A Simple Method for the Determination of Clostridium Thermosaccharolyticum in Sugar

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The micro-organisms which are of most concern in the spoilage of non-acid pack canned foods are the thermophilic spore bearing bacteria (1)[2]. Sugar was first suspected as a source of contamination by these organisms as early as 1926, and, as sugar is an essential ingredient in practically all canned foods, the National Canners' Association has established standards for its bacterial purity (2). To control the production of sugar to meet these specifications, the analysis of a large number of samples is necessary. It was the purpose of this investigation to devise a less cumbersome, and therefore a more efficient method, for the detection of a certain specific thermophilic anaerobes which may contaminate sugar.

Three major groups of bacteria have been shown to be most troublesome to canners: (a) the stearothermophilus of Donk (3), a facultative aerobic sporebearing thermophile which causes "flat sour" spoilage; (b) *Clostridium nigrificans* (4), an anaerobic organism which produces hydrogen sulfide and gives rise to "sulphur stinker" spoilage; and (c) *Clostridium thermosaccharolyticum* (5), an obligate anaerobe which produces gas, mainly carbon dioxide and hydrogen, but does not produce hydrogen sulfide, and causes spoilage known as "hard swells." It is this last group with which this study is concerned,

McClung (6) conducted extensive investigations of the physiological and cultural characteristics of the thermophilic anaerobes found in sugar, and suggested the name by which they are now known, *Clostridium thermosaccharolyticum.* He found them to be fastidious in their requirements, particularly with respect to anaerobiasis. Cameron, Williams and Thompson (7) conducted a study of the causes of spoilage in canned foods and devised methods for culturing these anaerobes routinely. Their medium was a liver infusion broth sterilized over bits of liver tissue and sealed for anaerobiases with a layer of nutrient agar. The medium was found to be well suited to the detection of these organisms, and is the medium recommended by the National Canners' Association. For routine analysis of sugar, the method is cumbersome and slow, and requires several lengthy operations to prepare the medium and to complete the test.

Several methods for the aerobic culture of anaerobes have been devised. Trenkmann (8) was the first to suggest that anaerobes would be cultured under aerobic conditions by reducing the oxygen tension with an alkaline sulphide. Quastel and Stevenson (9) were able to grow *Cl. sporogenes* in the presence of a small amount of a compound containing a sulfhydryl group. Brewer (10) showed that the addition of a small amount of agar combined with a reducing agent was of great value in the culturing of anaerobes. He showed also that agar in the amount of 0.05 percent together with a reducing compound such as sodium thioglycollate initiated growth of anaerobes. Marshall, Gunnison and Luxen (11) have also shown that a medium containing a substance similar to the medium of Brewer was satisfactory for the cultivation of anaerobes under aerobic conditions.

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[2] Numbers in parentheses refer to literature cited.

A study of the literature has shown no evidence that an aerobic medium has been utilized for the growth of *CI. thermosaccharolyticum,* nor has an attempt been made to utilize such a medium in the routine analysis of sugar samples for specification purposes. This investigation was conducted in an attempt to test various media for suitability as a routine testing medium to replace the cumbersome method now recognized by the National Canners' Association.

Methods and Materials

Sugar samples known to be contaminated with *CI. thermosaccharolyticum* were secured from two refineries which sporadically produced contaminated sugar. In addition, three samples of sugar were obtained from the National Canners' Association.

In order that the experiments be well controlled, all media tested were compared with the routine medium recommended by the National Canners' Association (2) and prepared according to the specifications outlined in the A.O.A.C. page 754, paragraph 40.14 (b) . The standard procedure of the A.O.A.C. for the detection of *CI. thermosaccharolyticum* was used in all tests, to wit:

> Twenty grams of sample sugar were placed in a sterile 250-ml. Erlenmeyer flask. Sterile water was added to a total volume of 100 mis. The sugar-water solution was then brought to boiling and allowed to boil for five minutes. Any loss by evaporation was replaced with sterile distilled water. Twenty mis. of the boiled solution were dispensed equally among six tubes each of the test media, and of the control medium. The liver extract broth tubes were overlaid with a sealing agar; the media of low oxygen tension were placed directly into the incubator after seeding. All tubes were incubated at 55° C. for 72 hours and then observed for the production of gas and other criteria distinctive for positive tests.

Results

Several media of low oxygen tension were investigated to meet the requirements of sugar analysis. These media were prepared using commercially dehydrated liver, liver fractions and combinations of these substances with enrichments of tryptone, peptone, dextrose, etc., both with an agar seal and at a reduced oxygen tension by the addition of thiogiycollic acid. Only negative to weakly positive results were obtained.

