Methods for Count of Microorganisms in Liquid Sugar

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During the past 25 years, and particularly since 1945, there has been a steady increase in the manufacture and use of liquid sugars. The demand has reached such proportions that liquid sugars now constitute a considerable portion of the total sugar production. With the increase in production of liquid sugars certain problems have developed. In the early days the liquid sugars were manufactured and consumed within a relatively short time, while now, with increased shipping distances and the greater demand, the time between manufacture and use has increased considerably.

Due to the nature of liquid sugar products they are far more susceptible to deterioration caused by spoilage organisms than dry sugars. While perfectly sterile products may be manufactured, they may be exposed to spoilage organisms in plant storage, during shipment and in the customer's tanks. The most scrupulous cleanliness is naturally a requisite for all liquid sugar equipment. In order to check the effectiveness of the cleaning procedure of equipment and preventative measures taken against contamination, a well planned microbiological control becomes the most effective weapon.

The organisms with which we are the most concerned are:

1. Mesophilic aerobic bacteria
2. Yeasts
3. Molds

All of these organisms may be airborne and while the optimum growth temperatures range from 28° to 40° C., some may be capable of resisting much higher temperatures for short periods.

Of the three groups of organisms the mesophilic aerobic bacteria are of least importance. They are rarely, if ever, responsible for deteriorative changes in sugars. Their presence, however, is an indication of the amount of airborne contamination occurring during storage and transportation of the liquid sugars. Yeasts and molds multiply rapidly when growth conditions are favorable and may cause serious damage to liquid sugars. The presence of these organisms, even in very small numbers, may eventually be a serious threat to the keeping quality of liquid sugars under unfavorable storage condiions.

The problems involved in a quantitative determination of a very low count do not appear to be generally understood. The difficulties in obtaining a small representative sample can readily be appreciated when we consider the following.

The organisms present in liquid sugar are not evenly distributed. With a very small population the chances for getting a representative count on a small sample are very remote, in particular from a tank which has been standing for some time. It has been demonstrated by Owen (1) that

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[2] Numbers in parentheses refer to literature cited.

yeast colonies have a tendency to migrate to the surface and that consequently, the main body of the syrup may show a low organism count while the surface would have a higher concentration than the average for the whole tank.

The above facts should be kept in mind when obtaining a sample of liquid sugars for microbiological examination. One sample from a freshly prepared syrup may be sufficient for the evaluation of its organism content. It would, however, be necessary to take samples from different levels of a tank which has been standing for some time. Thorough agitation of the syrup in the tank before sampling would make it possible to take less samples and expect a good evaluation. No specific instructions can be given to cover all cases, since each one must be considered by itself.

When the final sample is made up from one or more subsamples it must, with reasonable assurance, be representative of the whole tank of syrup. The final sample should not be too small, preferably at least one pint.

Additional problems come up when a portion is removed from the sample for analyses. A single portion of a few grams cannot represent the whole sample if the population is low. This situation is far more pronounced than that of the initial sampling of the whole tank.

At Spreckels Sugar Company's microbiological laboratory, five portions of the sample, each representing 20 grams of dry sugar, are weighed into five 250 ml. Erlenmeyer flasks.

Each flask is made up to 100 ml. with sterile water making sugar solutions of 20 percent strength W/V. From each of these flasks, five dextrose-tryptone agar plates for bacteria, and five wort agar plates for yeasts and molds are prepared. Two ml. of sugar solution is used for each plate. The original sample will be represented, therefore, by 25 plates for the count of bacteria, and 25 plates for the count of yeasts and molds. Twenty-five plates with 0.4 grams sugar on each represent 10 grams of dry sugar.

In a count of microorganisms we are dealing with a discontinuous distribution. With a limited number of microorganisms, 100 in 100 ml., we would find in 100 samples of one ml. each, a number containing no organisms and a number with one or more.

Among discontinuous distributions the Poisson series is probably of first importance. It has sometimes been called the law of small numbers.

If the probability of an event is exceedingly small, the number of occurrences of the event in a sufficiently large number of independent cases will be distributed in the Poisson series.

