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An Improved Agar Plate Method for Rapid Assessment of Chemical Inhibition to Microbial Populations

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Assessment of chemical toxicity is essential for effective management of the pollutants in the total environment. To meet such a demand, numerous bioassay procedures employing fish (SASTRY & AGRAWAL 1979), invertebrates (van DIJK et al. 1977), algae (WONG et al. 1979, JOUBERT 1980), protozoans (HONIG et al. 1977), and bacteria (MOWAT 1976, BABICH & STOTZKY 1977) have been developed. Bacteria have several attributes which make them attractive for use in such studies; for example, they are easily handled and require relatively little space. In addition, their short life cycle also means the readily availability of experimental results enabling the laboratory to process more samples. Consequently, bacteria are well suited for use as a primary biological monitoring system for detecting chemical toxicity (MOWAT 1976).

A chemical's toxicity to bacteria is normally measured in terms of inhibition of growth rate (NARKIS & ZUR 1979), oxygen consumption (BROUZES et al. 1978), ATP level (PATTERSON et al. 1970), dehydrogenase activity (RYSSOV-NIELSEN 1975, LIU 1981), and colony formation on agar plate (ANDERSON & ABDELGHANI 1980). The purpose of this paper was to describe the assessment of chemical inhibition to the mixed bacterial populations by a modified agar plate technique and to discuss the potential application of this method in other related areas.

MATERIALS AND METHODS

Chemicals: Phenol, 2-chlorophenol (MCP), 2,6-dichlorophenol (DCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) were obtained from Aldrich Chemicals, Inc., Milwaukee, WI. All chlorophenols, except phenol, were first dissolved in diluted NaOH, followed by neutralization to pH 7.0 with HCl and then made to volume in a 10-mL volumetric flask. Stock solutions were normally prepared at the concentration of 40 mg mL^{-1}. From these, various concentrations of the test chemicals were obtained by dilution with distilled water prior to the initiation of the experiment.

Medium: The growth medium was 0.08% nutrient broth, with the addition of 0.2 g each of glucose and sodium acetate per L of distilled water. Sterilization of the medium was accomplished by autoclaving at 121°C for 15 min. Agar at concentration of 15 g L^{-1} was added to the above liquid medium for making pour plate. The agar plates had a shelf life of approximately six weeks at 4°C in a sealed plastic bag and thus eliminated the need for frequent

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preparation of plates. The plates should be pre-dried before seeding to facilitate the adsorption of the liquid.

Culture: Either pure or mixed bacterial culture could be used to coat the agar plate. Under certain circumstances, the pure culture may be more desirable because of less variability involved in enterpreting the results. However, mixed culture was used because it could reflect the interaction between the toxicant and the microorganisms in the real environment (MOWAT 1976). This modified agar plate technique was originally intended to be used as a pre-screening toxicity test for the biodegradability determination which required the use of the mixed culture as the inoculum (OECD 1978). Consequently the mixed bacterial culture was adapted in the present investigation, which was developed by adding 0.1 mL of fresh domestic activated sludge to a 125-mL Erlenmyer flask containing 50 mL of the growth medium. After 18-20 h growth on a shaker (220 rpm) at room temperature (21°C), 0.1 mL of the culture was transferred into another flask containing fresh medium and the transfer was repeated three times so that an active and stabilized mixed culture was established. The cell concentration was adjusted to 0.10 O.D. (650 nm) with fresh medium just before the seeding of the agar plate.

Procedure: the bioassay procedure was designed to be simple and rapid. Few chemicals and only basic equipment were needed. To perform the toxicity assay, 1 mL of cell suspension (0.10 O.D.) was evenly spread onto the surface of the pre-dried agar plate. After 10-15 min or when the liquid was absorbed, a concentration gradient of the test toxicant were placed on the surface of the seeded agar plate by using a 10-μL Eppendorf micropipette. Usually seven different concentrations of the test toxicant plus one control (distilled water) could be accomodated on a standard 100 mm agar plate. The plates were incubated at room temperature for 18 h. The degree of chemical toxicity of the test substance to the mixed culture was judged by the minimal concentration (MC = μg/spot) of the toxicant required to produce a clear spot on the seeded agar plate in which the micoorganisms had failed to grow.

