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Comparison of dichloran 18% glycerol (DG18) agar with general purpose mycological media for enumerating food spoilage yeasts

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Abstract

Dichloran 18% glycerol (DG18) agar was originally developed to enumerate xerophilic foodborne moulds. However, some laboratories are using DG18 agar as a general medium to enumerate foodborne moulds and yeasts. A collaborative study, with the participation of seven laboratories, was undertaken to compare DG18 agar with dichloran rose bengal chloramphenicol (DRBC) agar, tryptone glucose yeast extract chloramphenicol (TGYC) agar, and plate count agar supplemented with chloramphenicol (PCAC) for enumerating 14 species of common food spoilage yeasts. Comparison of the mean values of populations of all yeasts recovered on each medium revealed no significant differences among DRBC agar, PCAC, and TGYC agar, while each of these media supported the development of significantly ($P \leq 0.05$) higher numbers of colonies than DG18 agar. However, differences were only 0.08 to 0.10 $\log_{10}$ cfu/ml, making the practical significance questionable. The overall coefficient of variation (CV) for within laboratory repeatability was 1.71%, while the CV for reproducibility of counts obtained among laboratories was 6.96%. Compared to DRBC agar, TGYC agar, and PCAC, yeast colonies were smaller on DG18 agar. Growth of *Brettanomyces anomalus*, *Cryptococcus albidus*, and *Rhodotorula mucilaginosa* was particularly retarded or inhibited on DG18 agar. Based on the performance of media in supporting colony development and ease of counting colonies, the use of DG18 agar as a general enumeration medium for foodborne yeasts cannot be recommended.

Keywords: DG18 agar; Food spoilage yeasts; Mycological media

1. Introduction

Dichloran 18% glycerol (DG18) agar was developed for the detection of xerophilic fungi (Hocking

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and Pitt, 1980) but in recent years has been used in some laboratories as a general purpose enumeration medium for both moulds and yeasts. Although a collaborative or interlaboratory study has not been done to assess the performance of DG18 agar as a general purpose mycological medium, several investigators have compared DG18 agar with other media to determine its performance. King (1992) reported that recovery of yeasts on DG18 agar was equivalent to that of media such as dichloran rose bengal chloramphenicol (DRBC) agar, oxytetracycline glucose yeast extract (OGY) agar, acidified or chloramphenicol-supplemented potato dextrose agar (PDA), or tryptone glucose yeast extract (TGY) agar. Others have reported that DG18 agar might support the development of lower numbers of yeast colonies than DRBC or TGY agars, depending on the composition of the food being analyzed and the profile of yeasts present (Deak, 1992; Nunez et al., 1996).

Studies using pure yeast cultures have shown that DG18 agar inhibited certain yeasts, particularly cryptococci and rhodotorulas, some candidas and others (Beckers et al., 1986; Nunez et al., 1996). The rates of growth and colony formation of some yeasts are reported to be retarded on DG18 agar (Seiler, 1992; Andrews et al., 1997). DG18 agar performs well for enumerating xerophilic yeasts, among them *Zygosaccharomyces rouxii* and *Debaryomyces hansenii* (Beuchat, 1993; Braendlin, 1996); however, in combination with various diluents, tryptone 10% glucose yeast extract agar (T10GY) agar and malt extract yeast extract 50% glucose (MY50G) agar produced higher counts than DG18 agar for enumerating *Z. rouxii* in foods with reduced a_w (Abdul-Raouf et al., 1994; Hernandez and Beuchat, 1995). MY50G and DG18 agars were considered less acceptable than TGY agar for enumerating *Z. rouxii* in high sugar foods (Beuchat et al., 1998).

Given this background, a collaborative study was organized to compare DG18 agar with general purpose mycological media, e.g., DRBC agar and TGY agar supplemented with chloramphenicol (TGYC agar), for enumerating food spoilage yeasts. In light of the results of a collaborative study to determine the performance of TGY agar (Deak et al., 1998), a further objective was to evaluate the effect of glucose concentration on recovery of yeasts. To this end, a fourth medium, plate count agar containing only 0.1% glucose and supplemented with chloramphenicol (PCAC), was also tested.