Tables have been prepared by F. G. Eis (2) to show the accuracy that may be expected in plate counts using 0.4 gram of sugar per plate. The tables give plate count per 10 grams, actual for 25 plates and calculated to 10 grams for other numbers. All values are based on the Poisson series and a technique of sampling and dilution affording a random distribution of organisms.

Table 1.—Percentage of the Time a Zero Count May Be Expected at Various Actual Average Organism Levels.

Actual Product Average Org./10 gr.	Number of Plates Counted (.4 gr. sugar, each)							
	1	2	5	10	25	50	100	200
1	96	92	82	67	37	14	1.8	0.03
5	82	67	37	14	0.7	.004		
10	67	45	14	1.8	.004			
20	45	20	1.8	0.03				
100	1.8	0.03						
200	0.03							

Example: Using 25 plates, each containing 0.4 grams dry sugar, a count of zero may be expected 37 percent of the time when there is an average of one organism per 10 grams of sugar. With an actual population of 10 organisms per 10 grams of sugar the chances for getting a zero count are practically nil.

Table 2.—Expected Count /10g. at Various Actual Organism Levels (Not more than 5 percent under minimum or 5 percent over maximum value listed).

Actual Product Average Org./10 gr.	Number of Plates Counted (.4 grams sugar, each)							
	1	2	5	10	25	50	100	200
1	0	0	0	0	0	0	0	1 Min.
	25	25	10	7	4	3	2	2 Max.
5	0	0	0	0	2	3	3	4 Min.
	50	38	20	15	10	8	7	6 Max.
10	0	0	0	3	5	6	7	8 Min.
	75	38	30	23	16	15	13	12 Max.
20	0	0	5	10	13	15	17 Min.	
	75	63	45	35	29	26	24 Max.	
50	0	13	25	33	39	42 Min.		
	150	113	100	73	63	59 Max.		
100	25	50	65	75	84 Min.			
	225	175	145	130	118 Max.			
200	100	125	150	165 Min.				
	350	300	260	240 Max.				

Example: Using 25 plates, each containing 0.4 grams dry sugar, a count of from 5 to 16 may be expected in 90 percent of trials when the actual product average is 10 per 10 grams. In 5 percent of trials the count may be expected to be less than 5 and in 5 percent of the trials the count may be expected to be greater than 16.

Table 3.—Expected Range of Average Number of Organism Per 10 gram at Various Plate Counts (Not more than 5 percent under minimum or 5 percent over maximum value listed).

Count Per 10 g.	Number of Plates Counted (.4 grams sugar, each)							
	1	2	5	10	25	50	100	200
0	0	0	0	0	0	0	0	0 Min.
	73	36	15	7	3	1	1	1 Max.
1					1	1	1	1 Min.
					5	3	2	2 Max.
3				1	1	1	2.	2 Min.
				12	8	6	5	4 Max.
5			1	1	1	2	3	4 Min.
			24	16	11	8	7	7 Max.
10			1	2	5	6	8	8 Min.
			31	23	16	13	13	12 Max.
25	1	1	7	12	17	20 Min.		
	118	78	53	40	34	32 Max.		
50	2	11	24	32	39 Min.			
	155	114	80	73	63 Max.			
100	23	41	63	75 Min.				
	228	180	145	130 Max.				
200	83	116	150	163 Min.				
	328	288	260	240 Max.				
400	233	288	325 Min.					
	575	501	480 Max.					

Example: In a sample, in which the organism count has been determined to be 25 per 10 grams, the actual product average may have been from 17 to 34 per 10 grams of sugar in 90 percent of trials, when 25 plates are used. In 5 percent of trials the product average may have been less than 17 and in 5 percent of trials more than 34.

The statistical treatment shows that 25 plates, containing 10 grams of sugar, give a fair approximation of the average number of organisms present per 10 grams of sugar. While 25 plates probably represent the practical limit when several samples are to be tested, it is also evident that more plates might be preferable for greater accuracy when the organism population is very low. It is not practical nor advisable to increase the sugar content per plate without making an adjustment in the nutritives medium. Such adjustment would in itself require considerable time and trials.

