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AGAR PLATE METHOD FOR RAPID SCREENING OF CHEMICAL TOXICITY

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EXECUTIVE SUMMARY

Biological monitoring of contaminants in the assessment of environmental quality have been recognized and accepted by various governmental agencies, such as US EPA. This is mainly due to the recognition of the inadequacy of using chemical analysis alone to safeguard the public health from the danger of exposure to environmental contaminants. Because of the vast number of existing and newly introduced chemicals in the environment, our laboratory has been actively involved in the development of various short-term bioassay procedures for assessing the toxicity potential of toxic substances. The agar plate technique developed in our laboratory in 1981 has been now widely used by American industries. The technique is based on the measurement of the interaction between the test toxicant and the microbial colony formation on the agar plate. To effect an efficient technology transfer to the private sector and governmental agencies, the agar plate method (1981) has been re-examined, modified, and prepared in a standard form (cook-book style). Thus most laboratory staff should be capable of performing this test without the need for additional references.

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RESUME

Divers organismes gouvernementaux, dont l'EPA aux Etats-Unis, ont reconnu l'importance de la surveillance biologique des contaminants dans l'évaluation de la qualité de l'environnement. En effet, il est apparu, entre autres choses, que les analyses chimiques ne pouvaient assurer seules la protection de la santé publique face à une exposition aux contaminants environnementaux. Etant donné le nombre très élevé des produits chimiques existants et nouvellement introduits dans l'environnement, nos laboratoires travaillent activement à mettre au point diverses méthodes de bio-essai à court terme pour évaluer la toxicité potentielle des substances toxiques. La technique de la plaque de gélose, mise au point dans nos laboratoires en 1981, est aujourd'hui largement utilisée par les industries américaines. Cette méthode est basée sur la mesure de l'interaction entre le produit toxique à l'essai et la formation de la colonie microbienne sur la plaque de gélose. Pour assurer un transfert efficace de la technologie vers le secteur privé et les organismes gouvernementaux, la méthode (1981) a été réévaluée, modifiée et diffusée sous forme normalisée (style livre de recettes). Ainsi, la plupart des membres du personnel de laboratoire devraient être en mesure d'effectuer cet essai sans références supplémentaires.

METHODE DE LA PLAQUE DE GÉLOSE POUR L'EVALUATION RAPIDE DE LA TOXICITÉ DES PRODUITS CHIMIQUES

MANAGEMENT PERSPECTIVE

Contaminants seldom exist in the environment in pure form or single species. The interactions between biota and toxicants are extremely complicated, because many processes and mechanisms such as biodegradation, biotransformation, syngerism, and antagonism are all involved in these interactions. Moreover, contaminants seldom exist in the environment in pure form or single species. Thus bioassays are the only sure way of providing reliable data about the potential impact of contaminants on environmental quality. The agar plate technique developed in our laboratory has been found wide use among American industries, mainly due to its rapidity and low cost. Consequently, this bioassay procedure was modified, and prepared in a standard format (I.S.O.) for publication in the new international journal of "Toxicity Assessment". Such an endeavour will, undoubtedly, continue to demonstrate ECD's active and leading role on the toxic chemical issues of national significance.

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PERSPECTIVE DE GESTION

Les contaminants se trouvent rarement à l'état pur ou d'espèce unique dans l'environnement. Les interactions entre le biote et les produits toxiques sont extrêmement complexes, étant donné que de nombreux processus et mécanismes, comme la biodégradation, la biotransformation, la synergie et l'antagonisme, entrent en jeu dans ces interactions. En outre, il est rare que les contaminants se trouvent dans l'environnement sous leur forme pure ou sous forme d'espèce unique. C'est pourquoi les bio-essais sont la seule façon sûre d'obtenir des données fiables sur l'impact éventuel de ces contaminants sur la qualité du milieu. La méthode de la plaque de gélose mise au point dans nos laboratoires est aujourd'hui largement utilisée dans les industries américaines, surtout à cause de sa rapidité et de son coût peu élevé. En conséquence, cette méthode de bio-essai a été modifiée et présentée sous un format standard (I.S.O.) pour fin de publication dans le nouveau journal international "Toxicity Assessment". Cette réalisation continuera sans aucun doute à témoigner du rôle dominant de la division des contaminants de l'environnement dans les questions portant sur les produits chimiques toxiques à l'échelle nationale.

