Techniques

Agarose Medium for Bioassay of Antimicrobial Substances

W. C. Ho and W. H. Ko

Graduate student and professor, respectively, Department of Plant Pathology, University of Hawaii, Beaumont Agricultural Research Center, Hilo 96720.
Journal Series Paper 2461 of the Hawaii Agricultural Experiment Station.
This study was supported in part by a grant from the East-West Center to the senior author.
Accepted for publication 2 February 1980.

ABSTRACT

HO, W. C., and W. H. KO. 1980. Agarose medium for bioassay of antimicrobial substances. Phytopathology 70:764-766.

Seven of eight media-solidifying agents tested contained nutrients sufficient to support spore germination of nutrient-dependent fungi. Agarose at 0.8% did not support germination of nutrient-dependent fungi, but supported complete germination of nutrient-independent fungi. The inhibitory effect of $AlCl_3$, $CuCl_2$, and $ZnCl_2$ on spore germination was greatly reduced in agar, but not in agarose medium. The inhibitory effect of streptomycin and neomycin against bacterial growth in agarose medium also was about the same as that in water. Results show that SeaKem agarose is an ideal solidifying agent for assaying antimicrobial activity of chemicals.

Agar media have been used commonly in screening microorganisms for antibiotic production (11) and also in assaying antimicrobial activity of chemicals (4,14). However, the presence of agar in a test medium has been shown to reduce the inhibitory effect of certain antibiotics (8,10) and other antibacterial substances (2,3,6,7,15,16) on the growth of bacteria, and to interfere with the activity of various chemicals against spore germination of fungi (12). The purpose of this investigation was to search for a solidifying agent with minimal effect on activity of antimicrobial substances. A brief account of this research has been reported (9).

MATERIALS AND METHODS

Conidia of *Bipolaris maydis* (Nishikado) Shoemaker, *Alternaria alternata* (Fr.) Keissler, *Penicillium frequentans* Westling, and *Mucor ramannianus* Moller were obtained by growing each fungus under continuous fluorescent light for 6–8 days at 24 C on V-8 juice agar (1). *Agrobacterium radiobacter* (Beijerinck & van Delden) Conn and *Xanthomonas campestris* (Pam.) Dows. were cultured on nutrient agar for 3 days at 24 C. *B. maydis* and *X. campestris* were supplied by M. Aragaki and A. M. Alverez, respectively.

Chemicals were added separately to 2% Bacto water agar, 0.8% SeaKem water agarose (SeaKem HGT-P Agarose; Marine Colloids Division, FMC Corporation, Rockland, ME 04841, USA), and distilled water before autoclaving. SeaKem agarose medium requires thorough shaking before pouring plates because of the high viscosity in the bottom portion after autoclaving. The gel strength of 0.8% SeaKem agarose is comparable to that of 2% Bacto agar. Water agarose and water were supplemented with 300 μg/ml glucose and 300 μg/ml peptone for growth of bacteria, and with 150 μg/ml glucose and 150 μg/ml peptone for spore germination of nutrient-dependent fungi. Twenty milliliters of solid medium in a 100-mm plate and 2 ml of liquid medium in a 60-mm plate were used. Spore suspensions were adjusted to give about 50 spores per square millimeter for *P. frequentans* and *M.*

0031-949X/80/08076403/$03.00/0

ramannianus and three spores per square millimeter for *B. maydis* and *A. alternata*. Inhibitory effect was compared at ED_{50} which was the amount of a chemical capable of reducing spore germination to 50% as determined from the dosage-response curve (12). Bacterial suspension was adjusted to about 10^5 cells per milliliter, and one drop (approximately 0.05 ml) of the suspension was spotted on each plate. Growth of bacteria was determined visually after 2 days of incubation at 24 C, and the minimum inhibitory concentration (MIC) was used to compare the antibiotic activity. The concentrations of antibiotics tested were 1, 5, 10, 50, 100, 200, 500, and 1,000 μg/ml. Two replicates were used and all experiments were done at least two times.

