Influence of Agar on the Effectiveness of Culture Media[1]

YISHAN LIN, *J. E. Siebel Sons' Company, Marschall Division, Miles Laboratories, Inc., Chicago, IL 60646*

ABSTRACT

A variety of agars were used in the preparation of culture media and studied for their influence on the effectiveness of each medium. Included in the studies were different kinds of agar, the same kind of agar purchased at different times, and agar from different companies. These agars in universal beer agar formulations did not have any effect on the growth of bacteria or yeasts. However, they greatly affected the effectiveness of Lin's medium and crystal violet medium in the detection of wild yeasts. The optimum concentrations of crystal violet in Lin's medium and in crystal violet medium varied in accordance with the different kinds of agar used in the medium. These agar variations did not alter the effectiveness of lysine medium in the detection of wild yeasts, but they did influence the colony sizes of some wild yeasts on this medium. They also affected the sporulation of yeasts on sodium acetate agar and on Kleyn's agar.

Key words: *Crystal violet, Wild yeast, Yeast sporulation.*

Agar is an agent used for the solidification of microbiological culture media, and is usually employed in concentrations of 1–2%. It should be free from starch and debris and capable of producing a clear solution when hot; the exact concentration needed to give the desired degree of solidity may vary with the degree of purification (10).

In the brewery several unique solid media are used in microbiological laboratories for the detection of brewery contaminants. In a previous study (6), we found that agar had some influence on the effective concentration of crystal violet in Lin's medium (LWYM) used to detect wild yeast. Therefore, in the present study, different types of agar, the same type of agar purchased in different years, and agars from different companies were used to determine what effect they might have on media used in breweries.

MATERIALS AND METHODS

Organisms

The bacterial cultures used in these studies were *Flavobacterium proteus, Lactobacillus brevis, L. delbruckii,* and *Pediococcus cerevisiae.* For inoculation, fresh inocula which had been subcultured in tomato juice broth with 1% peptonized milk at 25°C for 1–3 days were used. The yeast cultures used included *Saccharomyces uvarum* (lager yeast), *S. cerevisiae* var. *ellipsoideus, S. diastaticus, S. pastorianus, S. willianus, Hansenula anomala, Hansenula* sp., *Pichia membranaefaciens, Rhodotorula* sp., *Torula lactosa,* and *T. rosea.* All yeast cultures were maintained on YM agar (Difco 0712-01) slants. For inoculation, fresh inocula which had been subcultured on YM agar slants at 25°C for 1–3 days were used.

Media

Universal beer agar (UBA) was prepared according to the method of Kozulis and Page (5) except that distilled water was used instead of tap water. The medium was also prepared from commercially dehydrated UBA (Difco 0856-01) according to manufacturer's instructions.

Lysine medium was prepared according to the method of the ASBC Subcommittee for Microbiological Controls (1).

The method of Kato (3) was followed in the preparation of crystal violet medium except that various concentrations other than 20 mg/L of crystal violet were used.

Lin's medium was prepared as previously described (6).

Kleyn's agar was prepared according to the method of Kleyn (4), and sodium acetate agar was prepared by adding 1.5% agar to 0.5% sodium acetate solution.

Agar

The agars used and the year purchased are listed in Table I.

Plating

The inocula were diluted to suitable concentrations. Each plate received 0.1–0.3 ml inoculum dispersed over its surface with a bent glass rod. The cell concentration of the applied inoculum was determined with a hemocytometer just prior to inoculation. The incubation conditions for the inoculated plates are shown below. The UBA medium, bearing *L. brevis, L. delbruckii,* and *P. cerevisiae,* were incubated anaerobically, while those bearing *F. proteus* and yeasts were incubated aerobically.

UBA plates:	25°C, 3 days for aerobic 25°C, 5 days for anaerobic
LWYM plates:	25°C, 5 days for aerobic 30°C, 5 days for aerobic
Lysine medium plates:	25°C, 3–6 days for aerobic
Crystal violet medium plates:	30°C, 2 days for aerobic
Kleyn's agar plates:	25°C, 2 days for aerobic
Sodium acetate agar plates:	25°C, 2 days for aerobic

Sporulation

The yeast cells grown on YM agar for 24 hr were surface spread on sporulation medium. The concentration of inoculum used was 1×10^6 cells per plate. Following incubation, the yeast was examined for sporulation by staining according to the "cold" method of Bartholomew and Mitter (10).

Membrane Filtration

In membrane filtration experiments (7), 0.1 ml yeast suspension, containing a high concentration (1×10^5) of culture yeast and a low

TABLE I
Optimum Concentration of Crystal Violet in LWYM and in Crystal Violet Medium Prepared with a Variety of Agars

Agar	Source	Time Purchased	Optimum Concentration of Crystal Violet (mg/L)	
			LWYM	Crystal violet medium
Bacto-agar	Difco	Before 1972	0.40	...
Bacto-agar	Difco	1973	3.50	14
Bacto-agar	Difco	1974	0.40	8
Bacto-agar	Difco	1975	0.05	6
Bacto-agar (Lot 615838)	Difco	1976	0.40	8
Bacto-agar (Lot 621952)	Difco	1976	0.40	8
Agar granulated	BBL	1976	0.15	7
Agar flakes	Difco	1975	None	5
Agar purified	Difco	1975	1.0	10

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concentration (less than 200 yeast cells) of wild yeast was added to 30 ml sterile distilled water. The suspension was filtered through a Nuclepore polycarbonate membrane filter with 0.4 μ pore size (N040CPG04700). The membrane filter was then incubated on the medium.