RESULTS AND DISCUSSION

The idea of using agar plate technique for measuring the chemical inhibition to micoorganisms is not new. Researchers in the pharmaceutical field frequently use this test to determine the bacteriostatic potential of new drugs. To run such experiments, the test chemicals are normally impregnated into paper discs and the degree of bacterial inhibition is estimated by the clear zone around each disc after a certain period of incubation. Therefore, the modified agar plate technique, as described here, was evaluated against the paper disc method using phenol and chlorophenols as the test toxicants. To minimize the artifacts introduced by different seeding techniques in these experiments, the same surface plating technique as described in the Materials and Methods was employed in both methods. However, the procedures for applying the test chemicals were different. The chemicals were directly delivered onto the seeded plate via a 10- μL micropipette in the modified agar

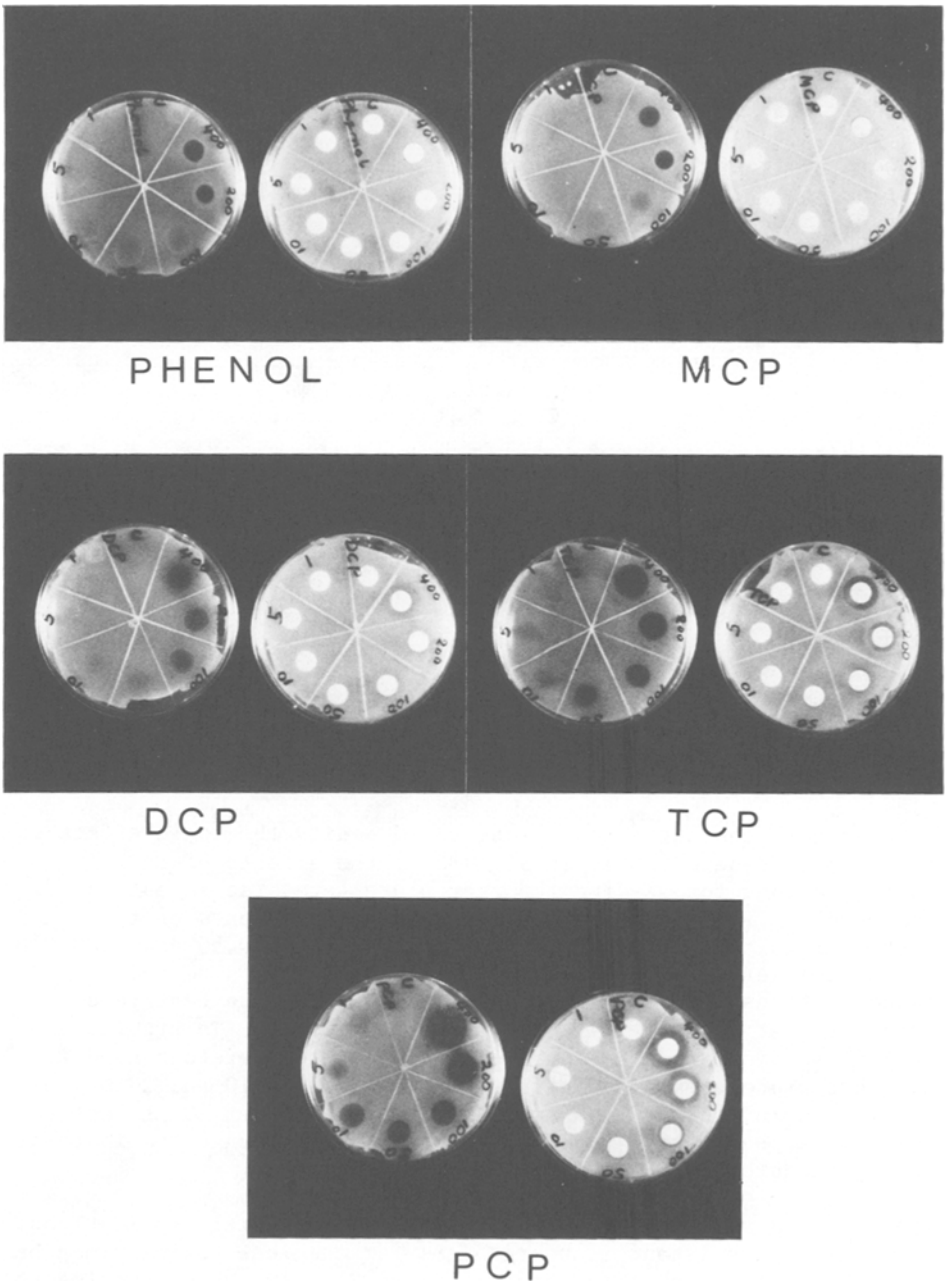

Figure 1. Typical clear spot formations on the modified agar plates after treatment with various toxicants. Plates on the right side using the conventional paper disc.

plate technique whereas 10 mm paper disc wetted with test chemicals were used in the paper disc method.

