Control of Molds During the Enumeration and Isolation of Yeasts from Soil and Plant Material[1]

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Most molds (higher fungi) and yeasts are sufficiently acid-tolerant to be easily separated from bacteria by the use of acidified media. However, the enumeration and isolation of yeasts from samples having large populations of molds has always been a problem. Since, as a group, molds grow more rapidly than yeasts and will quickly obscure the surface of an agar plate, it is desirable to have some agent or medium that will inhibit molds or greatly restrict their growth.

Hertz and Levine (1942) compared the effectiveness of sodium propionate and diphenyl in acidified malt extract agar for the separation of pure cultures of yeasts from molds. They noted that sodium propionate was unsatisfactory because concentrations which were sufficiently high to inhibit growth and development of most of the molds also inhibited growth of many of the yeasts. The use of 100 ppm (0.01 per cent) diphenyl in malt extract agar inhibited a large percentage of the pure cultures of molds studied and restricted the growth of only a few of the yeast species.

In contrast to the results of Hertz and Levine, Mrak and Phaff (1948) reported that a 5 per cent wort agar containing 2,500 ppm (0.25 per cent) sodium propionate was quite effective in the separation of yeasts and molds. More recently, this medium was used by Shihata and Mrak (1952) in the study of the intestinal yeast flora of Drosophila and of the yeast flora of plant material and soil.

The present investigation was undertaken with the view of improving existing cultural techniques for separating yeasts from molds in samples from natural sources, such as soil and plant material. Cultural media and techniques were desired that would permit population studies and isolation of the principal yeast species present without interference by mold growth. Since it is not possible to predict the species of yeasts and molds which are likely to be present in a sample, it seemed desirable to use material with unknown flora, as well as pure cultures, in attempts to devise various media which would restrict mold growth.

MATERIALS AND METHODS

Fourteen media were prepared by addition of various agents to dextrose agar (Difco)[5] as indicated in table 1.

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[5] Difco Laboratories, Detroit, Michigan. Mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture and the North Carolina Agricultural Experiment Station over similar products not mentioned.

TABLE 1. *Comparison of different cultural media for determining yeasts in soil*

Medium No.*	Chemical Added		Yeast Colonies per Plate					
			Soil sample No. 2 (1–10 dilution)			Soil sample No. 5 (1–100 dilution)		
			Days			Days		
			2	4	6	2	4	6
I								
A	None		M†	M	M	M	M	M
B	5% Sodium chloride		0	M	M	5	M	M
II	Sodium propionate							
A	0.15%		27	M	M	84	89	M
B	0.25%		9	11	M	84	86	M
C	0.35%		13	13	21	43	78	92
III	Sodium propionate	Sodium chloride						
A	0.15%	5%	0	9	10	0	1	1
B	0.25%	5%	0	7	12	0	0	0
C	0.35%	5%	0	0	3	0	0	0
IV	Diphenyl							
A	0.005%		70	M	M	21	M	M
B	0.010%		17	M	M	3	60	M
C	0.020%		0	0	M	0	12	M
V	Diphenyl	Sodium chloride						
A	0.005%	5%	0	M	M	0	M	M
B	0.010%	5%	0	M	M	0	0	0
C	0.020%	5%	0	0	0	0	0	0

* Basal medium of dextrose agar (Difco) acidified with 4 ml of 5 per cent tartaric acid per 100 ml of medium prior to pouring plates.

† M = Luxuriant mold growth; plates not usable for counting or picking yeast colonies.

Just prior to pouring plates, each medium was acidified to a pH of about 3.8–4.0 by the addition of 4 ml of sterile 5 per cent tartaric acid per 100 ml of medium. A number of soil samples were collected and plated on the various media. However, detailed results for two of these samples (No. 2, from under a grape vineyard, and No. 5, from under a fig tree) will serve the needs of this phase of the study. Dilutions of the soil samples were made in isotonic saline and the same dilutions were used for plating with all media. Yeast counts and observations on the efficacy of the various media in restricting mold growth were made after 2, 4 and 6 days incubation at 30 C.

In that portion of the work dealing with pure cultures, 18 species of yeasts were tested for the effect of sodium propionate on their growth. Suspensions of yeast cells from each species were made by washing the growth from young slant cultures and transferring 0.1 ml of this suspension into 99.9 ml of sterile saline. Cultures were seeded with 0.1 ml of this suspension. The plates were then poured with media I-A, II-A, II-B, and II-C (see table 1), incubated for 6 days at 30 C, and observed for the amount of growth.

