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Efficiency of the dispersion and differential centrifugation technique in the isolation of chitinolytic actinomycetes from soil

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Summary

Actinomycetes were isolated from an acidic Brazilian soil under cerrado (savanna) vegetation, previously amended with chitin. The efficiency of two isolation techniques, the conventional dilution plate and the dispersion and differential centrifugation (DDC) procedures, were compared and a mathematical computer analysis of the results was made. The DDC technique gave counts about nine times greater than the conventional one. Analysis of the derivative curves suggested the presence of two distinct groups of actinomycetes on the isolation plates, one able to induce chitinolytic enzymes during amendment and the other whose initial activity was delayed. Major differences in results with different isolation procedures suggest a more subtle ecological role for these microbes in terms of their location in the soil environment.

Introduction

Chitin is an insoluble linear β 1,4-linked polymer of N-acetylglucosamine, a common constituent of fungal cell walls and the arthropod exoskeleton (Flach *et al.* 1992), and an important substrate of the carbon cycle in soil. The ability to degrade chitin is a well known characteristic of soil actinomycetes, and these may comprise from 90% to 99% of the chitinoclastic organisms isolated from certain soils (Alexander 1977). Indeed, Lingappa & Lockwood (1962) have developed a chitin medium for the selective isolation and culture of these organisms. Enrichment of soils with chitin or, to a lesser degree, with fungal cell-wall components, is known to stimulate *Streptomyces* microflora in neutral soils (Mitchell & Alexander 1962; Mitchell 1963; Vruggink 1970), and also in acidic soils (Williams & Robinson 1981).

The addition of chitin to soil has been studied as a possible method for control of fungal pathogens. Amendment of soil with chitin would lead to a large increase in numbers of chitinolytic organisms that could degrade chitin from the fungus cell-wall, resulting in the suppression of the pathogen (Mitchell & Alexander 1962). Another possibility is to use the actinomycete directly as a chitinase producer acting against the cell-wall of the fungal pathogen (Hampson & Coons 1991 and Gupta *et al.* 1995).

In the present study the isolation of actinomycetes from a cerrado (savanna) soil using chitin-agar medium and soil amended with chitin was performed to: (a) compare two isolation procedures, the conventional dilution plate technique and the dispersion and differential centrifugation technique (DDC) (Hopkins *et al.* 1991) in terms of actinomycete recovery and (b) to test the ability of the isolates to degrade chitin on solid chitin media in order to select possible potential strains for biological control.

Materials and Methods

Soil

The soil was an acidic (pH 4.5) red-yellow latosol (oxisol) under cerrado (savanna) vegetation cover, located on the Central Plateau, Brasilia, Federal District, Brazil. Fifty grams sieved soil (< 2 mm) were thoroughly mixed with 4% colloidal chitin and kept for 30 days at a moisture content corresponding to 60% of its water-holding capacity, maintained by regular additions of distilled water.

Isolation procedure

In the conventional technique, actinomycetes were dissociated from soil particles by simply shaking a soil-water suspension (five grams of soil in 30 ml of distilled water) for 20 min at 140 rev/min. In the DDC

technique (Hopkins *et al.* 1991) the actinomycete dissociation was achieved in three successives stages, thus obtaining three distinct supernatants, briefly described as follows: S_1 – supernatant of the extraction and washing with buffer, after shaking, of a mixture of soil with sodium cholate and a chelating resin, submitted to centrifugation; S_2 – the above residue was ultrasonicated in a low-energy ultrasonic bath, in sodium cholate, then shaken and submitted to centrifugation; S_3 – the above residue was shaken in sterile distilled water and submitted to centrifugation. The supernatants obtained from the conventional technique and from the DDC technique were serially diluted in sterile water and plated on a selective medium of chitin-agar (Hsu & Lockwood 1975) with the following composition (g/l): colloidal chitin 4; K_2HPO_4 0.7; KH_2PO_4 0.3; $MgSO_4 \cdot 5H_2O$ 0.5; $FeSO_4 \cdot 7H_2O$ 0.01; $ZnSO_4$ 0.001; $MnCl_2$ 0.001; agar 20; pH 7.0. Inocula (0.1 ml of each dilution) were spread with the aid of a Drigalsky loop, on the surface of the solid medium. Ten replica plates were prepared from each dilution and each supernatant. After incubation at 28 °C, observations for growing colonies were performed at intervals for 23 days. Colonies were enumerated and results presented as number of colony forming units (c.f.u.) of actinomycetes/g dry weight soil.

