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PLATE-CLEARING TECHNIQUE TO SCREEN MIXED MICROBIAL POPULATIONS FOR PROTEIN DEGRADERS

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Summary—A plate-clearing assay was developed to screen for protein-degrading microorganisms from soil and activated sludge. An insoluble protein, with a substrate-bound dye (Azocoll®) was incorporated into a nutrient agar medium. Microbial enzymatic attack on the substrate produced zones of clearing around colonies with proteolytic capability.

INTRODUCTION

The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) pesticide registration guidelines (U.S. Environmental Protection Agency, 1978), require soil microbial function and activated sludge metabolism studies to determine the toxic effects of pesticides on protein degradation. Of primary concern to laboratories engaged in such testing is that assays be sensitive, rapid and adaptable to different matrices.

We have therefore developed a rapid and inexpensive screening technique whereby protein-degrading microorganisms can be plated directly from a sludge or soil matrix for quantification, isolation or further physiological testing. The plating media used for this assay was prepared from a nutrient medium amended with finely ground Azocoll® (Calbiochem-Behring Corp.) and layered over an agar or nutrient agar base. Azocoll® is an inexpensive, insoluble protein containing an adsorbed, red azo dye which is solubilized by the activity of numerous proteolytic enzymes. We have previously shown that the microbial proteolytic activity within both soil and activated sludge matrices can be measured (spectrophotometrically) using Azocoll® (Caplan and Fahey, 1980; Fahey and Caplan, 1981).

Dilutions of soil or activated sludge were spread-plated onto nutrient agar plates containing finely ground Azocoll®. After a suitable incubation (1–2 days) colonies with proteolytic capability produced distinct halos or clearing zones around them. Protein-degrading colonies could thus be enumerated in a heterogenous population. These colonies were then isolated and streaked for confirmation of proteolytic activity.

MATERIALS AND METHODS

Preparation of plates

Azocoll®-Nutrient Agar (ANA) plates were prepared by pouring an overlay onto standard nutrient agar with 0.1% added glucose (NA) or 15 g l^{-1} agar (WA) plates. Azocoll® was finely ground and brought into suspension with a minimal volume of 95% ethanol.

This suspension was then added to rapidly stirred, autoclaved nutrient agar (5 mg Azocoll® ml^{-1} ANA) which had been cooled to 50°C. Aliquots (10 ml) were poured on top of NA or WA for a final concentration of 50 mg Azocoll® per plate in the top layer. Residual ethanol was allowed to evaporate from the plates by incubating overnight at 37°C with the lids held slightly ajar. The net result was a semi-opaque, red film covering the surface of the agar.

Inoculation

The soil used for this experiment was a sandy loam obtained from Fairfax County, Virginia. It was sieved (<2 mm) and diluted into sterile saline (0.9% NaCl). The activated sludge used was obtained from Back River Sewage Treatment Plant, Dundalk, Maryland which contans *ca.* 15% industrial and 85% domestic wastes.

Soil and sludge aliquots were diluted by either adding 1 g soil or 1 ml sludge in 9 ml saline and diluting to final concentrations of 1×10^{-4} and 1×10^{-5}, respectively. Replicate aliquots (0.1 ml) of diluted sludge or soil were then spread-plated onto ANA plates and incubated for 2 days at 37°C. After incubation, plates were examined for colonial zones of clearing. Both protein degrading and non-degrading colonies were isolated and streaked alongside each other for comparison.

RESULTS AND DISCUSSION

The utility of ANA-plating in screening protein degraders from soil and activated sludge in shown in Fig. 1. The clear area surrounding many of the colonies is due to the disappearance of Azocoll® which is indicative of bacterial proteolytic activity. When colonies positive for zone-clearing were restreaked on ANA plates alongside colonies negative for zone-clearing, the distinction was more pronounced (Fig. 2).

