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Isolation of four aquatic streptomycetes strains capable of growth on organochlorine pesticides

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Abstract

Ninety-three wild-type isolates identified as actinomycetes were tested against 11 organochlorine pesticides (OPs): aldrin, chlordane, DDD, DDE, DDT, dieldrin, heptachlor, and heptachlor epoxides, lindane, and methoxychlor. Qualitative screening agar assays displayed 62–78% tolerance of strains to OPs. Four strains designed M4, M7, M9 and M15 were selected based on multi-OP-tolerance, and identified as members of the streptomycetes group. Different growth profiles were observed in cultures of the four selected streptomycetes cultured in synthetic medium containing 5–50 $\mu g l^{-1}$ aldrin or chlordane or lindane. Increase of aldrin removal by the selected microorganisms was concomitant with the 4.8–36.0 $\mu g l^{-1}$ pesticide concentration range. After 72 h of streptomycete M7 growth in synthetic medium containing 48.0 $\mu g l^{-1}$ aldrin, the remaining OP concentration in the supernatant was approximately 10% of the initial concentration. Also, in stationary growth phase less than 2.5 $\mu g l^{-1}$ aldrin residual concentration was detected in the medium.

Keywords: Streptomycetes; Organochlorine pesticides; Aldrin; Chlordane; Lindane

1. Introduction

Pesticides have been extensively used to protect and improve quality and quantity of food commodities, building materials, clothing, animal health, and to combat certain diseases transmitted by insect vectors to man and animals. However, indiscriminate use of pesticides has caused serious concern about toxic effects by residues on non-target organisms. As a consequence of extensive organochlorine pesticides (OPs) use, and because of their environmental problems and health hazards: aldrin, chlordane, dalapon, DDT, dieldrin, endosulfan, and lindane have been restricted or banned (Hassall, 1990). Aldrin was classified as highly dangerous and declared completely phased out in 110 countries (Hileman, 1999). Lindane and chlordane are considered moderately dangerous, but they have been largely used worldwide for control of agricultural and medical pests (De Cruz et al., 1996). Because of extensive aldrin, chlordane, and lindane use in the northwest of Argentina, residues are present in the main hydrographic system of Tucumán (Chaile et al., 1999). Pesticide residues in soils and their subsequent movements in the water–soil system are key aspects in their environmental behavior (Albanis et al., 1998). Consequently, reliable, cost effective method(s) for remediation of organochlorine pesticides is needed in order to minimize contaminated sites. This approach is dependent upon inoculation with microorganisms that are capable of degrading contaminant compounds (Shelton et al., 1996).

Metabolic pathways for pesticide biodegradation by gram negative bacteria have been more extensively studied than in gram positive bacteria (Singh and Kuhad, 1999). Additionally, little information has been reported about the ability of OP biotransformation by actinomycete species (De Schrijver and De Mot, 1999). Strains belonging to actinomycetes have been able to oxidize and/or partially dechlorinate aldrin, DDT,

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metalochlor, and PCNB. These previous works suggested that actinomycetes produce initial pesticide transformation, but no mineralization (Ferguson and Korte, 1977; Speedie et al., 1987; Liu et al., 1990, 1991). Also, it is not clear if halogenated organic compounds including organochlorine pesticides can participate on microbial growth and metabolism. The aim of this work was to describe the procedures for screening and selection of OPs tolerant and/or resistant actinomycetes strains, and to investigate the ability of wild-type strains to remove organochlorine pesticides from synthetic culture medium.

2. Methods

2.1. Samples

Sediment samples were collected from wastewater sediment of a copper filter plant located in an agricultural area of Tucumán, Argentina (Fig. 1). Each sample was aseptically collected using sterile test tubes, and kept at 5 °C until they were dried at 30 °C to constant weight. Samples were diluted with sterile 145 mM NaCl solution prior to inoculation onto isolation plates in duplicate.