For determining yeasts on plant material, a chemically defined medium[6] was used; it will be referred to herein as synthetic agar. This medium, acidified with 3 ml of 5 per cent tartaric acid per 100 ml, was compared to acidified dextrose agar (I-A) and acidified dextrose agar plus 0.35 per cent sodium propionate (II-C) as to its usefulness in the separation of yeasts from molds in plant material.

Thirty-eight samples of staminate and pistillate blossoms (25 blossoms per sample) from the cucumber plant (*Cucumis sativus*) were disintegrated in a Waring blendor, and dilutions streaked and plated on the various media. In most instances comparable counts were obtained with both techniques. However, the streaking technique was preferred, particularly where yeast isolations were to be made, because all colonies could be readily picked from the agar surface. This usually eliminates the contamination troubles which are so frequently encountered when picking subsurface yeast colonies from selective solid media.

RESULTS

Comparison of media for determining yeasts in soil. The yeast counts obtained for two soil samples on 14 different media are presented in table 1. To conserve space, the yeast counts of other dilutions of these two samples are not presented. However, the data presented are representative of all results.

Acidified dextrose agar with 0.35 per cent sodium propionate was the most satisfactory of the acidified dextrose agar media containing the different chemicals. Yeast counts on this medium were of about the same order as those with the lower levels of propionate. Also, molds were inhibited to a much greater degree by the higher concentration of propionate. Plates with the 0.35 per cent propionate agar were still suitable for picking yeast colonies after 6 days incubation at 30 C. Five per cent salt (NaCl) added to the propionate media resulted in complete control of mold growth but greatly reduced the yeast counts.

No level of diphenyl used was effective in inhibiting mold growth for the desired incubation period (6 days) and the highest concentration (0.02 per cent) greatly inhibited yeast growth. The addition of salt to the media containing diphenyl increased inhibition of both the yeasts and molds.

Because of marked inhibition of both molds and yeasts by salt and propionate when used together, platings were made of soil sample No. 5 using 0.1 per cent sodium propionate in combinations with 1, 2, 3, 4 and 5 per cent salt. With low salt concentration (1 and 2 per cent NaCl) molds covered the plates within 4 days while with 4 and 5 per cent the yeasts were

[6] Yeast nitrogen base broth (Difco) of Wickerham (1951); modified for our use as a solid medium by the addition of agar (2 per cent) and dextrose (2 per cent) as described by Etchells, Bell, and Jones (1953) under synthetic agar-B.

TABLE 2. *Influence of sodium propionate on the growth of certain species of yeast*

Yeast	FFL* Culture No.	Growth on Acidified Dextrose Agar Containing Different Concentrations of Sodium Propionate†			
		0.0 per cent	0.15 per cent	0.25 per cent	0.35 per cent
Brettanomyces					
B. versatilis	Y-12	4+	3+	3+	3+
B. sphaericus	Y-42	4+	3+	3+	3+
B. sphaericus (unnamed variety)§	Y-316	4+	3+	3+	3+
Candida					
C. krusei‡	Y-59	4+	4+	4+	3+
C. krusoides	Y-55	4+	3+	3+	3+
Debaryomyces					
D. membranaefaciens var. *Hollandicus*	Y-92	4+	3+	3+	3+
Endomycopsis					
E. ohmeri‡	Y-147	4+	2+	1+	1+
Hansenula					
H. subpelliculosa	Y-202	4+	3+	3+	3+
H. anomala		4+	3+	3+	3+
Pichia					
P. alcoholophila	Y-243	4+	3+	3+	3+
Saccharomyces (subgenus)					
S. fragilis	Y-258	4+	4+	4+	4+
S. globosus	Y-269	4+	3+	2+	2+
Torulopsis					
T. caroliniana	Y-300	4+	3+	3+	2+
T. holmii	Y-305	4+	3+	3+	3+
Torulaspora					
T. rosei	Y-376	4+	4+	4+	4+
Zygosaccharomyces (subgenus)					
Z. halomembranis	Y-471	4+	3+	2+	2+
Z. pastori		4+	4+	4+	4+
Rhodotorula					
R. glutinis‡	Y-251	4+	None	None	None

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† 4+ = Maximum growth of individual species on control medium (without propionate) after 6 days incubation at 30 C.; 3+ = slight reduction in growth; 2+ = definite reduction; 1+ = marked reduction; none = no colonies.