LA METHODE DE LA PLAQUE DE GELOSE POUR L'EVALUATION RAPIDE DE LA TOXICITE DES PRODUITS CHIMIQUES

1.0 OBJECTIVE

This method is intended to be used either as a screening procedure for detecting the presence of toxic chemicals in water and wastewater, and sediment extracts or as a qualitative test for ranking the toxicity potential of chemical compounds or formulations.

2.0 SCOPE

This method is applicable to the following types of samples:

(i) Polluted surface and ground waters

(ii) Domestic and industrial wasterwarers including leachates and sludge extract.

(iii) Most chemicals that are soluble or insoluble in water.

3.0 PRINCIPLE

3.1 Fresh and actively growing bacterial cells are, in general, more susceptible to growth inhibition by toxic chemicals, and the extent of this inhibition offers a convenient way of assessing a chemical's toxicity potential.

3.2 To perform the test, agar plates thinly coated with fresh bacterial culture (pure or mixed) are spotted with test chemicals, and the plates are incubated at room temperature for 18 hr.

3.3 The degree of toxicity of the test chemical to the bacterial culture is judged by the minimal concentration of the toxicant required to produce a clear spot on the seeded agar plate indicating the failure of the culture to grow.

4.0 REAGENTS, MATERIALS AND APPARATUS

4.1 Reagents

4.1.1 Growth Medium

Glucose	0.2 g
Sodium acetate	0.2 g
Nutrient broth	0.8 g
Distilled water	1 L

The medium is sterilized by autoclaving at 121^{o}C for 15 min. Agar at concentration of 15 g per liter is added to the above medium for making test plates. The agar plate has a shelf life of approximately 6 weeks at 4^{o}C in a sealed plastic bag. Thus eliminating the need for frequent plate preparation. The agar plates should be pre-dried (see section 6.1) before culture seeding to facilitate the adsorption of the liquid.

4.1.2 Test culture

Either pure or mixed bacterial cultures may be used to coat the agar plate. Under certain circumstances, a pure culture may be more desirable because of less variability involved in interpreting the test results. However the use of a mixed bacterial culture has the advantage of being able to approximate the interaction between the toxicant and microbial communities in the environment.

Bacillus cereus originally isolated from activated sludge is routinely used as the testing bacteria. The culture is grown in 100 mL of liquid medium contained in a 250-mL Erlenmyer flask on a rotary shaker (220 rpm) for 18 hrs at room temperature (21^{o}C). The culture should be transferred at least twice before using in the surface coating of the agar plates.

A mixed bacterial culture is usually developed by adding 0.5 mL of fresh domestic activated sludge to 100 mL of liquid medium contained in a 250-mL Erlenmyer flask. After 18 hrs growth on a shaker at room temperature, 0.5 mL of the culture is transferred into another flask containing fresh medium. The transfer is repeated at least three times so that an active and established mixed culture can be obtained.

4.2 Materials

Sterile 1-mL pipettes for culture inoculation and agar plate coating.

10-uL Eppendorf pipette for spotting the test chemicals.

250-mL Erlenmyer flasks.

Standard size (15 x 100 mm) petri dishes.

4.3 Apparatus

Colorimeter or spectrophotometer (400-700 nm).

Rotary shaker.

Microscope with magnification power of 400X.

Refrigerator for storing medium and agar plates.

Clean incubator (40-45^{o}C) for pre-drying agar plates.

5.0 CULTURE MAINTENANCE

Bacillus cereus can be easily isolated from the environment according to conventional microbiological procedures, or can be obtained from the author.