2.2. Isolation of microorganisms

Isolation of microorganisms was carried out in starch-casein medium (SC) containing per liter: starch, 10.0 g; casein, 1.0 g; K_2HPO_4, 0.5 g; agar, 15.0 g. The pH was adjusted to 7.0 prior to sterilization. The medium was supplemented with 10.0 $\mu g\,ml^{-1}$ nalidixic acid (NA), and cycloheximide final concentrations to inhibit growth of gram negative bacteria and fungi, as previously reported for actinomycetes isolations (Ravel et al., 1998). Plates were incubated at 25 °C and colonies were purified without the antibiotics by streaking on agar medium. Colonies on SC medium supplemented with NA, with tough, leathery characteristics of actinomycete vegetative mycelia (and in some cases with aerial mycelia and spore formation). Microbial selection was based on colony morphology, color and presence of diffusible pigments of isolates according to the Bergey's Manual (Lechevalier, 1989). Selected colonies were screened for OPs tolerance.

2.3. OP tolerance assays

Preliminary qualitative screening experiments were carried out in square plates containing SC agar medium supplemented with 10 $\mu g\,l^{-1}$ of OPs. Aldrin (1,2,3,4,10-10-hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-1,4-*endo–exo*-5,8 dimethanonaphthalene), chlordane (1,2,4,5,7,8,8-octachloro-3*a*,4,7,7*a*-tetrahydro-4,7-methanoindane), DDD (2,2′-bis(*p*-chlorophenyl)-1,1-dichloroethane), DDE (2,2′-bis(*p*-chlorophenyl)-1,1-dichloroethylene), DDT (1,1,1-trichloro-2-2-bis(*p*-chlorophenyl)ethane), dieldrin (1,2,3,4,10,10-hexachloro-6,6-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydro-1,4-endo,exo-5,8-diphenyl methane), heptachlor (1,4,5,6,7,8,8-heptachloro-3*a*,5,7,7*a*-tetrahydro-4,7-methanoindene), heptachlor epoxide A and B (1,4,5,6,7,8,8-heptachloro-2,2-epoxy-3*a*,4,7,7*a*-tetrahydro-4,7-endomethanoindene), lindane (γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane), and methoxychlor (1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane) were purchased from ULTRAScientific (North Kingstown, RI) and tested.

In plate assays, troughs were made in the center of petri dishes by aseptically removing a strip of agar which

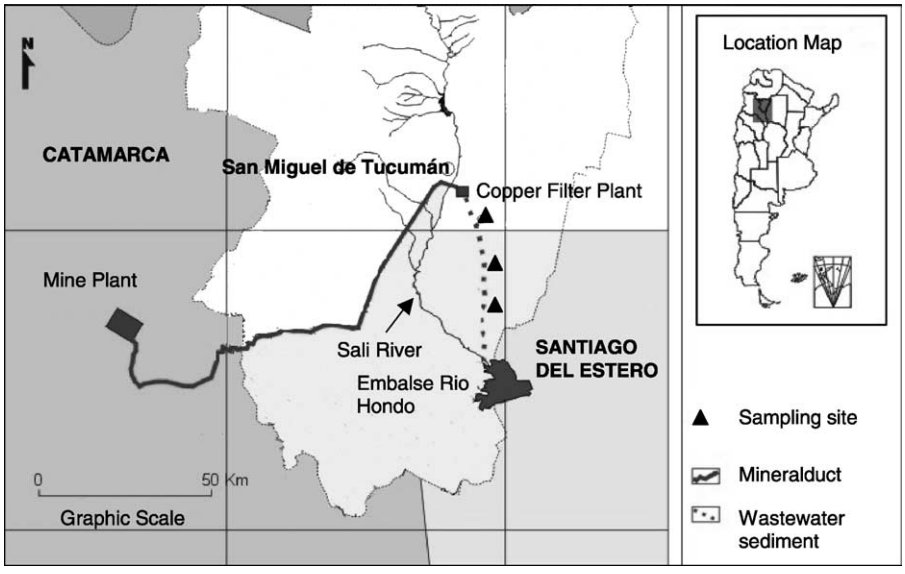

Fig. 1. Location of sampling site of wastewater sediment of a copper filter plant located in an agricultural area of Tucumán, Argentina.

were filled with the OP solutions. Isolates were inoculated by streaking 100 μl of spore suspension perpendicularly to the troughs. OP tolerance was qualitatively estimated by growth after 7 days at 30 °C.

