, a toxic metabolite, and IMHP or oxypyrimidine, a persistent, less toxic product [5, 24]. In our study, the analysis of diazinon showed a peak attributed to IMP (data not shown). However, we need to make further investigations on this. According to Robert and Hudson [4], diazinon is hydrolysed to IMP as the primary step, followed by oxidation and oxidative dealkylation of the methyl and isopropyl groups, which must eventually lead to the total breakdown of the pyrimidine ring, and ultimately to CO_2.

When immobilized microbial cells are used, the efficacy of biodegradation is often improved [20, 26]. Therefore, we evaluated the ability of immobilized

Streptomyces sp. AC1-6 to remove diazinon. We used calcium alginate as the model support because this technique is simple and inexpensive [14]. As per the results obtained in our studies, the use of encapsulated *Streptomyces* could be a promising alternative for the treatment of diazinon residues. Although a higher diazinon removal was observed with the free biomass after 24 h of incubation, this response was reversed after 96 h, at which point an approximately 60% higher diazinon removal (with respect to that obtained with the free biomass) was obtained with the encapsulated biomass. Changes in cell physiology, an increase in cell permeability, and optimal diffusion of contaminants in the alginate beads with a diameter similar to that used in this study have been associated with this response [27, 28]. However, in our case, the higher diazinon removal by the immobilized actinobacteria was coincident with a release of the cells from the support into the medium (approximately 10^5 cfu ml^{-1}). Before 96 h of incubation, the amount of cells in the liquid medium was negligible. These results indicate that the cells were leaking from the alginate beads, most likely due to limited space for growth, which suggests the viability of the leaked cells based on the increase in the cell number in the culture media. Similar results were reported by Plangklang and Reungsang [29] while evaluating the removal of carbofuran using *Burkholderia cepacia* immobilized on agricultural residues. Studies conducted with immobilized actinobacteria for pesticide degradation are scarce. Recently, removal of lindane by pure and mixed cultures of immobilized actinobacteria was investigated by Saez *et al*. [20], who found that the lindane removal by the immobilized microorganisms was significantly higher than that obtained with free biomass. Similarly, mixed cultures of *Streptomyces* spp., when assayed either as free or immobilized cells, showed chlorpyrifos removal rates of 40.17 and 71.05%, respectively, and of 5.24 and 14.72%, respectively, for pentachlorophenol, suggesting better removal of both pesticides by using immobilized cells in Ca-alginate beads [16].

One important advantage of an immobilized biomass system is the extended or repeated use of the catalytic activity in sequential batches [27]. In this study, the best diazinon removal (56%) was obtained after 96 h in the second reuse. The increase in diazinon degradation during successive batches indicates that the cells become better adapted with repeated batches, which shows the feasibility of using immobilized actinobacteria cells for continuous diazinon removal. Saez *et al*. [20] demonstrated the reusability of actinobacteria during three 96-h periods for lindane removal by pure and mixed cultures.

It is noteworthy that the reaction medium was completely replaced for each batch and that the inoculated alginate beads containing the cells were stable in each one. In contrast, the abiotic removal of the pesticide from the control was negligible throughout the study, whereas 16% of the initial diazinon was adsorbed onto the alginate beads without biomass after 96 h of incubation. This finding confirms the hypothesis that the greater diazinon removal observed is due to the immobilized actinobacteria and that this material may be an attractive support for the entrapment of microbial cells. Our results demonstrate that the immobilization of actinobacteria is a promising biotechnological technique for the removal of diazinon wastes. However, future studies are necessary to further our knowledge of these bacteria and immobilization supports and to optimize the immobilization system, to obtain higher and faster removal of pesticides from contaminated environments.

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References

[1] Garfitt, S.J., Jones, K., Mason, H.J., Cocker, J., 2002. Exposure to the organophosphate diazinon: data from a human volunteer study with oral and dermal doses. Toxicol. Lett., **134**, 105–113.

[2] Ashauer, R., Wittmer, I., Stamm, C., Escher, B.I., 2011. Environmental risk assessment of fluctuating diazinon concentrations in an urban and agricultural catchment using toxicokinetic–toxicodynamic modeling. Environ. Sci. Technol., **45**, 9783–9792.

[3] Fenlon, K., Andreou, K., Jones, K.C., Semple, K.T., 2011. The formation of bound residues of diazinon in four UK soils: implications for risk assessment. Environ. Pollut., **159**, 776–781.

[4] Robert, T., Hudson, D., 1999. Metabolic Pathways of Agrochemicals. II. Insecticides and Fungicides, The Royal Society of Chemicals, UK, 1447

[5] Karpouzas, D.G., Singh, B.K., 2006. Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular basis. Adv. Microb. Physiol., **51**, 119–225.

[6] Abo-Amer, A.E., 2011. Biodegradation of diazinon by *Serratia marcescens* DI101 and its use in bioremediation of contaminated environment. J. Microbiol. Biotechnol., **21**, 71–80.