The growing colonies capable of producing a clear zone on the chitin agar medium were selected and transferred to malt extract-yeast extract-agar medium (Shirling & Gottlieb 1966) for purification. The pure cultures were maintained as a spore suspension in glycerol 10% at −20 °C.

Mathematical analysis

A computer program was specially developed for mathematical analysis of the experiment. First the number of c.f.u. of actinomycetes/g was plotted against time. As there were only nine observation points during the entire experiment, an interpolation routine (Ruckdeshell 1984) was used to obtain a continuous curve, so that the first derivative (Ruckdeshell 1984) of the curve could be taken. The original curve and the derivative one, for each step of the process, was plotted against time.

Selection of chitinolytic actinomycetes

Pure cultures were spot-inoculated on chitin agar plates (Hsu & Lockwood 1975). In order to increase the number of possible positive strains, different values of pH (5.0, 6.0, 7.0 and 8.0) and temperature (28 °C and 37 °C) were tested. After 15 days, plates were examined for evidence of chitinolysis expressed as clear zones around individual growth. Two separate trials were made to confirm the activity.

Statistical analysis

All analysis were run in ten replicates, mean values and standard deviations calculated (SD $<$ 4.8).

Results and Discussion

Using the computer program developed, it was possible to construct continuous curves showing the number of c.f.u. of actinomycetes appearing each time in both isolation procedures (Figure 1), and also the derivative curves showing the rate of appearance of the colonies with time (Figure 2).

In the DDC technique, first described by Hopkins *et al.* (1991), the aim was to disperse soil aggregates and dissociate microorganisms from soil particles, thus making sampling more efficient. Later Macnaughton & O'Donnell (1994), demonstrated the usefulness of the procedure in enhancing the recovery of tuberculostearic-acid-containing actinomycete propagules from soil. In the present paper, the efficiency of the technique was confirmed for isolation and enumeration of chitinolytic actinomycetes from soil amended with chitin. Counts obtained by the conventional technique were compared with those obtained by DDC (Figure 1). After 23 days, there were, respectively, 4.8×10^3 and 43×10^3 ($S_1 + S_2 + S_3$) c.f.u./g of dry weight soil, the DDC procedure being approximately nine times more efficient than the conventional one. Among the three supernatants, the best results were obtained with S_1, counts corresponding to 58% of the total ($S_1 + S_2 + S_3$) (Figure 1).

From the derivative curves (Figure 2), it can be seen that on plates derived from the three supernatants of the DDC procedure, colonies began to appear after the sixth day, while in the conventional procedure this occurred on the eighth day. Between the fourteenth and seventeenth days, the rate of colony appearance was very high on supernatant S_1. Thus, it may be suggested that in the experimental conditions used, counts and/or isolations should begin only after the seventeenth day of incubation.

Chitinase production is generally induced by chitin, and its low molecular weight derivatives, although low levels of chitinase may also be produced in the absence of chitin (Neugebauer *et al.* 1991; Gupta *et al.* 1995). Amendment of soil with chitin for 30 days, and later, isolation on chitin medium, should induce chitinolytic soil actinomycetes (Mitchell & Alexander 1962; Mitchell 1963; Williams & Robinson 1981). In our experiment, the derivative curves revealed, in both procedures, the presence of two humps, corresponding to two different rates of appearance of colonies on plates (Figure 2), the second hump being specially high in DDC-S_1. It can be suggested that, in the amendment conditions of the experiment, some actinomycete propagules still need extra time to be able to grow on a medium containing chitin as sole C and N sources. Apparently there are two groups of actinomycetes on the isolation plates: the first one, able to induce enzymes for chitin degradation during amendment (first hump), and the second one, whose initial activity was delayed (second hump). During the isolation, the first group was able to grow promptly in the chitin medium, while the later-appear-