‡ See figure 1 for photographs of culture plates.

§ ETCHELLS, J. L., COSTILOW, R. N., AND BELL, T. A. 1952 Identification of yeasts from commercial cucumber fermentations in northern brining areas. Farlowia, 4, 249–264.

greatly inhibited. The medium with 3 per cent salt inhibited mold growth for 6 days, but the yeast count was somewhat less than when dextrose agar containing 0.35 per cent sodium propionate alone was used.

Effect of sodium propionate on various yeast species. Eighteen species of yeasts representing 11 genera were seeded into acidified dextrose agar containing 0.0, 0.15, 0.25 and 0.35 per cent sodium propionate. After 6 days incubation at 30 C, the relative growth of the yeasts in the various media was noted (table 2).

Thirteen of the 18 species tested were not affected to any great extent by the sodium propionate. They

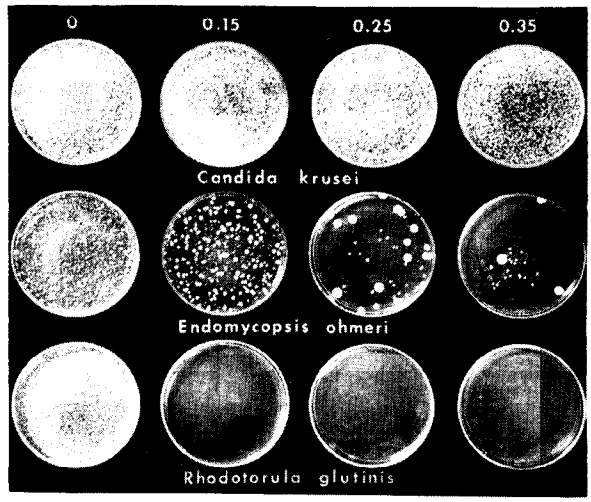

FIG. 1. Effect of sodium propionate concentration (0, 0.15, 0.25, and 0.35 per cent) in acidified dextrose agar on the growth of *Candida krusei*, *Endomycopsis ohmeri*, and *Rhodotorula glutinis* after 6 days' incubation at 30 C.

gave growth response of 3+ or better on all media; *Candida krusei* is an example of this group (see figure 1). Growth of *Saccharomyces globosus*, *Torulopsis caroliniana* and *Zygosaccharomyces halomembranis* was definitely reduced by the higher concentrations of propionate. However, many colonies of these yeasts were present even on 0.35 per cent propionate agar plates.

Marked inhibition of *Endomycopsis ohmeri* was evident with increasing concentrations of sodium propionate, and *Rhodotorula glutinis* failed to grow at even the lowest concentration used (figure 1). Therefore, it is not likely that cultures comparable to these two species would be isolated by the use of propionate agar. Also, other species which were inhibited to a lesser extent in the heavily seeded platings used in this study might well be completely inhibited when present only in small numbers.

Determination of yeasts on plant material. Comparative studies were made of the usefulness of acidified dextrose agar, acidified dextrose agar plus 0.35 per cent sodium propionate and acidified synthetic agar in the enumeration and isolation of yeasts from plant material. A summary of these studies is presented in table 3. Although a slightly higher mean yeast count was obtained by the use of dextrose agar as compared to synthetic agar, luxuriant mold growth covered many plates with the former medium. Such plates are entirely unsatisfactory for the isolation of yeasts and at best make counting very difficult. The inclusion of 0.35 per cent sodium propionate into the dextrose agar controlled molds but inhibited many of the yeasts. The mean yeast count of 17 samples on the propionate agar was less than one-tenth the counts of the same samples obtained with the other two media. In a large percentage of the samples no yeast colonies grew on

TABLE 3. *Comparison of three cultural media for determining yeasts in plant material*

Item	Culture Medium Used*		
	Dextrose agar	Dextrose agar plus 0.35 per cent sodium propionate	Synthetic agar
Streaking technique†			
Number of samples examined	38	36	34
Percentage of samples:			
Without yeast colonies	13	86	0
With excessive mold growth	45	0	6
Suitable for picking yeasts	42	14	94
Plating technique‡			
Number of samples examined	38	36	None
Percentage of samples:			
Without yeast colonies	11	30	—
With excessive mold growth	63	0	—
Suitable for picking yeasts	26	70	—
Mean yeast count of samples§	32,000/g	2,500/g	26,000/g
Number of yeast isolates obtained¶	222	64	225
Number of *Rhodotorula* isolates obtained¶	94	2	132

* Media were acidified with 3 to 4 ml of 5 per cent tartaric acid per 100 ml prior to pouring plates.