[7] Seong, C.N., Choi, J.H., Baik, K., 2001. An improved selective isolation of rare actinomycetes from forest soil. J. Microbiol., **39**, 17–23.

[8] Castillo, M., Felis, N., Aragón, P., Cuesta, G. et al., 2006. Biodegradation of the herbicide diuron by streptomycetes isolated from soil. Int. Biodeter. Biodegrad., **58**, 196–202.

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

[10] Fuentes, M.S., Benimeli, C.S., Cuozzo, S.A., Amoroso, M.J., 2010. Isolation of pesticide-degrading actinomycetes from a contaminated site: bacterial growth, removal and dechlorination of organochlorine pesticides. Int. Biodeter. Biodegrad., **64**, 434–441.

[11] Lin, Q.S., Chen, S.H., Hu, M.Y., Hul Haq, M.R. et al., 2011. Biodegradation of cypermethrin by a newly isolated actinomycetes HU-S-01 from wastewater sludge. Int. J. Environ. Sci. Technol., **8**, 45–56.

[12] Briceño, G., Fuentes, M.S., Palma, G., Jorquera, M.A. et al., 2012. Chlorpyrifos biodegradation and 3,5,6-trichloro-2-pyridinol production by actinobacteria isolated from soil. Int. Biodeter. Biodegrad., **73**, 1–7.

[13] El Fantroussi, S., Agathos, S.N., 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr. Opin. Microbiol., **8**, 268–275.

[14] Anisha, G.S., Prema, P., 2008. Cell immobilization technique for the enhanced production of alpha-galactosidase by *Streptomyces griseoloalbus*. Bioresource Technol., **99**, 3325–3330.

[15] Briceño, G., Pizzul, L., Diez, M.C., 2013. Biodegradation of pesticides by actinobacteria and their possible application in biobed systems, in: Amoroso, M.J., Benimeli, C.S., Cuozzo, S.A. (Ed.), Actinobacteria. Application in Bioremediation and Production of Industrial Enzymes, CRC Press Taylos & Francis Group, Florida, USA, 165–191.

[16] Fuentes, M.S., Briceño, G.E., Saez, J.M., Benimeli, C.S. et al., 2013. Enhanced removal of a pesticides mixture by single cultures consortia of free immobilized Streptomyces strains. Biomed Res. Int., **2013**, 1–9.

[17] Lechevalier, H., 1989. A practical guide to generic identification of actinomycetes, in: Williams, S.T. (Ed.), Bergey's Manual of Systematic Bacteriology, Vol. **4**. Williams and Wilkins Company, Baltimore, 2344–2347.

[18] Amoroso, M.J., Castro, G.R., Durán, A., Peraud, O. et al., 2001. Chromium accumulation by two *Streptomyces* spp. isolated from riverine sediments. J. Ind. Microbiol. Biotechnol., **26**, 205–210.

[19] Peace, T., Brock, K., Stills, H., 1994. Comparative analysis of the 16 rRNA gene sequence of the putative agent of proliferative ileitis of hamsters. Appl. Environ. Microbiol., **44**, 832–835.

[20] Saez, J.M., Benimeli, C.S., Amoroso, M.J., 2012. Lindane removal by pure and mixed cultures of immobilized actinobacteria. Chemosphere, **89**, 982–997.

[21] Fuentes, M.S., Sáez, 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.

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

[23] Cycoń, M., Żmijowska, A., Wójcik, M., Piotrowska-Seget, Z., 2013. Biodegradation and bioremediation potential of diazinon-degrading *Serratia marcescens* to remove other organophosphorus pesticides from soils. J. Environ. Manage., **117**, 7–16.

[24] Cycoń, M., Wójcik, M., Piotrowska-Seget, Z., 2009. Biodegradation of the organophosphorus insecticide diazinon by *Serratia* sp. and *Pseudomonas* sp. and their use in bioremediation of contaminated soil. Chemosphere, **76**, 494–501.

[25] Drufovka, K., Danevčič, T., Trebše, P., Stopar, D., 2008. Microorganisms trigger chemical degradation of diazinon. Int. Biodeter. Biodegrad., **62**, 293–296.

[26] Lin, Q., Donghui, W., Jianlong, W., 2010. Biodegradation of pyridine by *Paracoccus* sp. KT-5 immobilized on bamboo-based activated carbon. Bioresource Technol., **101**, 5229–5234.

[27] Ha, J., Engler, C.R., Wild, J.R., 2009. Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads. Bioresource Technol., **100**, 1138–1142.

[28] Yañez-Ocampo, G., Sanchez-Salinas, E., Jimenez-Tobon, G.A., Penninckx, M. et al., 2009. Removal of two organophosphate pesticides by a bacterial consortium immobilized in alginate or tezontle. J. Hazard. Mater., **168**, 1554–1561.

[29] Plangklang, P., Reungsang, A., 2009. Bioaugmentation of carbofuran residues in soil using *Burkholderia cepacia* PCL3 adsorbed on agricultural residues. Int. Biodeter. Biodegrad., **63**, 515–522.