Semi-quantitative screening for OP tolerance was carried out in the presence of aldrin, chlordane, DDD, DDE, DDT, dieldrin, heptachlor and their epoxides A and B, lindane, and metoxychlor, 0.75–50.00 $\mu g l^{-1}$ in SC agar medium diffusion plate tests. OP solutions were placed in the wells on the surface of petri dishes containing SC medium previously inoculated with spore suspensions of the 15 selected strains to be tested. Diameter of the growth inhibition was considered as a measure of OP sensitivity after incubation at 30 °C for 7 days.

2.4. Batch cultures in synthetic medium supplemented with OP

Pre-cultures of four selected strains were carried out in a liquid-defined medium (MM) containing (in grams per liter): glucose, 10.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7H_2O$, 0.01 (Amoroso et al., 2001). Pre-culture spore suspensions were inoculated in MM medium without glucose, and supplemented with 5–50 $g l^{-1}$ of aldrin, chlordane and lindane, respectively.

Selected strains were cultured on a rotator shaker (100 rpm) at 30 °C, for 96 h. Samples were taken and centrifuged (3000*g*, 10 min). Biomass was estimated by washing the pellets with 25 mM Tris–EDTA buffer (pH 8.0) and drying to constant weight at 105 °C. Since the toxicity of chosen pesticides, aldrin was selected for further degradation tests with the four selected wild type streptomycete strains. Strain M7 was also selected for determining the aldrin consumption and biomass every 24 h, during 96 h under initial aldrin concentration of 48 $g l^{-1}$. Relative growth was calculated considering microbial growth in MM medium without OP.

2.5. Chemotaxonomic analysis

Four selected strains: M4, M7, M9, and M15, were analyzed by thin layer chromatography (TLC) for isomeric diaminopimelic acid configurations (A2pm isomer), and for whole-cell sugar composition (Lechevalier, 1989). In order to verify the taxonomic characterization of the selected strains, the cell wall amino acids were analyzed (Kummer et al., 1999). For morphological observations, the strains were cultured at 25 °C on SC agar and on artificial soil (AS) agar for morphological observations (Vobis, 1992) and microscopic examinations were carried out as previously described by Cross (1989). The strains were identified to generic level on morphological criteria using the practical guide of Lechevalier (1989).

2.6. Gas chromatography analysis

Supernatant samples of centrifuged cultures (5000*g*, 30 min, at 4 °C) were used to determine residual aldrin by gas chromatography. Aldrin was assayed in filtered supernatants by gas chromatography (Hewlett-Packard 6890, Wilmington, DE) using HP 5 capillary column (30 m × 0.53 mm × 0.35 m) and equipped with ^{63}Ni ECD detector and split/split less injector HP 7694, and ChemStation Vectra XM software. Quantitative analysis of samples was performed using appropriate OP calibration standards (ULTRAScientific, North Kingstown, RI).

2.7. Statistical analysis

Each experiment was done in triplicate and the results are arithmetic means. For statistical evaluation, Student's *t*-test and one way ANOVA for the analysis of variance were used.

3. Results and discussion

Ninety-three indigenous colonies belonging to the actinomycetes group were isolated from wastewater sediment samples of a copper filter plant collected at a depth of 1 m (Fig. 1). Qualitative analysis of growth in presence of 11 OPs was systematized using 4 categories: from no-growth strain (no-pesticide-tolerant strain) to very good growth (highly-pesticide-tolerant strain). Strain growth controls were performed using medium without pesticide. Sixty-two to 78% of the strains displayed tolerance depending on the pesticide tested (Table 1). Based on multiple OP tolerances to eleven organochlorine pesticides tested, the best fifteen strains were selected.

No growth inhibitions for the 15 selected strains were found by semi-quantitative pesticide-tolerance assays carried out in SC agar medium supplemented with OPs concentrations ranging from 0.75 to 50.00 $\mu g l^{-1}$. These results would indicate that the OPs concentrations used were not toxic for these actinomycete strains under our experimental conditions.