The statistical treatment has shown the accuracy with which low counts can be determined by plating techniques. Maximum limits placed on a product, in a range where methods of counting are not accurate, may be subject to question as to the actual necessity for such limits. If such low limits are an actual necessity, then a different approach to the quantitative determination of organism levels is necessary.

A new approach to the estimation of the population of microorganisms in liquids and gases has been proposed by manufacturers of a new filter membrane. This membrane is stated to be composed of cellulose esters with a porosity of 80-85 percent, the porosity being defined as the ratio of voids or pore volume to the volume of structural elements. Two basic types are available, one with a pore size of 0.45 micron, recommended for hydrosol assay and one with a pore size of 0.8 micron, recommended for aerosol assay

The filter membrane may be sterilized or may be obtained in a sterile package. The sample of liquid or gas is passed through the membrane placed in a suitable holder. The membrane is then incubated in a Petri dish at a specific temperature on a pad containing a selective medium. The organisms, favored by the selective medium, develop in a much shorter time than that required in conventional methods and may be stained and counted directly from the pad.

Membrane filters have been used for the direct count of bacteria since 1933 or earlier. During the last five years, an increasing use has been found for molecular membrane filters for the examination of water and sewage and in the dairy industry.

Two bottling companies, the Pepsi Cola Bottling Company and Belfast Beverages, Incorporated of San Francisco and Oakland are currently using the membrane filter method in conjunction with other microbiological methods.

To our knowledge membrane methods have not otherwise been widely used on sugar products. Trials conducted at the Central Quality Control Laboratory of the Spreckels Sugar Company at Woodland, California, indicate that the filter membrane may permit the use of larger samples and an increase in the number of samples which can be handled by the microbiological laboratory. Due to the low flow resistance of the membrane filter it is possible to filter 150 grams of liquid sugar (100 grams of dry sugar), diluted to about 20 percent sugar, in about 3 to 4 minutes.

The filter holder, which is shown in Figure 1, is manufactured by the A. G. Chemical Company, of Pasadena, California. It consists of two sections. The upper is made of stainless steel with an anodized aluminum base which locks onto the lower section. The lower section, also made of stainless steel, contains a porous carbon filter disk on which the membrane filter is placed. Before use the filter assembly may be sterilized by autoclaving, dry heat or flaming. However, the most convenient method is probably sterilization by the incomplete combustion of methyl alcohol.

For this method a special sterilizing base is used. This base contains a porous carbon disk which is saturated with methyl alcohol. The alcohol is ignited and the upper section of the holder assembly is placed on the base. After a few seconds the lower section of the assembly is placed in an inverted position over the funnel. The formaldehyde, formed by the incomplete combustion of the methyl alcohol, is sufficient to completely sterilize the assembly in 15 minutes.

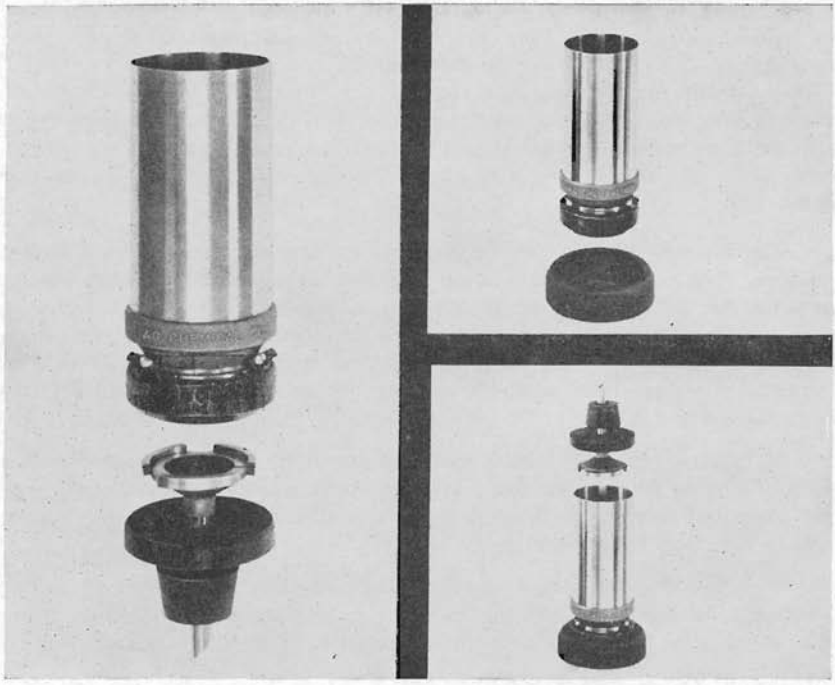

Figure 1.—Filter holder assembly.