RESULTS

Universal Beer Agar

Universal beer agar plates were prepared with Difco bacto-agar purchased in differnt years, with agar purified, agar flake, agar granulated and commercial UBA. The bacterial species tested on these plates were *F. proteus, L. brevis, L. delbruckii,* and *P. cerevisiae*. The yeast species tested were *S. uvarum, S. diastaticus, S. willianus,* and *T. lactosa*. The purpose of this was to determine whether the different agars would affect the growth of these organisms when used in UBA. The data show that all bacterial and yeast species grew very well on all UBA plates tested, each organism showing similar recovery levels and colony size.

Lin's Medium

In LWYM, crystal violet was used at 0.4 mg/L. The LWYM plates were prepared with bacto-agar purchased during different years. These plates were then used for the detection of wild yeasts in mixtures of wild yeast and culture yeast, which were plated directly on the medium or retained on Nuclepore membrane filter and incubated with the membrane on the medium. The wild yeasts used included *S. cerevisiae* var. *ellipsoideus, S. diastaticus, S. pastorianus, S. willianus, C. mycoderma, Rhodoturla* sp., and *T. rosea*. These species have been reported to be detectable by LWYM (6). The results showed that the media containing bacto-agar purchased in 1974 and 1976 or before 1972 were satisfactory in suppressing the growth of culture yeasts while supporting the growth of wild yeasts. However, when bacto-agar purchased in 1973 was used in the medium, both culture yeast and wild yeast grew and made detection of the latter difficult. If bacto-agar purchased in 1975 was used in the medium, the growth of culture yeast and some LWYM detectable wild yeasts were suppressed. These results indicate that bacto-agar purchased at different times had a variable effect on LWYM when the medium contained 0.4 mg/L of crystal violet.

For satisfactory suppression of culture yeast growth and support of wild yeast growth, the levels of crystal violet in LWYM had to be adjusted for each bacto-agar purchased at different times. The optimum levels of crystal violet in the medium for each sample of agar are shown in Table I.

Bacto-agar purchased in 1973 and 1974 had been used in LWYM under laboratory conditions immediately after purchase or from time to time during two years of storage. Results showed that the storage of these agars did not affect the required optimum level of crystal violet in LWYM. In other words, the storage time or the freshness of the agar was not a factor in causing the variations found in the optimum concentration of crystal violet in LWYM made with agar purchased at different times.

Purified and granulated agar were also found to act differently with respect to the optimum crystal violet concentration in LWYM (Table I), requiring adjustments in crystal violet addition. However, when agar flake was used, crystal violet suppressed the growth of culture yeasts and some LWYM detectable wild yeasts even at extremely low concentrations, *i.e.*, 0.01 mg/L. Hence, agar flake was not found to be suitable for use in LWYM.

Crystal Violet Medium

Crystal violet medium prepared with agar of different kinds, or purchased at different times, showed variable results in the detection of wild yeasts if crystal violet was used at the level of 8 mg/L. When prepared with bacto-agar purchased in 1974 and 1976, the medium was suitable for use in the detection of wild yeasts. However, when prepared with agar purified or bacto-agar purchased in 1973, the medium permitted the growth of culture yeast and interfered with the growth of wild yeasts. When the medium was prepared with agar flakes or bacto-agar purchased in 1975, the growth of some crystal violet medium-detectable wild yeasts was suppressed. Therefore, the level of crystal violet had to be adjusted with the variations of agars. Table I shows the optimum level of crystal violet found for wild yeast detection; it fluctuated because of the differences in both the type of agar and the time of its purchase.

Lysine Medium

In contrast to LWYM and crystal violet medium, bacto-agar purchased in different years did not cause significant differences in the recovery or colony sizes of wild yeasts on lysine medium. However, when different kinds of agar were used in lysine medium, a comparison showed some significant variations in the colony sizes of some yeast species. *Rhodotorula* sp. developed significantly smaller colonies (1/2 size) on lysine medium prepared with agar flakes than on the same medium prepared with other kinds of agar. *Hansenula* sp. and *P. membranaefaciens* also showed smaller colonies (1/4 size and 1/2 size, respectively) on the medium prepared with agar purified. *C. pseudotropicalis, H. anomala, T. lactosa,* and *T. rosea* were not affected by the difference in agar. Though these agars in lysine medium had some effect on the colony sizes of some wild yeasts, they did not change the effectiveness of such a medium for wild yeast detection. Even the smaller wild yeast colonies were still large enough to be detected.