5.1 Stock Culture Maintenance (Agar Slant Technique)

Prepare agar slants in 20 x 150 mm plugged culture tubes using the growth medium (supplemented with 15 g of agar per liter) as noted in Section 4.1.1. Transfer 1 loop of active culture

onto the agar slant and incubate at room temperature (21°C) for approximately 18 hrs to develop a healthy culture on the agar slant. The slant is stored at 4°C and is transferred at two month intervals.

5.2 Stock Culture Maintenance (Liquid Broth Technique)

Transfer 1 mL of culture (pure or mixed) into 100 mL of growth medium as noted in Sections 4.1.1 and 4.1.2. The culture is incugated at room temperature on a rotary shaker for approximately 18 hrs. The culture is stored in a cold room or refrigerator (4°C) and is thereafter transferred at two month intervals.

5.3 All test cultures (pure or mixed) must be transferred at least twice before using in the toxicity screening procedure. This practice is to ensure that only the actively growing cells are used as the test organisms.

6.0 TESTING PROCEDURE

The agar plate bioassay procedure is designed to be simple and rapid. Consequently, only very basic equipments are required to perform this testing procedure.

6.1 Pre-dry agar plates in a clean oven (40-45°C) for approximately one hour to facilitate the rapid absorption of bacterial seeding and test toxicants.

6.2 Adjust the bacterial culture with fresh growth medium (broth) to a final concentration of 0.10 O.D. (625nm) using a spectrophotometer or colorimeter, prior to seeding the agar plates.

6.3 With the aid of a rotary auto-plater (Lab-Line cat. No. 1580), 1 mL of the above bacterial suspension is evenly spread onto the

surface of the pre-dried agar plate. Alternatively, a bent glass rod can be used to manually spread the 1 mL of culture evenly across the agar plate.

6.4 After 5-10 min or when the liquid is completely absorbed, the seeded agar plates are ready for the toxicity bioassay.

6.5 Ten uL of solution containing 10, 50, 200, 500, and 1000 ug of test toxicant plus one control (distilled water) are placed onto the surface of the seeded agar plate by using a 10-uL Eppendorf micropipette.

6.6 To avoid any confusion that may arise in performing the bioassay procedure, the control should be always placed at the 12 o'clock position on the agar plate, which is followed clockwise by the the test toxicant arranged in a concentration gradient pattern.

6.7 The agar plates are incubated at room temperature for 18 hrs for observation of any microbial growth inhibition by the test toxicant.

7.0 EXPRESSION OF RESULTS

7.1 The degree of toxicity of the test chemical compound to the bacterial culture is judged by the minimal concentration (MC = ug/spot) of the test toxicant required to produce a clear spot on the seeded agar plate on which the microorganisms have failed to grow.

7.2 For sediment extract, wastewater, and leachate, the MC values may be expressed in equivalent to uL/spot or ug sample/spot.

7.3 The toxicity data generated by the agar plate method are qualitative in nature. However they could be used conveniently in ranking the toxicity potential of the test chemicals or environmental samples.

7.4 The relative toxicities of chemical compounds may be also obtained by comparing their toxicities with a reference chemical. Thus the toxicity of chlorophenols may be reported in terms of phenol toxicity. For instance, pentachlorophenol is reported as 40 times the phenol toxicity.

8.0 PERSONAL REMARKS

8.1 In general, fresh actively growing, log phase cultures are more responsive than stable older cultures to the toxic effect of chemicals are. Consequently, only actively growing cells (18 hrs or less) should be used in seeding the agar plate.

8.2 The agar plate technique has many potential applications in various environmental and toxicological studies. For example, microorganisms resistant to specific toxicants could be easily isolated by this method, simply by re-incubating the agar plates for 48 hrs or longer. Colonies developing within the cleared area are probably resistant to the concentration of toxicant used, or may be mutant forms naturally occurring or due to plasmid transfer.