Considering multiple OP-tolerance, actinomycete strains M4, M7, M9, and M15 were selected for quantitative OPs tolerance tests. Macroscopic and microscopic observation of these selected strains revealed formation of ample aerial mycelium and spore chains, which indicated their belonging to the genus *Streptomyces* (Locci, 1989). This was supported by the analysis of chemotaxonomic parameters. The cell wall was for all cases type I with LL-diaminopimelic acid. Mycolic acid was absent, in contrast to teichonic acid. Glucose and

Table 1
Qualitative assays of growth of 93 wild-type actinomycete strains in presence of pesticides

Pesticide	Strains (%)			
	(−)	(+)	(++)	(+++)
Aldrin	12	11	2	75
Chlordane	10	4	8	78
DDD	10	10	3	77
DDE	18	8	2	72
DDT	10	3	16	71
Dieldrin	14	4	19	63
Heptachlor	11	9	6	74
H. epoxide A	12	8	10	70
H. epoxide B	9	14	10	67
Lindane	11	8	4	77
Metoxichlor	13	10	15	62

(−) Non-growth; (+) limited growth; (++) moderate growth; (+++) abundant growth.

ribose were present in their cell walls. Menaquinone composition was predominantly MK-9(H_8) and MK-9(H_6).

In the Salí River, which flows parallel of the wastewater sediment site (Fig. 1), presence of 0.03–0.50 $\mu g\,l^{-1}$ of aldrin, 0.1–2.0 $\mu g\,l^{-1}$ of lindane, and 0.02–1.0 $\mu g\,l^{-1}$ of chlordane were detected (Chaile et al., 1999). The four selected strains were not able to grow in MM medium with L-asparagine as a sole carbon source, but growth was observed in the presence of aldrin, lindane and chlordane in the medium for all strains, indicating possible OPs degradation and metabolization by the

