Determination of Microorganisms in Sugar Products by the Millipore Method

H. E. Halden, D. D. Leethem and F. G. Eis[1]

Received for publication February 12, 1960

Molecular filter membranes, which are manufactured in this country and in Europe, have found recognized applications in laboratories for precise particle separation from liquids and gases. The successful use of the membranes for the separation of microorganisms from air and liquids is also well established. Due to the wide range of porosities offered by the manufacturers and the remarkably low variance in the stated pore size, it is possible to select a membrane which is capable of trapping certain organisms while allowing a maximum filter flow. These properties, combined with subsequent incubation, looked attractive for the microbiological analyses of sugar products. The apparent advantages visualized over the conventional plating methods were, principally:

1. Possibility of larger sample size.
2. Less equipment required.
3. Less time required for preparation of sample.
4. Less chance for contamination.
5. Shorter incubation time, as had already been experienced in water analyses.
6. Staining and counting the trapped organisms without incubation.

Some of these apparent advantages have been realized, in particular the first four. There has, however, been some doubt raised regarding the reliability of the membrane method. Such doubt was probably caused by the frequent lack of agreement between the membrane method aand the conventional plating methods. When such disagreement occurred there has frequently been an unfounded tendency to consider the plate method to be the correct one.

Older methods used for the enumeration of microorganisms in sugars were well established and approved by canners and bottlers. When the same methods were used by different laboratories good agreement in results could be expected on the same sample. The possibility of the presence of spoilage organisms, other than those found, was not always recognized or was overlooked. This attitude was not nearly as dangerous in the days of dry sugars only, as it is now when a very considerable portion of the sugar production is in the form of liquid sugars.

[1] Supervisor, Central Testing Laboratory, Food Technologist, and Head Research Chemist, respectively, Spreckels Sugar Company, Woodland, California.

Our present knowledge indicates that the organisms we should be mostly concerned with in liquid sugars are the yeasts and molds. Of the two, the yeasts appear to be the most troublesome.

Several investigators within the sugar, canning or bottling industries have expressed the opinion that the term yeast, as now used in standards for sugars, must be further clarified. Yeasts found in sugar products may be of several types with different growth requirements. Variations in growth requirements indicate a need for different means of detection and enumeration of specific yeast types.

The research department of the Spreckels Sugar Company has conducted a series of comparative tests for the enumeration of yeasts in liquid sugars by the filter membrane method and the ABCB method.

Two porosities of filter membranes were used, 0.45 μ and 0.80 μ. The membranes are manufactured and distributed by the Millipore Filter Corporation, Bedford, Massachusetts, under the trade name MF.

The ABCB method is that tentatively adopted by the American Bottlers of Carbonated Beverages.

The media used were:

ABCB method	Mycophil Agar with low pH (BBL)[2]
MF method	Mycophil Broth with low pH (BBL)[2]
or	Osmophilic Broth (de Whalley medium without agar) (1)[3]

Tests were run on sterile liquid sugars inoculated with pure cultures of various yeasts isolated from liquid sugars obtained from sources outside the company. These yeasts have not been positively identified and are designated herein as A, B, and C.

It was decided to run the tests on pure yeast cultures in order to avoid the necessity of identifying types and also to avoid the interference of molds. In most cases, except in very low counts or when gross contamination occurs, the yeasts found in liquid sugars are predominantly of one type. The use of pure cultures was only an extra precaution.

Tests were run on 20 grams dse (dry sugar equivalent) portions of liquid sugar. The ABCB method, which specifies the use of five grams dse on four plates, was run in four sets of four plates, each, for a total of 20 grams dse.

The period of incubation was three days in all cases at a temperature of 31° to 33° C. The conventional 100 mm ×

[2] Baltimore Biological Laboratory, Inc.
[3] Numbers in parentheses refer to literature cited.

15 mm glass Petri dishes were used in the ABCB method. Tops or bottoms of the 60 mm × 15 mm glass Petri dishes, covered during incubation with "Parafilm," were used in the MF method. The actual manipulation of the MF membrane method, such as filtration of sample, use of absorbent pad, etc. have been described by others (2) (3).