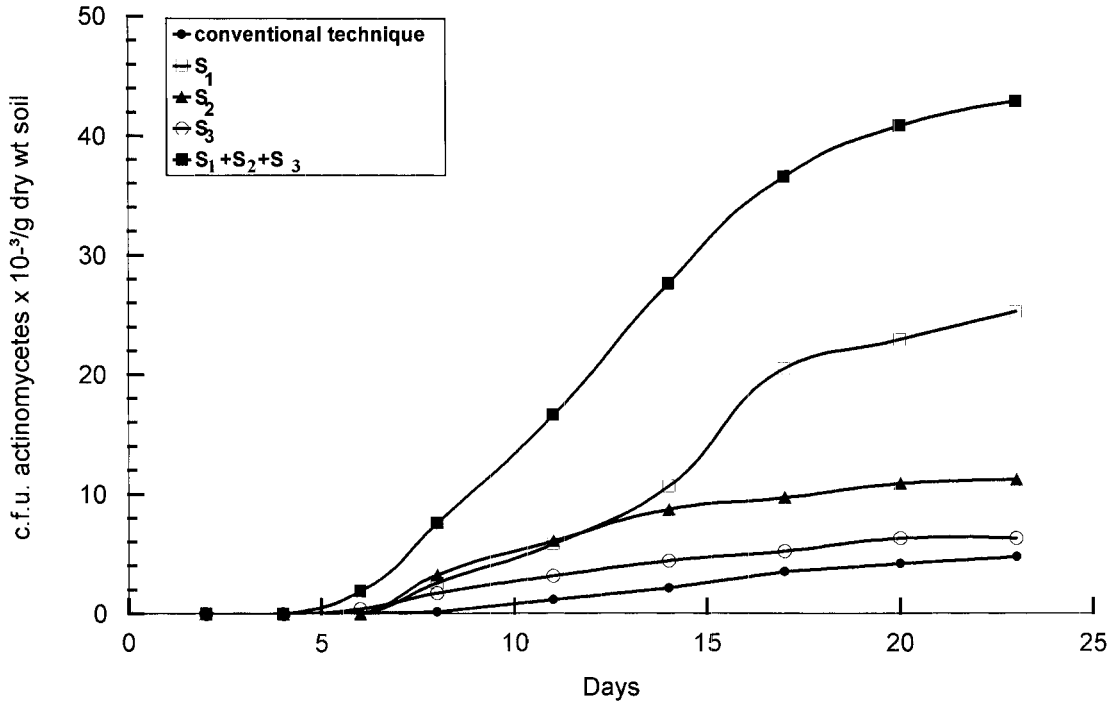

Figure 1. Number of actinomycete colonies appearing on chitin-agar plates each time, in each supernatant (S_1; S_2; S_3) and its sum (S_1 + S_2 + S_3), obtained by the DDC procedure, and by the conventional technique. Supernatants: S_1 – extraction with sodium cholate + chelating resin; S_2 – extraction with sodium cholate and ultrasonication; S_3 – water extraction.