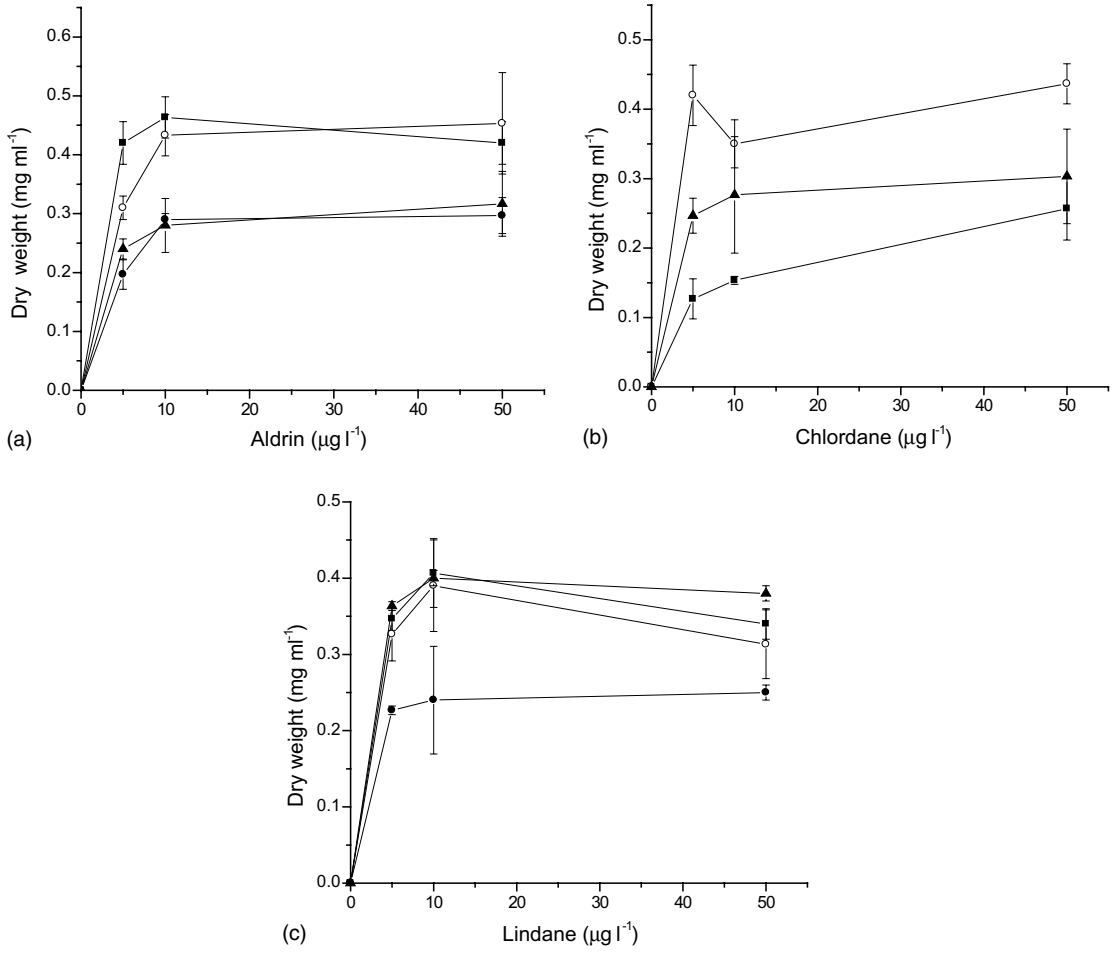

Fig. 2. Growth of four streptomycete strains in presence of aldrin (a), chlordane (b) and lindane (c) as unique carbon source. Symbols: (○) M4; (■) M7; (●) M9; (▲), M15. Error bars represent standard deviations.

microorganisms in order to support the microbial growth.

Different growth profiles in each pesticide for the four strains were observed in liquid-defined medium (MM). Similar growth was observed on streptomycete M4 in presence of 5.0–50.0 $\mu g\,l^{-1}$ ranges of the three OPs tested. Streptomycete M15 did not show significant growth changes in the presence of aldrin, lindane and chlordane. Streptomycete M9 showed similar growth profiles in the presence of aldrin and lindane, while no growth was detected in the presence of chlordane. Streptomycete M7 displayed similar growth profiles in the presence of aldrin and lindane, but growth was inhibited between 40% and 60% in the presence of chlordane, compared with the two OPs ($P < 0.05$). Different growth profiles of the four selected strains at the chlordane concentrations assayed were observed (Fig. 2b). However, the growth profiles of the four strains in presence of aldrin or lindane displayed two different behaviors with a half to one third of growth between both groups (Fig. 2a and c).

Experiments using initial aldrin concentrations of 4.8, 8.0, and 36.0 $\mu g\,l^{-1}$ in MM medium without glucose were performed respectively. Cultures of streptomycetes M4 and M9 displayed an increase of aldrin removal correlated with the pesticide concentration. On the other hand, aldrin removal by streptomycete M7 was about 80% at all aldrin concentrations tested, while streptomycete M15 displayed higher than 60% pesticide concentration under similar experimental conditions (Fig. 3).

Aldrin removal by streptomycete M7 and M15 increased continuously with pesticide concentration in the medium, sequestering a maximum of 31.3 and 22.3 $\mu g\,l^{-1}$ pesticide, respectively. Also, aldrin removal was approximately 3.5 and 7.0 times higher than the other two lower pesticide concentrations tested, respectively (Fig. 3).

In the case of streptomycete M9, positive trends on pesticide removal and microbial growth were observed. Aldrin removal was 2.4, 4.8 and 30.4 $\mu g\,l^{-1}$, representing 47%, 59%, and 82% of initial pesticide concentrations in the medium. Comparing aldrin accumulation and/or utilization by M9 cells, they were 13 and 6 times higher at maximum pesticide concentration tested than the other two OP concentrations.

Aldrin removal by streptomycete M4 was in the 40–77% range at the tested concentration (Fig. 3). From the experiments displayed in Figs. 2a and 3 it is possible to conclude that aldrin removal for the four selected strains increased with pesticide availability in the medium, but concomitantly slight changes of growth patterns were observed for each strain according to the enhanced medium toxicity.

However, the two different profiles of growth in presence of aldrin represent not only different sensitivities to the highly toxic pesticide, but also may account