2. Materials and methods

2.1. Yeast strains

Fourteen yeast species representing 11 genera were used: *Brettanomyces anomalus* NRRL 1415, *Candida parapsilosis* ATCC 22019, *Cryptococcus albidus* DSS197, *D. hansenii* NRRL 7268, *Hanseniaspora uvarum* CFSQE 77, *Issatchenkia orientalis* NRRL 7179, *Kluyveromyces marxianus* UCD 61293, *Pichia anomala* NRRL 3668, *P. membranifaciens* UCD 5722, *Rhodotorula mucilaginosa* CFSQE 63, *Saccharomyces cerevisiae* KE 162, *Torulaspora delbrueckii* CFSQE 73, *Zygosaccharomyces bailii* NRRL 7256, and *Z. rouxii* ATCC 52519. *D. hansenii* and *Z. rouxii* are the most tolerant and *R. mucilaginosa* perhaps the least tolerant to reduced a_w. Yeasts were grown in tryptone glucose yeast extract (TGY) broth (pH 6.0), which consists of 5.0 g of tryptone (Difco, Detroit, MI, USA), 5.0 g of yeast extract (Difco), 100 g of anhydrous glucose, and 1 l of deionized water. Broth was sterilized by heating at 121°C for 10 min. Yeasts grown at 30°C for 3 days on TGY agar (TGY broth plus 1.5% agar) slants at the University of Georgia were sent to each collaborating laboratory. Upon receipt, cultures were stored at 5–8°C until used. A detailed experimental protocol was also provided to each collaborator.

2.2. Enumeration media

DG18 agar (Oxoid CM 729, pH 6.5) (Oxoid, Basingstoke, UK), DRBC agar (Oxoid CM 727, pH 5.6), TGYC agar (TGY agar supplemented with 100 mg of chloramphenicol per liter, pH 6.0), and PCAC (Oxoid CM 325 or Difco 0479 supplemented with 100 mg of chloramphenicol per liter, pH 7.0) were evaluated for their performance in supporting colony development by test yeasts. DG18 agar, DRBC agar, and PCAC were sterilized by heating at 121°C for 15 min; TGYC agar was sterilized at 121°C for 10 min. Media (15–20 ml) were poured into 90-mm diameter Petri dishes and held 1–2 days at room temperature to facilitate the removal of excess surface water.

2.3. Preparation of inoculum and application to agar media

Yeasts grown in TGY broth at 25°C for 48–72 h were serially diluted in sterile 0.1% peptone water and surface plated (0.1 ml in duplicate) on DG18, DRBC, TGYC, and PCAC media. Plates were incubated in an upright position in the dark at 25°C for 5 days. Plates on which 15–150 colonies developed were selected for counting colonies. Subjective observations on differences in size, colour, general appearance, and ease of counting colonies on the test media were recorded.

2.4. Statistical analysis

Each laboratory plated duplicate samples of each yeast on all test agars. Means of populations of each yeast recovered on each medium were calculated using data provided by all collaborators. Data were $\log_{10}$ transformed and evaluated by analysis of variance using the general linear models procedure of the SAS System (SAS Institute, Cary, NC, USA). Significant differences between means are presented at ($\alpha = \leq 0.05$).

3. Results and discussion

Only two of the seven collaborating laboratories reported counts for *B. anomalus* on all four recovery media. This yeast is perhaps the most fastidious among those tested. In five laboratories, no colony development occurred on DG18 agar or PCAC. Observations from the two laboratories reporting counts on all four enumeration media indicated that the size of colonies formed on DG18 agar and PCAC was very small. None of the data obtained on *B. anomalus* was subjected to statistical analysis.

Analysis of variance of data obtained from 13 yeasts revealed that counts differed significantly between yeasts, enumeration media, and laboratories (Table 1). There were also significant interactions between these parameters. The largest variance was among yeasts and the yeasts * laboratories interaction. Because of this significant interaction, data were also analyzed within laboratory.