The culture yeast *S. uvarum,* when plated alone at low concentrations (less than 200 cells per plate) on lysine medium, developed colonies much smaller than those of wild yeasts. The size of this culture yeast also varied as different agars were used in the preparation of the medium. When the medium was prepared with agar purified, this culture yeast developed significantly smaller colonies (only 1/3 the size of others) than on the medium prepared with other kinds of agar.

Kleyn's Agar

When Kleyn's agar was prepared with bacto-agar purchased in different years to test the sporulation of four yeast species, *S.*

TABLE II
Percentage of Yeast Sporulation on Kleyn's Agar Prepared with Differtnt Kinds of Agar[a]

Yeast	Bacto-Agar	Agar Purified	Agar Flakes	Agar Granulated
S. cerevisiae var. *ellipsoideus*	13.8	11.5	9.8	6.5
S. diastaticus	13.2	28.0	4.5	0
S. pastorianus	14.4	15.8	15.2	0.2
S. willianus	14.5	13.2	11.8	4.3

[a]Average of six replicates.

TABLE III
Percentage of Yeast Sporulation on Sodium Acetate Agar Prepared with Different Kinds of Agar[a]

Yeast	Bacto-Agar	Agar Purified	Agar Flakes	Agar Granulated
S. cerevisiae var. *ellipsoideus*	0.9	0.5	1.0	0.2
S. diastaticus	17.7	16.3	16.0	2.5
S. pastorianus	28.8	23.5	23.0	12.3
S. willianus	8.0	6.3	4.5	1.1

[a]Average of four replicates.

cerevisiae var. *ellipsoideus, S. diastaticus, S. pastorianus,* and *S. willianus,* the results revealed similar sporulation percentages for each yeast species.

However, all four yeasts sporulated poorly when agar granulated was used, as well as *S. diastaticus* when agar flake was used. An exception was *S. diastaticus* which sporulated best when agar purified was used (Table II).

Sodium Acetate Agar

Bacto-agar purchased at different times was used in the preparation of sodium acetate agar to test the sporulation of four yeast species, as mentioned above. For each yeast species, similar sporulation percentages were observed, regardless of the time of bacto-agar purchase. However, agar granulated did affect the sporulation of these four yeast species. Table III shows these results.

DISCUSSION

Bacto-agar purchased at different times affected the optimum crystal violet concentration needed in LWYM and crystal violet medium. This variation could have been caused by the variable purity of agars manufactured at different times. Hence, it is necessary that the optimum crystal violet concentration in these two media be determined with every purchase of agar. However, it was not found necessary to vary the concentration of the fuchsin-sulfite mixture, the other growth inhibitor in LWYM. This indicates that crystal violet is very sensitive to the agar while fuchsin-sulfite mixture is not.

Walters and Thiselton (11) reported that culture yeasts failed to grow in a liquid synthetic medium containing lysine as sole source of nitrogen, but that many wild yeasts were able to grow in this medium. Morris and Eddy (9) added agar as a solidification agent to liquid lysine medium which suppressed the growth of culture yeast and supported the growth of wild yeasts. It was also reported that when culture yeasts were inoculated on lysine agar at low concentrations they developed colonies about 0.4 mm in diameter (8). This was assumed to be caused by a trace amount of nitrogen from sources other than lysine. In the present study culture yeasts developed much smaller colonies on lysine agar prepared with agar purified than when prepared with other agars. Agar purified is highly purified and is recommended for use in microbiological nutritional studies (2). As such, it contains a smaller amount of nitrogen than bacto-agar, agar flake, or agar granulated. This probably explains the difference in the results.

The growth response of wild yeasts to the different kinds of agar varies with the yeast species. This may be caused by the presence of different kinds or amounts of impurities in the agars. The exact nature of these impurities is not known at the present time.

It has also been mentioned that when agar granulated was used in the preparation of Kleyn's agar and sodium acetate agar yeast sporulation was extremely low. The reason for this reduction in sporulation is not known.

Literature Cited

1. AMERICAN SOCIETY OF BREWING CHEMISTS. Report of Subcommittee on Microbiological Controls. *Proc. 1963,* p. 221.
2. DIFCO SUPPLEMENTARY LITERATURE. Difco Laboratories, Detroit, MI, 1972.
3. KATO, S. *Bull. Brew. Sci. (Japan) 13*: 19 (1967).
4. KLEYN, J. G. *Wallerstein Lab. Commun. 17*: 91 (1954).
5. KOZULIS, J. A., and PAGE, H. E. *Amer. Soc. Brew. Chem., Proc. 1968,* p. 52.
6. LIN, Y. *Amer. Soc. Brew. Chem., Proc. 1974,* p. 69.
7. LIN, Y. *Amer. Soc. Brew. Chem., Proc. 1975,* p. 124.
8. LIN, Y. *J. Inst. Brew. 81*: 410 (1975).
9. MORRIS, E. O., and EDDY, A. A. *J. Inst. Brew. 63*: 34 (1957).
10. SOCIETY OF AMERICAN BACTERIOLOGISTS, Committee on Bacteriological Technic. Manual of Microbiological Methods, p. 21, p. 42. McGraw-Hill, New York, 1957.
11. WALTERS, L. S., and THISELTON, M. R. *J. Inst. Brew. 59*: 401 (1953).

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