The yeast colonies were counted by 9X magnification, using a stereoscopic microscope with reflected light.

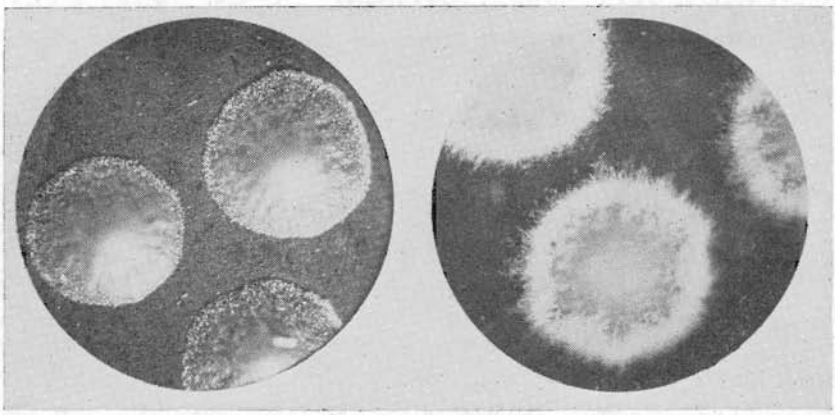

Figure 1.—Yeast colonies grown on 0.45 micron and 0.80 micron MF membranes (magnified from a field of 2mm diameter).

The yeast colonies grew to approximately the same size on all three media. Those grown on .80 μ membranes were generally more mature and better defined than those grown on either the .45 μ membrane or the agar medium. Figure 1 shows colonies of yeast B grown on .45 μ and .80 μ membranes, respectively. The better yeast definition on the .80 μ membrane may allow a shorter incubation time than was used in the present tests.[4]

Table 1 is a summary of results. The results obtained allow the following observations:

1. There is no significant difference between the results obtained by .45 μ and .80 μ membranes when the same medium is used. As mentioned above, the organisms are

[4] In previous tests conducted by the research department of Spreckels Sugar Company the possibility was indicated that the .80 μ membrane might permit the development of a higher number of organisms than the .45 μ membrane. The tests were run on samples containing a mixed microbiological flora, i.e., possibly more than one type of yeast or mold. Some of the yeasts may have been inhibited on the .45 μ membranes and therefore were incapable of developing in the same time period as those on the .80 μ membrane.

better defined on the .80 μ membrane, which also allows faster filtration of the sample. This latter feature may permit the use of larger sample size than 20 grams dse.

2. Yeasts A and B grow equally well on either of the nutrient broths. Results are comparable for all three methods except in one test where definitely lower results were obtained by the ABCB method.

3. Yeast C shows a definite preference for Mycophil broth (low sugar content) and its growth may possibly be inhibited on Mycophil agar (ABCB method) at least at high counts. It is definitely inhibited when grown on osmophilic broth (40% sugars).

Table 1.—Yeast Counts in Liquid Sugars Obtained by the Use of Three Methods. Twenty Grams dse Sample Size Was Used in All Tests. Results Are Calculated to 10 Grams dse.

	Mycophil Broth		Osmophil Broth		Mycophil Agar
	MF .45 μ	MF .80 μ	MF .45 μ	MF .80 μ	Plate ABCB
Yeast A	95	95	70	83	108
	115	86	90	81	95
	51	41	44	45	41
	21	15	27	28	10
	24	26	26	26	25
	63	68	62	63	62
	34	37	30	37	7
	3	11	—[a]	—[a]	6
	66	—[a]	49	71	85
	—[a]	—[a]	49	49	31
	—[a]	—[a]	48	56	46
	62	—[a]	72	76	65
Yeast B	91	88	98	87	79
	19	19	18	22	17
	158	—[a]	154	—[a]	156
	76	—[a]	75	—[a]	74
Yeast C	238	251	168	163	106
	152	149	104	101	69
	56	66	25	25	51
	53	38	26	27	56
	31	25	18	18	29

Missing data:

[a] Membranes ruptured

[b] Samples not run

The agreement in results obtained by the three methods is in a large part due to a larger than usual sample size (20 grams dse). Table 2 shows the agreement that would have been obtained if based on the individual results of the ABCB method

Table 2.—Yeast Counts Obtained by Plating Four Sets, Each Four Plates of 1.25 Grams dse.