RESULTS AND DISCUSSION

Conidia of *P. frequentans* and *M. ramannianus* are nutrient dependent and cannot germinate in the nutrient-free medium (13). With the exception of SeaKem agarose, all of the solidifying agents tested contained sufficient nutrients to support complete or partial germination of both fungi (Table 1). None of the conidia of *P. frequentans* and *M. ramannianus* germinated on 0.8% SeaKem agarose. Conidia of *B. maydis* and *A. alternata* which are nutrient independent, germinated completely both in distilled water and on 0.8% SeaKem agarose. Both *P. frequentans* and *M. ramannianus* germinated completely when SeaKem agarose was amended with 150 μg/ml glucose and 150 μg/ml peptone. This suggested that failure of *P. frequentans* and *M. ramannianus* to germinate on SeaKem water agarose was due to the absence of nutrients, rather than the presence of inhibitors.

TABLE 1. Effect of solidifying agents on spore germination of nutrient-dependent fungi

Solidifying agent	Spore germinations (%)[a]	
	Penicillium frequentans	*Mucor ramannianus*
Bacto agar (2%)	99	99
Bacto purified agar (2%)	99	100
Bacto Noble agar (2%)	99	100
Bacto gelatin (12%)	98	100
Calbiochem agarose, grade A (2%)	97	74
Calbiochem agarose, grade B (2%)	95	49
NBC agarose (2%)	46	37
SeaKem agarose (0.8%)	0	0
Water, control	0	0

[a] Data were from one of two experiments with similar results. More than 200 spores were counted in each treatment for each experiment.

Bacto agar greatly reduced the inhibitory effect of $AlCl_3$, $CuCl_2$, and $ZnCl_2$ on spore germination of *A. alternata, B. maydis* and *P. frequentans* (Table 2). However, the inhibitory effect of these compounds in SeaKem water agarose was about the same as that in water. For example, when *A. alternata* was used the ED_{50} values for $AlCl_3$ in Bacto water agar, SeaKem water agar, and water were 370, 1.2, and 0.6 μg/ml, respectively. For the three fungi tested, the ED_{50} ratio of $AlCl_3$ in Bacto water agar and in water ranged from 100 to 617, while that in SeaKem water agarose and in water ranged from 1 to 2. Antibiotic activity of streptomycin and neomycin towards *A. radiobacter* and *X. campestris* also was decreased markedly by Bacto agar (Table 3). Inactivation of antibiotic activity was insignificant when Bacto agar was replaced by SeaKem agarose. For instance, when *A. radiobacter* was used as the test organism, the MIC values for neomycin in Bacto water agar, SeaKem water agarose and water were 1,000, 5, and 5 μg/ml, respectively. For both *A. radiobacter* and *X. campestris*, the MIC ratio of neomycin and streptomycin in Bacto water agar and in water ranged from 100 to 200, while that in SeaKem water agarose and in water ranged from 1 to 2. Our results showed that the antimicrobial activity of antibiotics and other chemicals tested in SeaKem water agar was about the same as that in water. Therefore, SeaKem HGT-P agarose is considered an ideal solidifying agent for bioassay of antimicrobial substances.

According to Duckworth and Yaphe (5), complete agar consists of three extremes of polysaccharides. These are neutral agarose, charged pyruvated agarose with little sulphation, and sulphated galactan. The first two are capable of forming gel, but the last one is not. Since addition of agar granules to solution also reduced the antimicrobial activity, binding of chemicals by the ionic portion of agar was considered to be responsible for chemical inactivation

TABLE 2. Effect of different chemicals in 2% Bacto water agar, 0.8% SeaKem water agarose and water on spore germination of fungi

Chemical and test fungus	ED_{50} (μg/ml)[a]			ED_{50} ratio[b]	
	Water agar	Water agarose	Water	Water agar/Water	Water agarose/Water
Aluminum chloride					
Alternaria alternata	370	1.2	0.6	617	2
Bipolaris maydis	500	2	1.5	333	1
Penicillium frequentans	350	2.5	3.5	100	1
Cupric chloride					
A. alternata	70	0.3	0.3	233	1
B. maydis	28	0.3	2.3	12	0.1
P. frequentans	20	4	3	7	1
Zinc chloride					
A. alternata	700	4	34	21	0.1
B. maydis	650	13	5	130	3
P. frequentans	250	10	12	21	1

[a] For *Penicillium frequentans* 150 μg/ml glucose and 150 μg/ml peptone were added to water agarose and water.
[b] Ratio was calculated to the nearest whole number. Data are from one of two experiments with similar results.