Table 1
Summary of analysis of variance of populations ($\log_{10}$ cfu/ml) of 13 yeasts recovered on four media by seven collaborating laboratories

Source	*df*	Sum of squares	Mean squares	*F* value	Pr > *F*
Replicates	1	0.3270	0.3270	22.96	0.0001
Laboratory	6	12.7485	2.1247	149.15	0.0001
Yeasts	12	120.2133	10.0178	703.24	0.0001
Media	3	1.0249	0.3416	23.98	0.0001
Yeasts * Media	36	1.0212	0.0283	1.99	0.0007
Lab * Media	18	0.7578	0.0421	2.96	0.0001
Lab * Yeasts	72	125.6751	1.7458	122.53	0.0001

A comparison of the means of populations of yeasts recovered on enumeration media indicated that *T. delbrueckii. P. anomala*, and *P. membranifaciens* were consistently higher than other yeasts (Table 2). The two Basidiomycetous yeasts, *C. albidus* and *R. mucilaginosa*, formed the lowest numbers of colonies on test media. It is interesting to note that Laboratory 4 reported the highest mean population for *H. uvarum* while Laboratory 6 reported the highest mean population for *Z. rouxii* and the lowest population for *H. uvarum*. In two laboratories, counts for *D. hansenii* were less than those of Basidiomycetous yeasts. These differences are undoubtedly due to differences in populations of yeasts in 24-h-old TYG broth cultures plated on recovery media in various laboratories as well as differences in the performance of recovery media in supporting colony development. A comparison of mean values of counts of all yeasts on each enumeration medium revealed no significant differences among DRBC, PCAC, and TGYC. However, mean populations recovered on these media were significantly ($P \leq 0.05$) higher than the mean population of yeasts recovered on DG18 agar. The practical significance of this difference, however, is minimal and perhaps meaningless, since the mean count on DG18 agar was only 0.08–0.10 $\log_{10}$ cfu/ml less than counts on the other three test media.

Seldom would only one yeast species be present in food in which yeasts are the cause of spoilage. By determining mean composite populations of all 13 yeasts recovered on each medium, an overall performance of each medium can be assessed. Differences

Table 2
Mean populations of yeasts recovered on four media by collaborators in seven laboratories

Yeast	Population ($\log_{10}$ cfu/ml)				
	Recovery medium[a]				Mean[b]
	DG18	DRBC	PCAC	TGYC	
Can. parapsilosis	7.08 a	7.20 a	7.17 a	6.54 b	7.14 e
C. albidus	6.00 b	6.11 ab	6.17 a	6.16 a	6.11 i
D. hansenii	6.63 b	6.78 a	6.77 a	6.64 ab	6.70 g
H. uvarum	6.98 b	7.13 ab	7.12 ab	7.18 a	7.10 e
I. orientalis	6.90 b	7.06 a	7.07 a	7.03 a	7.02 f
K. marxianus	7.04 a	7.14 a	7.17 a	7.15 a	7.13 e
P. anomala	7.44 a	7.52 a	7.51 a	7.50 a	7.49 b
P. membranifaciens	7.35 a	7.34 a	7.35 a	7.32 a	7.34 c
R. mucilaginosa	6.31 a	6.30 a	6.41 a	6.35 a	6.34 h
S. cerevisiae	6.85 b	7.03 a	7.06 a	7.07 a	7.00 f
T. delbrueckii	7.59 a	7.57 a	7.61 a	7.63 a	7.60 a
Z. bailii	6.97 a	6.98 a	6.98 a	6.96 a	6.97 f
Z. rouxii	7.25 a	7.26 a	7.29 a	7.24 a	7.26 d
Mean:	6.95 b	7.03 a	7.05 a	7.03 a	

[a]Mean populations of yeasts recovered on each medium; values in the same row that are followed by the same letter are not significantly different ($\alpha \leq 0.05$).

[b]Mean populations of 13 yeasts recovered on four media; values followed by the same letter are not significantly different ($\alpha \leq 0.05$).

Table 3
Mean populations of 13 yeasts recovered on four media by each of seven collaborating laboratories

Laboratory number	CV%[a]	Population ($\log_{10}$ cfu/ml)				
		Recovery medium[b]				Mean[c]
		DG18	DRBC	PCAC	TGYC	
1	1.47	7.22 a	7.24 a	7.27 a	7.24 a	7.24 a
2	0.99	6.83 c	6.84 bc	6.88 a	6.87 ab	6.85 d
3	1.60	7.03 c	7.13 a	7.12 ab	7.06 bc	7.09 b
4	1.14	6.94 c	7.02 b	7.08 a	7.04 ab	7.02 c
5	1.01	7.00 c	7.06 b	7.02 bc	7.11 a	7.05 bc
6	2.37	6.76 b	6.82 ab	6.86 a	6.85 a	6.82 d
7	1.77	6.90 c	7.12 a	7.14 a	7.03 b	7.05 bc
Mean:		6.95 b	7.03 a	7.05 a	7.03 a	

[a]Coefficient of variation (repeatability within laboratory).