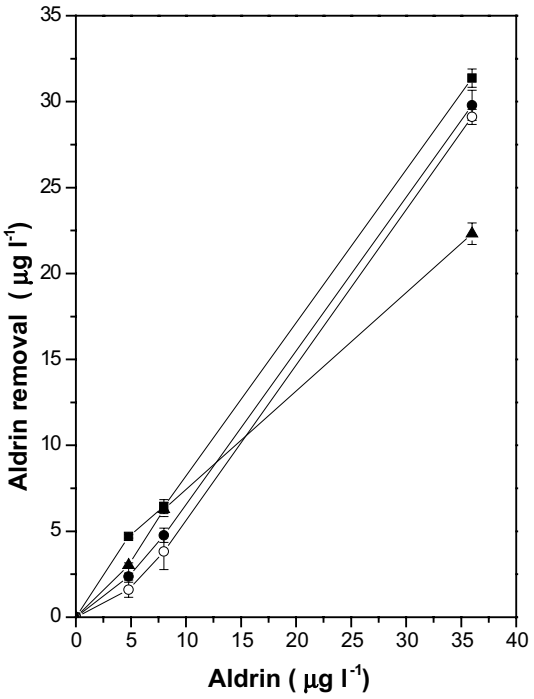

Fig. 3. Aldrin removal by streptomycete strains as unique carbon source. Symbols: (○) M4; (■) M7; (●), M9; (▲) M15. Error bars represent standard deviations.

for different mechanisms of aldrin removal. A white-rot fungus, *Trametes hirsutus*, was reported to accumulate lindane intracellular without degrading it, suggesting that no intracellular enzymes are involved in pesticide degradation, at least in initial stages (Singh et al., 1999). In other reports, strains of different genera of actinomycetes, including *Micromonospora*, *Mycobacterium*, *Nocardia*, and *Streptomyces*, have been shown to transform aldrin to its epoxide exo-dieldrin form (Ferguson and Korte, 1977). However, in the four selected streptomycete strains, no secondary products of aldrin microbial transformation were detected under the current experimental conditions. This fact is very important for bioremediation purposes since complete pesticide degradation or mineralization is desirable. Pesticide biotransformation to more toxic products and refractory compounds by microorganisms has also been reported (Shelton et al., 1996).

The time-course of streptomycete M7 microbial growth in the presence of 50 $\mu g\,l^{-1}$ aldrin is shown in Fig. 4. Microbial growth was exponential until 72 h, and along with aldrin concentration decreased exponentially to less than 10% of initial concentration in the supernatant. The growth rate was $5.5 \times 10^{-2}\ g\,l^{-1}\,h^{-1}$ and after 72 h when the stationary growth phase was reached. Cellular lysis was observed to some extent on streptomycete M7 microbial culture associated with a biomass decrease of approximately 27%. However, aldrin removal

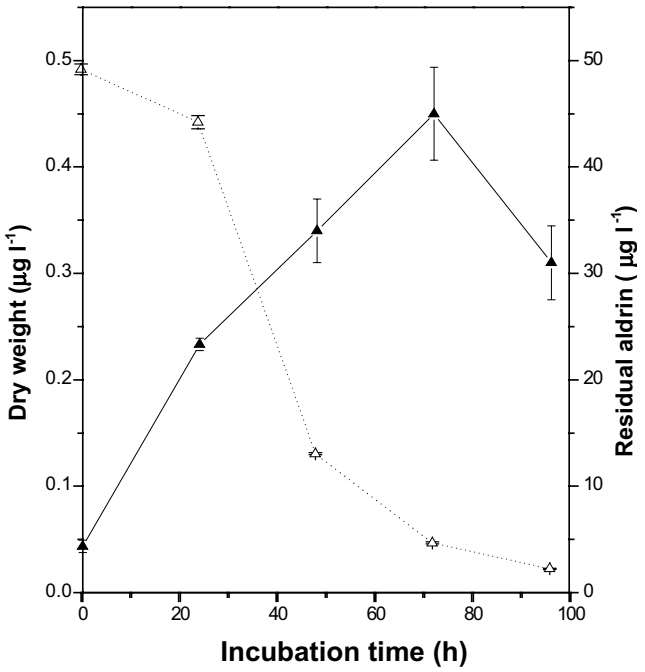

Fig. 4. Determination of growth and residual aldrin of streptomycete M7 culture for 96 h. Symbols: (▲), dry weight; (△), residual aldrin concentration. Error bars represent standard deviations.

continued to less than 5% residual, and no pesticide release into the medium was observed after cell lysis.

This result concludes that streptomycete M7 possesses optimal growth capabilities for removing aldrin from the synthetic medium under the described experimental conditions. Also, the results presented here support the hypothesis of the two means for aldrin removal: one for cellular metabolism and the other for bioaccumulation by cell components and not related to the soluble fraction of the cells.