TABLE 3. Effect of antibiotics in 2% Bacto water agar, 0.8% SeaKem water agarose, and water on growth of bacteria

Antibiotic and test bacterium	MIC (μg/ml)[a]			MIC ratio	
	Water agar	Water agarose	Water	Water agarose/Water	Water agarose/Water
Neomycin					
Agrobacterium radiobacter	1,000	5	5	200	1
Xanthomonas campestris	500	5	5	100	1
Streptomycin					
A. radiobacter	1,000	10	5	200	2
X. campestris	1,000	10	5	200	2

[a] Water agarose and water were supplemented with 300 μg/ml glucose and 300 μg/ml peptone. Data are from one of two experiments with similar results.

(12,15). The practical definition of agarose is a mixture of agar molecules with lowest charge content and therefore greatest gelling ability (5). Commercial preparations of agarose contain different numbers of charged groups. According to the analytical values of Marine Colloids, Inc., SeaKem HGT-P agarose contains relatively few charged groups and has very high gel strength. This may be the reason why it has little effect on antimicrobial activity of chemicals. SeaKem agarose may also be useful in nutritional studies of microorganisms.

LITERATURE CITED

1. ARAGAKI, M. 1964. Relation of radiation and temperature to the sporulation of *Alternaria tomato* and other fungi. Phytopathology 54:565-569.
2. BARKER, H. E. R. 1948. Cationic-anionic incompatibility and ointments containing cation active antiseptics. Aust. J. Pharm. 29:801-807.
3. BARKER, H. E. R. 1949. The effect of agar on the bacteriostatic activities of acridine. Aust. J. Pharm. 30:573.
4. COLLINS, C. H., and P. M. LYNE. 1970. Microbiological Methods. Butterworth & Co., Ltd., London. 454 pp.
5. DUCKWORTH, M., and W. YAPHE. 1971. The structure of agar. Part I. Fractionation of a complex mixture of polysaccharides. Carbohyd. Res. 16:189-197.
6. GREENBERG, J. 1969. A factor in agar which reverses the antibacterial activity of 1-methyl-3-nitro-1-nitrosoguanidine. Nature 88:660.
7. HANUS, F. J., and E. O. BENNETT. 1964. The effect of agar on the inhibitory activities of fatty amines. Antonie van Leeuwenhoek J. Microbiol. Serol. 30:412-416.
8. HANUS, F. J., J. G. SANDS, and E. O. BENNETT. 1967. Antiobiotic activity in the presence of agar. Appl. Microbiol. 15:31-34.
9. HO, W. C., and W. H. KO. 1979. SeaKem agarose as a solidifying agent for studying chemical activity. (Abstr.) Phytopathology 69:1031-1032.
10. IYER, R., and V. IYER. 1960. Effect of agar on the inhibition of *Micrococcus pyogens* var. *aureus* by chlortetracycline and other antibiotics. Antibiot. Chemother. 10:409-413.
11. JOHNSON, L. F., and E. A. CURL. 1972. Methods for research on the ecology of soil-borne plant pathogens. Burgess, Minneapolis, MN 247 pp.
12. KO, W. H., J. T. KLIEJUNAS, and J. T. SHIMOOKA. 1976. Effect of agar on inhibition of spore germination by chemicals. Phytopathology 66:363-366.
13. KO, W. H., and J. L. LOCKWOOD. 1967. Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57:894-901.
14. NEELY, D. 1969. The value of in vitro fungicide tests. Ill. Nat. Hist. Surv., Biol. Notes 64:1-8.
15. QUISNO, R. A., I. W. GIBBY, and M. J. FOTER. 1946. The effect of agar upon the germicidal efficiency of the quaternary ammonium salts. J. Am. Pharm. Assoc., Sci. Ed. 35:317-319.
16. SANDS, J. G., L. A. GOERS, and E. O. BENNETT. 1963. The effect of agar on the inhibitory activity of phenols. Antonie van Leeuwenhoek, J. Microbiol. Serol. 29:386-392.