The membrane filters are supplied with a protective paper cover and an absorbent pad which may be obtained with or without a nutritive medium. The absorbent pad is placed in a 60 mm. Petri dish and saturated with 2.3 ml. of sterile water (for prepared pads) or the same amount of a nutritive medium. The protective pad is not removed from the filter until it is placed in the holder. After filtration the filter is placed on the absorbent pad. The dish is then covered and incubated in an inverted position. To prevent loss of moisture from evaporation the dish may be sealed with a plastic film or tightly wrapped in aluminum foil. The incubation period is 24 to 36 hours at 30° C. for yeast and 12 to 18 hours at 37° C. for aerobes.

The organisms may be stained before viewing them microscopically.

If it is desired to express the count of organisms on the basis of 10 grams of dry sugar, the total count of organisms on the filter is divided by 10 if a total of 100 grams dry sugar (150 grams liquid sugar) has been used.

Figure 2 shows a comparison of the accuracy which may be expected by using 100 grams of sugar in the membrane filter method instead of 10 grams in the 25 plate method.

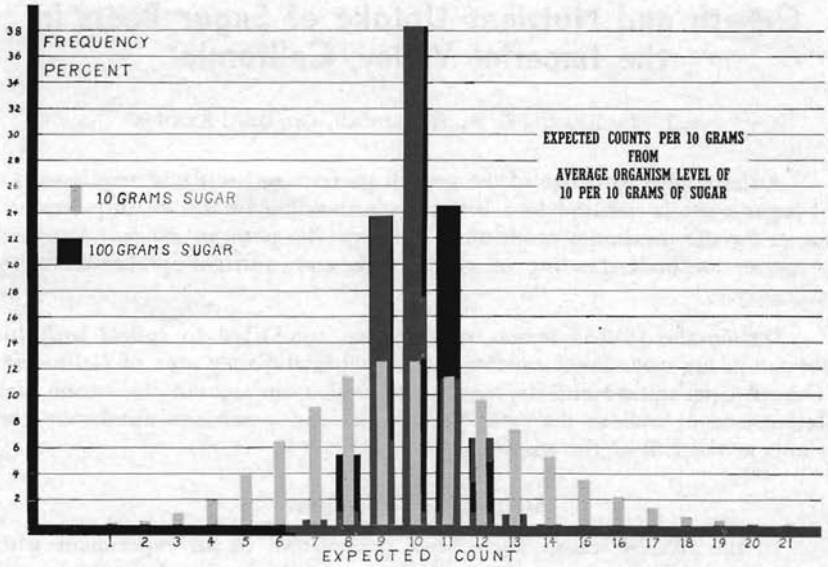

Figure 2.—Expected counts per 10 grams from average organism level of 10 per 10 grams of sugar.

Summary and Conclusions

Liquid sugar products are more susceptible to deterioration caused by spoilage organisms than dry sugar. Accurate quantitative determination of very small numbers present is considered to be important for at least two reasons:

1. Accurate counts are necessary for a well planned microbiological control program during storage and shipment.

2. Accurate counts of small numbers of organisms are necessary so that specific limits may be established for consumers acceptance. Limits based on inaccurate determinations may work hardships on both the producer and the consumer.

A statistical evaluation of the plating technique has shown that an excessive number of plates are required for accurate counts at low average organism levels. A new approach has been proposed by manufacturers of a new filter membrane. This new approach may result in a better control program and allow an accurate estimation of the number of organisms in a sample.

References

(1) OWEN, WILLIAM L. 1949. The microbiology of sugars, syrups and molasses. Burgess Publishing Company, Minneapolis, Minnesota.
(2) EIS, F. G. Personal Communication.