In conclusion, bearing in mind that actinomycetes are highly metabolically diverse, they can act on chemically different and toxic substrates. Based on the results presented here, aquatic streptomycete strains appear to have great potential as bioremedial agents for organochlorine pesticides on contaminated environments. Uptake, accumulation, and biochemical processes of organochlorine pesticides by streptomycetes are presently under study in our laboratory.

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References

Albanis, T.A., Hela, D.G., Sakellarides, T.M., Konstantinou, I.K., 1998. Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. J. Chromatog. A 823, 59–71.

Amoroso, M.J., Castro, G.R., Durán, A., Oliver, G., Hill, R.T., 2001. Chromium cation uptake by aquatic actinomycetes. J. Ind. Microbiol. Biotechnol. 26, 210–215.

Chaile, A.P., Romero, N., Amoroso, M.J., Hidalgo, M. del V., Apella, M.C., 1999. Organochlorine pesticides in Sali River. Tucumán—Argentina. Rev. Bol. Ecol. 6, 203–209 (in Spanish).

Cross, T., 1989. Growth and examination of actinomycetes—some guidelines. In: Williams, S.T. (Ed.), Bergey's Manual of Systematic Bacteriology, vol. 4. Williams and Wilkins, Baltimore, pp. 2340–2343.

De Cruz, I., Locroix, G., Mougin, C., Grolleu, G., 1996. Residues of chlorinated pesticides in eggs of grey heron (*Ardea cinerea* L.); contribution of capillary gas chromatography ion-trap mass detection. J. H. Res. Chromatog. 19, 62–64.

De Schrijver, A., De Mot, R., 1999. Degradation of pesticides by actinomycetes. Crit. Rev. Microbiol. 25, 85–119.

Ferguson, J.A., Korte, F., 1977. Epoxidation of aldrin to exo-dieldrin by soil bacteria. Appl. Environ. Microbiol. 34, 7–13.

Hassall, K.A., 1990. Organochlorine insecticides. In: The Biochemistry and Uses of Pesticides. Structure, Metabolism, Mode of Action and Uses in Crop Protection, second ed. VCH, Weinheim, p. 157.

Hileman, B., 1999. Paring of persistent pollutant progresses. Chem. Eng. News 77, 9.

Kummer, C., Schumann, P., Stackebrandt, E., 1999. *Gordonia alkalinovorans* sp. nov., isolated from tar-contamined soil. Int. J. Syst. Bacteriol. 49, 1513–1522.

Lechevalier, H.A., 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, Baltimore, MD, pp. 2344–2347.

Liu, S.Y., Lu, M.H., Bollag, J.M., 1990. Transformation of metachlor in soil inoculated with *Streptomyces* sp. Biodegradation 1, 9–17.

Liu, S.Y., Freyer, A.J., Bollag, J.M., 1991. Microbial dechlorination of the herbicide metolachlor. J. Agric. Food Chem. 39, 631–636.

Locci, R., 1989. Streptomyces and related genera. In: Williams, S.T., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 4. Williams and Wilkins, Baltimore, MD, pp. 2451–2508.

Ravel, J., Amoroso, M.J., Colwell, R.R., Hill, R.T., 1998. Mercury-resistant actinomycetes from Chesapeake Bay. FEMS Microbiol. Lett. 162, 177–184.

Shelton, D.R., Khader, S., Karns, J.S., Pogell, B.M., 1996. Metabolism of twelve herbicides by *Streptomyces*. Biodegradation 7, 129–136.

Singh, B.K., Kuhad, R.C., 1999. Biodegradation of lindane (γ-hexachlorocyclohexane) by the white-rot fungus *Trametes hirsutus*. Lett. Appl. Microbiol. 28, 238–241.

Singh, B.K., Kuhad, R.C., Singh, A., Lal, R., Tripathi, K.K., 1999. Biochemical and molecular basis of pesticide degradation by microorganisms. Crit. Rev. Biotechnol. 3, 197–225.

Speedie, M.K., Pogell, B.M., Mac Donald, M.J., Kline, R., Huang, Y.I., 1987. Potential usefulness of *Streptomyces* for the detoxification of recalcitrant organochlorines and other pollutans. The Actinomycetes 20, 315–335.

Vobis, G., 1992. The genus *Actinoplanes* and related genera. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K. (Eds.), The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Springer, New York, pp. 1030–1060.