[b]Mean populations of all yeasts recovered on each medium; values in the same row followed by the same letter are not significantly different ($\alpha \leq 0.05$).

[c]Mean populations of all yeasts recovered on four media by each collaborating laboratory; values followed by the same letter are not significantly different ($\alpha \leq 0.05$).

in mean populations of all test yeasts recovered by the seven collaborating laboratories on each enumeration medium were small but, in some instances, significant (Table 3). The difference between the highest and lowest mean counts reported by laboratories was 0.42 $\log_{10}$ cfu/ml, or 2.6 cfu/ml. The within-laboratory repeatability coefficient of variation ranged from 0.99 to 2.37, with an overall coefficient of variation of 1.71%. The reproducibility of counts obtained among laboratories was good, as the coefficient of variation was only 6.96%. While counts obtained on DG18 agar were generally lower than counts on the other enumeration media, in only Laboratories 4 and 7 were counts on DG18 agar significantly ($P \leq 0.05$) lower than counts on all other media. Data reported by Laboratory 1 showed no significant differences in yeast counts obtained on the four enumeration media.

In addition to determining the number of colonies formed on recovery media, collaborators were asked to make subjective evaluations concerning the size, colour, and appearance of colonies, as well as the ease of counting. Three laboratories ranked the four enumeration media according to the diameter of colonies on plates on which cultures with the same dilution were applied. The medium supporting the largest colonies was assigned a rank score of 1, while media on which successively smaller colonies formed were signed scores of 2, 3, or 4. Considering the size of colonies developed by all yeasts, the mean rank values were 1.51, 2.18, 2.75, and 3.44 for TGYC agar, DRBC agar, DG18 agar, and PCAC. Results of non-parametric statistical evaluation of rankings using the Genmod procedure of the SAS system are shown in Table 4. Analysis revealed that larger colonies developed on TGYC agar. The small-

Table 4
Contrast analysis of performance of yeast enumeration media

Contrast	*df*	Chi-square	Pr > Chi-squre
DG19 vs. DBRC	1	12.19	0.0005
DG18 vs. PCAC	1	0.88	0.3488
DG18 vs. TGYC	1	67.72	< 0.0001
DRBC vs. PCAC	1	18.64	< 0.0001
DRBC vs. TGYC	1	32.22	< 0.0001
PCAC vs. TGYC	1	77.17	< 0.0001

est colonies were formed on PCAC, although colonies did not differ significantly in size compared to colonies formed on DG18 agar.

The highest ranking of TGYC agar, compared to other enumeration media, is attributed in part to the observation that several yeasts, e.g. *P. membranifaciens*, *I. orientalis*, and *H. uvarum*, clearly grew better on this medium. *C. albidus* and *H. uvarum* formed very small colonies on DG18 agar. Both *Zygosaccharomyces* species grew poorly on PCAC. *Z. bailii* also grew poorly on DG18 agar, whereas *Z. rouxii*, because of its preference for an environment with reduced a_w, grew best on DG18 agar. *R. mucilaginosa* and *C. albidus* produced mucous and frequently confluent colonies, making counting difficult. Rapidly spreading colonies of *I. orientalis* and *P. membranifaciens* also occasionally made counting of colonies difficult.

In summary, compared to DRBC agar, PCAC, and TGYC agar, DG18 agar was inferior in supporting colony development of the food spoilage yeasts examined in this study. Growth of some of these yeasts, e.g., *B. anomalus* and Basidiomycetous species, was retarded or inhibited on DG18 agar. Thus, the use of DG18 agar to analyze foods such as wine, other fermented fruit juices, beer, or salad vegetables, which are apt to harbour these rather than other yeast species, is discouraged. However, as recommended by Beuchat and Hocking (1990), DG18 agar performs well in supporting colony development by some yeasts, e.g. *Z. rouxii*, which prefer a reduced a_w environment for growth. DG18 agar was originally designed by Hocking and Pitt (1980) to enumerate xerophilic fungi and remains the medium of choice for this purpose. Its use as a general enumeration medium for yeasts and molds was never intended and, as evidenced by the results of this study, cannot be recommended for enumerating a wide range of food spoilage yeasts.

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