The medium of Cameron, et al (7) was modified by using thiogiycollic acid as a reducing agent instead of an agar seal. This also gave sporadic and unreliable results. The medium which finally proved to be the most satisfactory was the commercial preparation "Fluid Thioglycollate, Difco." This broth is prepared by adding 29.5 grams of the dry ingredient to 1 liter of distilled water, boiling for a few minutes to dissolve, and tubing in 150 mm. x 18 mm. tubes. Each tube of medium contained a 75 mm. x 10 mm. gas tube. The medium was autoclaved in the tubes at 121° C. for 20 minutes at 15 lbs. pressure. All data pertaining to the dehydrated product may be obtained from the Difco manual, eighth, edition. The final pH of the broth is 7.1.

The results obtained comparing the standard medium with the thioglycollate method are shown in Table 1.

A composite sample was made of several of the sugar samples listed in Table 1 to obtain a larger homogeneous mixture for an investigation of

Table 1.—Results Obtained from Comparative Tests of Liver Extract Broth and Thioglycolate Broth in 6 Tube Tests.

Sample Number	Liver Broth	Thioglycollate Broth	Sample Number	Liver Broth	Thioglycollate Broth
Utah 1	++++--	+++++	Utah 13	+++++	+++++
Utah 2	+++++	+++++	Utah 14	+++++	+++++
Utah 3	++----	++++--	Oregon 1	+++++	+++++
Utah 4	+++++	+++++	Oregon 2	+----	+++---
Utah 5	++++--	+++++	Oregon 3	++----	++----
Utah 6	-----	++----	Oregon 4	+++++	+++++
Utah 7	+++++	+++++	Oregon 5	-----	++----
Utah 8	+++++	+++++-	Oregon 6	+----	-----
Utah 9	+++++	+++++-	NCA 7087	++----	----
Utah 10	+++----	+++++	NCA 7021	++++--	+++++-
Utah 11	+++++	++----	NCA 1949	+++----	+++++-
Utah 12	+++++	+++++-			

the comparative sensitivity of the media. To maintain a constant composition of the innoculum a dilution of this composite sample was made using as a diluent a sugar known to be free of thermophilic anaerobes; thus, the total weight of each sample amounted to 20 grams to make the sample comparable to the standard procedure. The procedure was otherwise as outlined for the standard in a previous paragraph. It may be observed from Table 2 that the thioglycollate medium is equally as sensitive as the liver extract broth.

Table 2.—Results of Tests Showing Comparati : Sensitivity of Liver Extract Broth and Thioglycolate Broth in Single Tube Tests.

Dilution	Liver Broth	Thioglycollate Broth
1:5	+	+
1:7.5	+	+
1:10	+	+
1:15	+	+
1:20	+	+
1:30	+	+
1:40	-	+
1:60	-	-
1:80	-	-

Table 3 presents the results of tests made using National Canners' Association reference culture T.A. 3814. This culture was diluted with sterile water to the dilutions shown in the table, and 1 nil. of each dilution was seeded into a tube containing sugar equivalent to a standard test. Here again, it was observed that while the two media at a dilution of 1:80 were only weakly positive, each showed equal amounts of turbidity and gas production.

Throughout the investigation it was noted that best results were obtained when the liver extract broth and the thioglycollate medium were seeded as soon as possible after removal from the autoclave, and while the innoculum was still near the boiling point. Results were more consistent if the fluid thioglycollate was made fresh at least every other day. It is the practice in our laboratories to heat both media under pressure 5 to 10 minutes before seeding with the sugar solution.

Conclusions

It is evident that the liquid thioglycollate medium, Difco, is equally as sensitive for the detection of thermophilic anaerobes as the liver infusion medium which is used at present as standard procedure by the National Canners' Association. This being the case the thioglycollate medium has much to recommend its use as standard procedure. The preparation of the medium is much simpler, and if the Difco product is utilized a much more standard preparation is obtained than could be had in the preparation of the liver medium in the laboratory.

Table 3.—Results Using NCA Reference Culture T.A. 3814 in Two Tubes Tests.

Dilution	Liver B Broth	Thioglycollate Broth
1:5	++	++
1:10	++	++
1:20	++	++
1:40	++	++
1:80	+-	+-
1:160	+-	+-

The Utah Idaho Sugar Company has adopted the thioglycollate method for the routine detection of thermophilic anaerobes, and is justified by the standards it maintains in the control of its sugar. As a routine procedure in the laboratory, any sample showing one tube positive for thermophilic anaerobes rejects that lot of sugar for canners' specification.

The method is recommended to the National Canners' Association as an alternate method for its standard procedure for the detection of thermophilic anaerobes.

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