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**Modified Resazurin Test for Estimating Bacterial Population
in Maple Sap**

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The resazurin test for bacterial counts in milk has been modified for estimating the bacterial cells in maple sap. This simple test provides an approximate bacterial cell count in 2-3 hr.

The sanitary quality of maple sap is of primary importance in the manufacture of maple sirup, because the color and flavor of maple sirup are determined largely by the bacterial population of the sap from which it is made. Sirup made from fermented sap is dark in color and may have "off" flavors and, in extreme cases, a poor (ropy) texture. Conversely,

sirup made from sap with low populations of bacteria and yeast is light amber, has a delicate maple flavor, and commands the highest price. Maple sap evaporator plant operators have recognized the need for controlling bacterial growth in maple sap and have instituted plant sanitation programs designed to maintain sap in good sanitary condition, from the time it is received at the plant until it is processed into standard density sirup. However, the evaporator plant operator has little control over the sanitary quality of sap delivered to the plant. At present there is no satisfactory, rapid test that can be used to

determine whether or not the sap is suitable for conversion to sirup. A method is urgently needed which will provide a quick estimation of the bacterial population in maple sap and give an indication of the degree of fermentation and thereby the extent of deterioration. The method should permit identification of sap samples having bacterial populations in excess of 1×10^6 cells/ml but need not provide exact quantitative data.

The resazurin reduction test, a standard method used to determine the sanitary quality of raw milk (1), offers a potential means of determining the sanitary quality of maple sap. The test is based on the reduction of resazurin to resorufin by bacterial action, which is marked by a color change from purple to pink. This method, if applicable to maple sap, offers the following advantages: (1) the resazurin dye can be obtained in standardized tablet form certified by the Biological Stain Commission; (2) relatively little equipment is needed; (3) the analysis can be performed easily by technicians; and (4) the analysis can be completed in a short time.

In the past year a study of the resazurin reduction test was conducted to determine its applicability to the measurement of the bacterial population in maple sap. This method was first tested with maple sap by the same procedure as that used for milk. The color change of the dye was sharp and easily discernible, but the incubation time required to achieve the full color change was too long. A bacterial population of 1.0×10^7 cells/ml required 4-hr incubation to produce a strong, positive color change. This did not compare favorably with resazurin reduction times reported for raw milk, where bacterial populations as low as 5×10^5 reduced the resazurin dye in 2 hr (2).

The procedure was therefore re-examined. Probably a shorter time is required for the reduction of the dye by bacteria in milk because milk is a much better culture medium than maple sap, which has a low nitrogen content. Thus, the sap would not produce the rate of growth of bacteria during the incubation period necessary to bring about as rapid reduction of the dye as in the resazurin milk test. To stimulate the rate of bacterial growth in sap during the resazurin test the effects of

the addition of nutrients to the sap and of increasing the temperature of incubation were studied. The best results for pink color development were obtained when 10 ml of the maple sap was fortified with 1 ml of a nutrient broth and the mixture was incubated 15 min prior to addition of the dye solution. After addition of the dye the sap-dye mixture was incubated at 37.5°C , 2° above the temperature used in the resazurin milk test. The modified resazurin test for measuring the bacterial populations in maple sap is given below.

METHOD

Apparatus and Reagents

(a) *Pipets, serological.*—To deliver 1 ml and 10 ml, with 1.0 ml graduations (sterilized).

(b) *Test tubes.*— 150×16 mm, screw-top with molded plastic caps (sterilized).

(c) *Test tube racks.*

(d) *Water bath.*—Constant temperature, capable of holding temperature at $37.5 \pm 0.5^\circ\text{C}$.

(e) *Bottles.*—200 ml amber, glass-stoppered.

(f) *Nutrient broth.*—10-fold. Combine 30 g beef extract and 50 g peptone, and make to 1 L with distilled water. Sterilize by autoclaving 15 min at 15 psig.

(g) *Resazurin dye.*—To 200 ml distilled water in an amber bottle, sterilized in an autoclave 15 min at 15 psig, add 1 standard resazurin dye tablet, using clean, dry forceps. Shake to insure complete solution of the tablet before the water cools.

Procedure

To sterile test tube, transfer 1 ml nutrient broth and 10 ml of the sap to be tested. Mix sap and nutrient broth by capping and inverting tube several times, and place tube in 37.5°C water bath. After 15 min, remove tube from water bath and, with sterile pipet, add 1.0 ml resazurin dye. Cap tube, invert, and shake to mix dye thoroughly. Replace tube in 37.5°C water bath and heat sap-dye mixture, removing tube at 30-min intervals to note time of end point (pink). After dye is added to tube, keep water bath covered with aluminum foil or other opaque cover to prevent light from affecting the rate of dye reduction.

Determine bacterial cell population in the sap from a curve relating time of color development to cell count.

This report of the Associate Referee was presented at the 82nd Annual Meeting of the Association of Official Analytical Chemists, Oct. 14–17, 1968, at Washington, D.C.

Results and Discussion

The bacterial cell population-time of color development curve (Fig. 1) was constructed from the results obtained from 37 determinations by the modified resazurin test. The reduction of the resazurin and the color change were unaffected by the addition of nutrient broth.

The modified resazurin test for maple sap has met the requirements for a rapid, simple method for estimating bacterial cell counts in maple sap. For sap containing approximately 1×10^7 cells/ml, the time required for the test was $1\frac{1}{2}$ hr. Maple saps with smaller bacterial populations would require longer incubation periods. Thus, sap with approximately 1×10^5 cells/ml would require $2\frac{1}{2}$ to 3 hr for reduction, and sap with 1×10^4 cells/ml would require more than 4 hr for the test.

The resazurin reduction test is well suited to the determination of the bacterial populations in maple sap because (1) the nutrient-sap solutions are clear and pale amber so that the change in the color from the purple of the resazurin to the pink of its reduced form are easily detected, and (2) the higher the bacterial populations the shorter the time required to make the test. The latter is an important factor since it makes possible the early identification of heavily contaminated sap.

It should be noted that the pink developed in sap is not the same as the pink developed in milk because of the effect of the white color of milk. The pink developed in sap, therefore, does not match the Munsell 5P7/4 color standard used in the milk resazurin test.

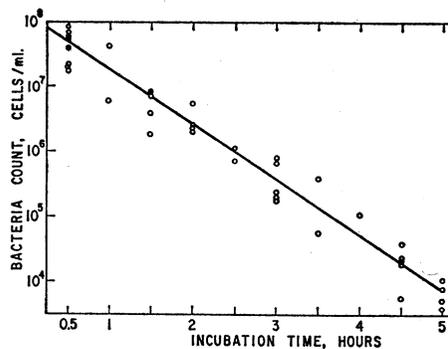


FIG. 1—Time for reduction of resazurin by different bacterial populations in maple sap. The solid curve was fitted by least squares.

Recommendations

It is recommended—

(1) That the study on the modified resazurin test for bacterial populations in maple sap be continued.

(2) That the method for determining the yeast count in maple sirup, reported and adopted as official first action at the 1967 meeting of the AOAC, be adopted as official final action since in the interim no questions have been raised regarding this method.

REFERENCES

- (1) *Standard Methods for the Examination of Dairy Products*, 11th Ed., The American Public Health Association, New York, N.Y., 1960.
- (2) Johns, C. K., *J. Milk Food Technol.* **17**, 369-371 (1954).

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D and were adopted by the Association. See *This Journal* **52**, 334 (1969).