The diameter of zone-clearing about the colony may be used as an indicator of proteolysis, however, caution should be used when interpreting these results quantitatively (Sylvestre, 1980). In other plate-clearing assays (Montecourt and Eveleigh, 1977), overgrowth by filamentous or fast-growing microorganisms can obscure plate-clearing zones, thus requiring the use of inhibitors. Our plate-clearing assay is rapid enough so

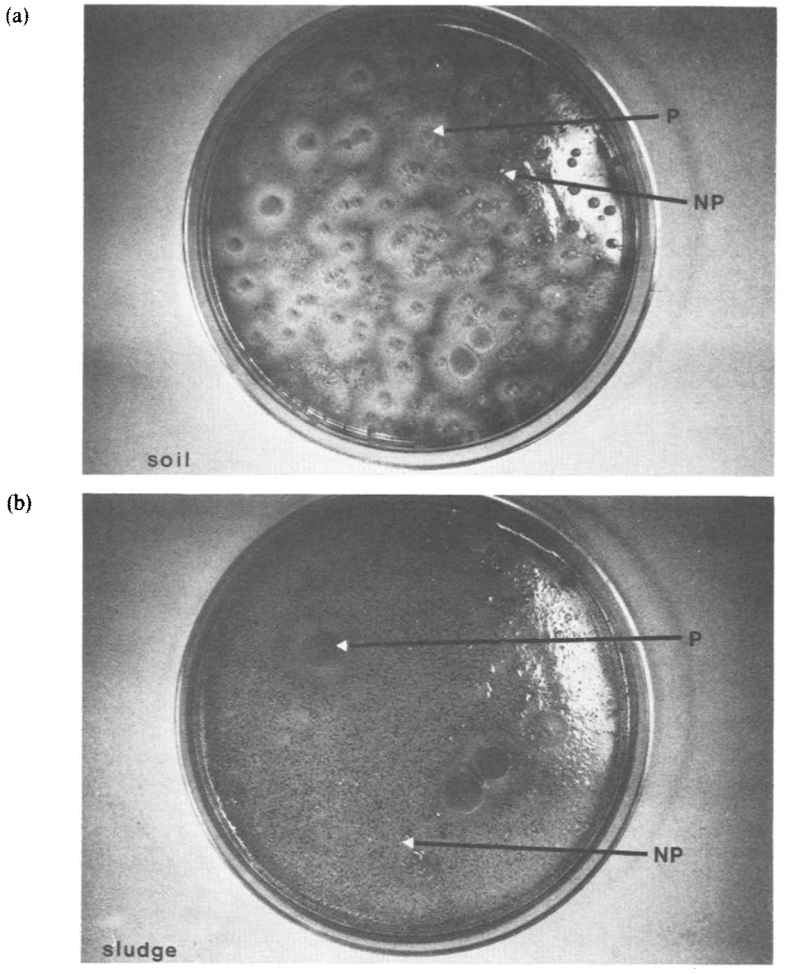

Fig. 1. Plates (ANA) inoculated by serial dilution and spread-plating from soil and activated sludge. Proteolytic (P) or non-proteolytic (NP) colonies are detected by the presence or absence of zone-clearing, respectively.

that microbial overgrowth was not a problem. We were, however, able to limit the growth of filamentous-type, spreading colonies by nutrient limitation, using a WA base rather than a NA base.

In conclusion, we have developed a simple test to screen for protein degraders in soil and activated sludge. This method may be adaptable to other environmental or clinical matrices. Its utility lies in its

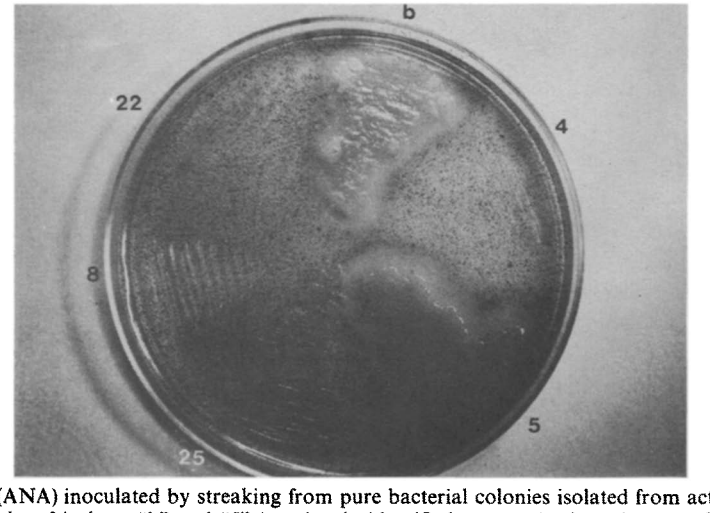

Fig. 2. Plates (ANA) inoculated by streaking from pure bacterial colonies isolated from activated sludge and soil. Streaks of isolates "b" and "5" (previously identified as protein degraders) produced clearing zones on ANA whereas isolates 4, 8, 22 and 25 did not.

ability to rapidly and non-destructively screen large numbers of microbial colonies for proteolytic activity.

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