The results show that the modified agar plate technique had a higher sensitivity in detecting the chemical toxicity than the paper disc method (Figure 1). PCP toxicity could be demonstrated at concentrations as low as 5 μg/spot in the former, while the latter required 50 μg PCP to exhibit a visible toxicity. Similarly, phenol show no apparent toxicity at 400 μg/spot in the paper disc test, a distinct inhibition was observed at 200 μg/spot for the modified agar plate technique. The lower sensitivity of the paper disc test could be due to the retention of the toxicant by the paper and thus diminished the toxicity effect. However, the modified agar plate method did not provide quantitative toxicity data, although information on relative toxicities of chemicals could be easily obtained by comparing those with a reference chemical, such as phenol (Table 1). Thus the toxicity data could be reported in terms of phenol toxicity; e.g., TCP toxicity is four times the phenol toxicity.

Table 1. Relative toxicity of chlorophenols expressed in terms of phenol toxicity

Toxicants	Relative phenol toxicity
phenol	1
2-chlorophenol	1
2,6-dichlorophenol	2
2,4,6-trichlorophenol	4
pentachlorophenol	40

As high cell concentrations alone could modify the toxic effect in a bioassay procedure (HONIG et al. 1980), the effects of cell concentrations for seeding the agar plate were also investigated. One mL of cell suspensions containing various amounts of the mixed bacterial culture (0.01, 0.02, 0.05, 0.10, 0.20, 0.50 and 1.00 O.D. at 650 nm) were employed to coat the agar plates. The cell concentrations at 0.10 O.D. were found to yield the best results in terms of test sensitivity and coating uniformity. To provide an optimal seeding, such cell concentrations were therefore used for all test procedures. The effect of the size (5, 10 and 20 μL) of the test solution was also examined and the size of 10 μL test solution appeared to give the best spot and was suggested in the test procedures.

The modified agar plate technique has many potential applications. For example, resistant microorganisms for chemical toxicity may be easily isolated with this method (Figure 2). In these experiments, various amounts of PCP were applied to the seeded plates. After 20-24 h incubation at room temperature, bacterial colonies resistant to PCP toxicity were observed to develop at the spots having received 5 and 10 μg of PCP. Further incubation of the plates resulted in the formation of two colonies in the spot receiving higher PCP treatment (50 μg). Subculture of these colonies into

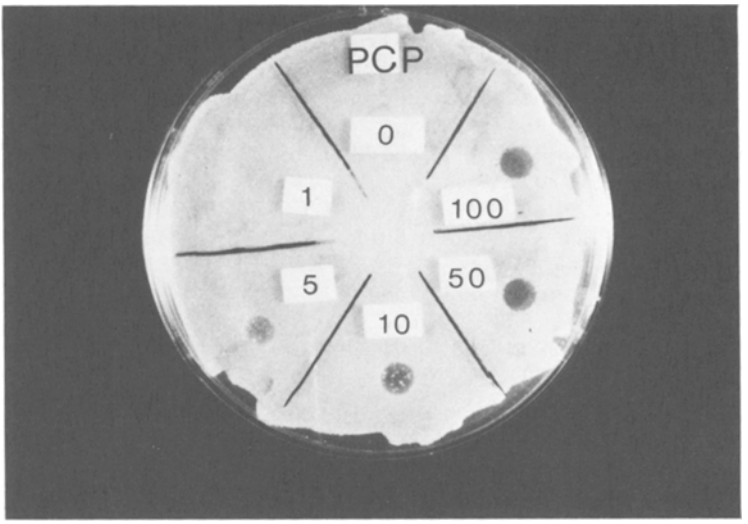

Figure 2. Development of PCP resistant colonies after 20-24 h incubation.

liquid growth medium containing 20 ppm of PCP demonstrated their tolerance to PCP toxicity. Therefore, the modified agar plate technique could be readily used in both toxicity and biodegradation studies. Another promising aspect of this test is its applicability in the assessment of chemical toxicity to fungi. Fungi tend to form clumps during growth in liquid medium which limits the usefulness of the conventional biomass technique such as turbidity and dry weight determinations in the study of chemical toxicity to fungi. To demonstrate the versatility of the modified agar plate technique for use in such a toxicity study, the fungus, Peacilomyces sp. was grown as a stationary culture in 100 mL of Sabouraud dextrose broth (DIFCO) for four days at room temperature (21°C) and the contents of the culture flask were homogenized in a Waring blender at low speed (powerstat at 30/120 v) for 3 min. Then 1 mL of the suspensions (0.20 O.D. at 650 nm) was used to seed the pre-dried Sabouraud dextrose agar plate prior to the application of the test toxicant (PCP) onto the plates. After 48 h incubation, the chemical toxicity of PCP to Peacilomyces sp. was determined to be about 20 µg/spot.

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