† Samples ground in blendor and dilutions streaked with standard loop (0.01 gram capac.) on surface of poured plates of three test media.

‡ Samples ground in blendor and dilutions plated with two test media.

§ Data based on the 17 samples where yeast counts could be made on all 3 media.

¶ Represents isolates obtained by streaking and plating techniques from all samples yielding plates suitable for picking colonies.

the propionate agar, even after a prolonged incubation period of 3 weeks.

Synthetic agar was much more satisfactory than either of the other two media, both for counting and isolation of yeasts from plant material. Yeasts grew well on this medium and mold colonies were restricted from spreading. Also, only 6 per cent of the plates gave mold growth considered to be excessive and the yeast populations were comparable to those obtained on acidified dextrose agar. Another observation resulting from this part of the study was the pronounced inhibition of yeast species in the genus Rhodotorula by the use of propionate. This is clearly illustrated by the fact that 42 per cent of the yeast isolates from dextrose agar and 59 per cent of those from synthetic agar were Rhodotorula, whereas only 3 per cent of the yeasts obtained from the propionate agar belonged to this genus.

DISCUSSION

The results of this study show the limitations of either sodium propionate or diphenyl in media for determining yeasts. The use of these chemicals may give data that are inaccurate both as to the total populations and the predominating species present.

Strains of a given species may differ appreciably as to their resistance to sodium propionate. Hertz and Levine (1942) observed this when testing a number of strains of *Saccharomyces cerevisiae* var. *ellipsoideus*. The strain of *Zygosaccharomyces pastori* tested by these workers was greatly inhibited, but 0.2 per cent propionate while the strain used in the present study was not affected by 0.35 per cent. Finally, *Rhodotorula glutinis* was found to be completely inhibited by 0.1 per cent propionate in our study, but Shihata and Mrak (1952) reported the isolation of this species from Manzanita and Juniper berries by the use of 0.25 per cent propionate in wort agar. This type of species variation in response to propionate makes it impossible to predict the extent of growth restriction of yeast flora by this chemical.

Synthetic agar proved to be the medium of choice for counting and isolating yeasts from plant material. It restricts the mold colonies from spreading rather than inhibiting them from forming. In samples where mold populations are expected to be many times higher than yeasts, propionate agar may be of some value; however, the results obtained by the use of propionate should be qualified, and the use of an additional medium such as synthetic agar as a control would be desirable.

SUMMARY

Three levels of sodium propionate (0.15, 0.25, and 0.35 per cent) and three levels of diphenyl (0.005, 0.01 and 0.02 per cent) in acidified dextrose agar and acidified dextrose agar with 5 per cent salt were tested for their ability to restrict mold growth yet permit the rapid growth of yeasts from soil samples.

No level of diphenyl tested in acidified dextrose agar was found to inhibit mold growth satisfactorily, and the use of 0.02 per cent of this agent resulted in marked inhibition of the yeasts. While the use of diphenyl in combinations with salt (NaCl) agar was superior to diphenyl alone in controlling mold growth, yeasts were even more inhibited.

Acidified dextrose agar plus 0.35 per cent sodium propionate was the most effective medium tested in separating yeasts from molds in soil samples. However, this chemical, even in low concentrations, greatly inhibited the growth of *Endomycopsis ohmeri* and completely inhibited *Rhodotorula glutinis*. Furthermore,

the use of propionate agar resulted in very low yeast counts in samples of plant material as compared to the populations obtained with acidified dextrose agar and synthetic agar.

Acidified synthetic agar proved to be the medium of choice for the enumeration and isolation of yeasts from plant material. Counts obtained on this medium were comparable to those on acidified dextrose agar and mold growth was satisfactorily controlled because of restricted colonial development on the synthetic medium.

ADDENDUM

After this manuscript was prepared for publication, J. J. Miller and N. S. Webb (Soil Science, **77** (3), 197–204. 1954) reported on the use of lactic acid, rose bengal, and oxgall in media to determine yeasts in soil. The latter two agents were considered to be the most useful in the isolation studies because they restricted the growth of spreading fungi. The effectiveness of the rose bengal appeared to be related to the nature of the plating medium.

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