	Average Calculated to 10 Grams dse	Individual Sets of 5 Grams dse Calculated to 10 Grams dse				Poisson Distribution 95% Assurance Based on 5 Grams dse Calculated to 10 Grams
		1	2	3	4	
Yeast A	108	94	108	110	120	80-138
	95	78	92	102	106	70-124
	41	34	34	38	58	26-60
	10	6	8	10	14	2-20
	25	12	18	28	40	12-40
	62	48	56	64	74	42-84
	7	2	4	12	10	0-16
	6	0	0	8	16	0-14
	85	76	82	84	96	62-112
	31	28	30	32	34	18-48
	46	36	42	46	60	28-66
	65	46	52	74	86	44-90
Yeast B	79	56	98	94	66	56-106
	17	22	18	18	10	6-30
	156	170	156	152	146	122-188
	74	70	84	72	66	52-98
Yeast C	106	90	128	122	84	78-136
	69	74	78	54	72	48-94
	51	54	46	50	52	34-72
	56	48	48	74	54	36-78
	29	30	24	42	20	16-46

using a sample size of five grams dse. The MF method permits the use of a sample size greater than 20 grams dse with resultant increased accuracy.

We have as yet found no yeast in liquid beet sugar products which shows a preference for high concentrations of sugar. It is, however, very likely that such a yeast may be present at times. It seems, therefore, necessary to test sugar products, in particular liquid sugars, for osmophilic as well as non-osmophilic yeasts. In order to arrive at a numerical evaluation it might be necessary to identify the yeasts as it could be expected that certain types, while inhibited somewhat by either medium, might develop under osmophilic as well as non-osmophilic conditions.

Three pure yeast cultures, supplied through the courtesy of Dr. Herman J. Phaff of the Department of Food Science and Technology, University of California at Davis, have very effectively shown the anticipated necessity of employing more than one medium.

These yeasts are:

Saccharomyces cerevisiae (low sugar tolerance)
Saccharomyces mellis (high sugar tolerance)
Saccharomyces rouxii (low and high sugar tolerance)

On tests run at the Spreckels Sugar Co. research laboratory it was found that the count of *S. cerevisiae* would be from three to five times greater on Mycophil broth than on osmophilic broth. *S. mellis* showed a definite preference for osmophilic broth and did not grow on Mycophil broth. *S. rouxii* grew vigorously on both media with possibly a slight preference for Mycophil broth.

The results obtained with the low and high sugar tolerant yeasts suggest the possibility that standards for yeasts may not have been devised on a completely rational basis. It seems likely that standards may have been set at unreasonably low values because of falsely low counts obtained when using a particular method. Even these lower standard values cannot give the desired protection if the yeast does not grow on the medium. Far better protection would appear possible, even with higher tolerances, if two types of media were used in the tests.

Summary

The use of molecular filter membranes for the detection and enumeration of yeasts in sugar products has not been completely accepted. This has principally been due to frequent lack of agreement with older established methods. It appears, however, that if due consideration is given to the growth requirements of the yeasts in regard to the selection of media, that equal or more accurate results may be obtained by the membrane method. Various yeasts found in sugar products may vary widely in growth requirements. It may, therefore, become necessary to differentiate between the yeasts which show optimum growth in low sugar solutions and those which prefer high sugar concentrations.

Literature Cited

(1) DE WHALLEY, H. C. S. and SCARR, M. P. 1946. Microorganisms in raw sugars, affination syrups and molasses. Int. Sug. Jour. 48: 180-183.

(2) COLEMAN, M. C. and BENDER, C. R. 1957. Microbiological examination of liquid sugar using molecular membrane filters. Food Tech. 11: 398-403.

(3) A. G. CHEMICAL COMPANY, Pasadena, California. Tech. Bulletin No. 15, Sept. 1956.