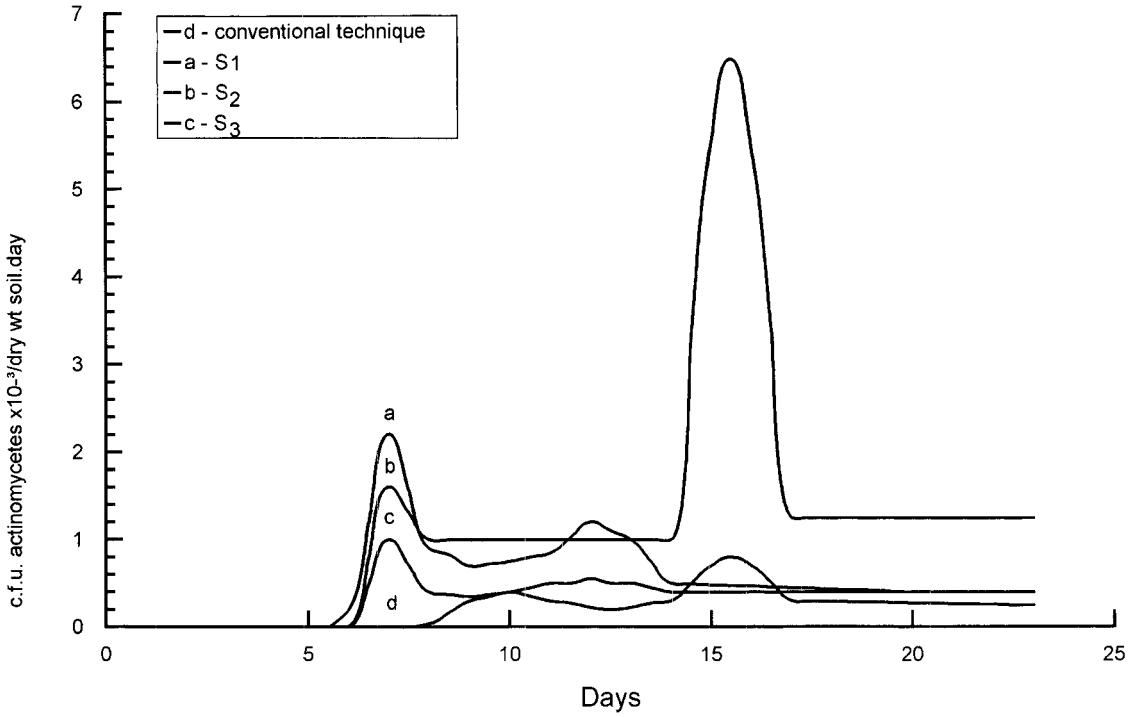

Figure 2. Growth rate of actinomycete colonies appearing on chitin-agar plates each time, in each supernatant S_1; S_2; S_3 obtained by the DDC procedure, and by the conventional technique. Supernatants: S1 – extraction with sodium cholate + chelating resin; S_2 – extraction with sodium cholate and ultrasonication; S_3 – water extraction.

ing second group had to be adapted first, and grew later. It is possible that a portion of soil actinomycetes, included in the second group, might be hidden in microenvironments in the interior of soil aggregates, and did not have access to chitin during amendment. However, after the DDC procedure, or even after the less efficient conventional extraction, these could be separated and then, during contact with chitin in the medium, the induction process could finally take place (Figure 2).

Forty-one actinomycetes were isolated from the cerrado soil amended with 4% chitin. All of them were able to produce, originally, a clear zone around growth, eighty five percent showing activity at both temperatures tested. Most of the isolates showed activity in a neutral to alkaline pH range, although some isolates were able to grow and produce a clear zone also at pH 5.0 and 6.0.

According to Williams and Robinson (1981), successions from acidophilic to neutrophilic actinomycetes may occur when chitin is added to an acidic soil. Initially, acidophiles may be active but as ammonification proceeds, and the pH rises, neutrophilic numbers may significantly increase whereas acidophile numbers may drop. On the other hand, acidic soils may contain, besides the expected acidophiles, neutrophile actinomycetes also, due to periodic occurrence of microsites of higher pH, produced by ammonification of substrates such as amino acids or chitin, initiated by acidophilic or acidouric soil microbes (Williams & Mayfield 1971). Coupled with this, spores of neutrophiles are tolerant to acidity (Flowers & Williams 1977).

Our results demonstrating that most of the strains are able to grow and show chitinolytic activity at a neutral to alkaline pH range, and only two at a strictly acidic range (pH 5 and 6) may be also in accordance with these authors.

In conclusion, the highly significant increase in the numbers of the two populations, evidenced by the DDC procedure, when compared to the conventional isolation technique, suggests the ecological value of this technique in terms of the location of microorganisms in the soil environment. As a consequence, a greater part of soil actinomycete population could be isolated, and tested for chitinolytic activity for further application on biological control.

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