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Aerobic Plate Counts of 100-ml Samples in Plastic Bags[1]

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ABSTRACT

Counts from samples that contained low numbers of bacteria were determined by mixing the samples with double-strength agar media in 42-oz (1.2-L) Whirl-Pak bags. The bag-plate method was compared with other direct-plating methods and the most-probable-number and membrane-filtration procedures. Results obtained by using the bag method were as reliable as methods commonly used for analysis of samples that contain low numbers of viable bacteria.

Microbiologists sometimes need to determine the viable counts of samples that contain low numbers of microorganisms (i.e., less than 1/ml).

Concentration of the bacteria on various types of filters *(3,8,13)* or by flocculation *(16)* before cultivation are suitable under some circumstances, but these methods are cumbersome.

To accommodate large sample volumes, Weiss and Hunter *(22)* invented and Clark *(4,5)* popularized the "presence-absence" (P-A) test. In the P-A test, a large sample is added to concentrated medium in a container and the presence or absence of bacteria is noted after incubation. Quinn *(18)* devised an MPN test for coliforms based on this principle. Similar methods were proposed to predict the shelf-life of poultry *(14)*, determine the commercial sterility of heated foods *(10)*, test the sterility of infusion fluids *(20)*, detect salmonellae in rinse waters from poultry carcasses *(2)*, and isolate bacteria from dialysis patients with peritonitis *(6)*.

As early as 1896, Metchnikoff and coworkers *(15)* cultivated bacteria in collodian sacs. The most significant advance since Metchnikoff's work was the invention by Bladel and Greenberg *(1)* of pouches constructed from gas-impermeable plastic film for the cultivation of anaerobes. Other investigators proposed the use of plastic pouches for plating small volumes of sample *(11,17)*, but sterile, gas-permeable bags in a variety of sizes became routinely available only in recent years.

In the present study, 100-ml volumes of sample were mixed with double-strength agar media in sterile Whirl-Pak bags. The agar was allowed to solidify while the bags were placed on a flat surface. Incubation and colony enumeration were performed as usual.

MATERIALS AND METHODS

All samples were purchased or collected locally. Media were purchased from Difco, except that Trypticase Soy Agar was purchased from BBL.

The samples were examined by using standard *(9,19)* or recommended *(21)* most-probable-number (MPN), plating, and membrane-filtration (MF) procedures. In addition, 100-ml volumes of appropriate dilutions were added to sterile, 42-oz (1.2-L) Whirl-Pak plastic bags (Nasco, Fort Atkinson, WI) while the bags were held upright in a Whirl-Pak bag holder or a small test-tube basket. A 100-ml volume of double-strength, tempered (45-46°C) agar medium was poured into each bag, the bag was closed loosely, and the contents of the bag were mixed by holding the bag in one hand and massaging the bag for 30 s with the other hand. The bags were sealed by rolling the opened end 10-12 times and were placed on a flat surface. After the agar had solidified, the bags were transported to an incubator shelf and were opened (Fig. 1). The bags, and corresponding plates and tubes of the same samples, were incubated for 48 h at 35°C (water samples) or 32°C (food samples). A light box and colony counter lens (Fig. 2) were used to facilitate colony enumeration.

The data were analyzed for significant differences at the 0.1, 1, 5, and 10% levels by using the sign test *(7)*.

RESULTS AND DISCUSSION

One shortcoming of growing bacteria in the anaerobic pouches of Bladel and Greenberg *(1)* is that "spreaders" (motile bacteria) tend to form large, diffuse colonies in the liquid film at the agar-plastic interface. We experienced the same difficulties when closed, 24-oz (0.7-L), gas-permeable Whirl-Pak bags were used. Therefore, longer, 42-oz bags were selected and 5- to 15-g quantities of various materials were added adjacent to the solidified agar before incubation in attempts to absorb the water of syneresis that formed in the bags. Powdered gum arabic, powdered 325-mesh clay, diatomaceous earth, silicic acid, beaded silica gel, and powdered silica gel were unsatisfactory (data not shown). The addition

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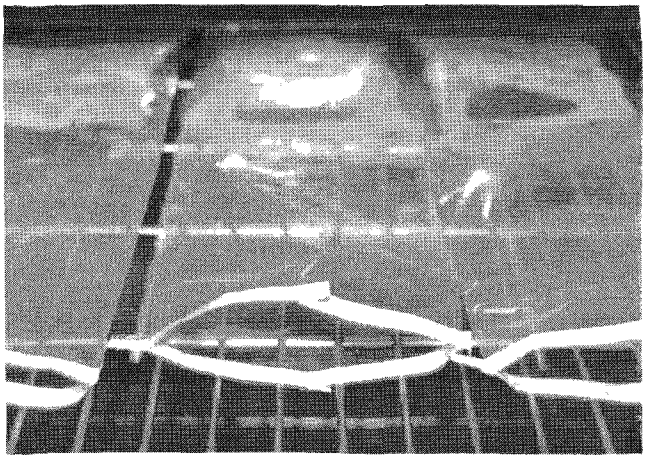

Figure 1. *Opened bag-plates on incubator shelf.*

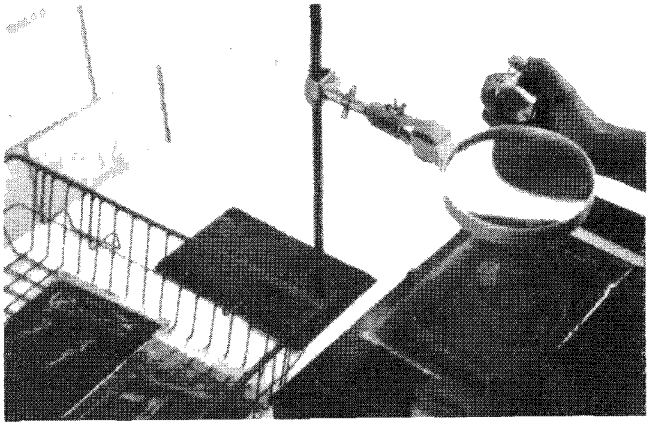

Figure 2. *Colony enumeration on a bag-plate. The bag rests on a Plexiglas insert in a rectangular opening cut out of a metal box containing a light. The magnifying lens was obtained from a Quebec colony counter.*

TABLE 1. *Mean percentage yields[a] and statistical evaluation[b] of water samples as determined by using six different methods.*

Method[c]	Surface waters	Other waters
Bag	52	54
MPN	59	39
Pour-plate	47	52
Spread-plate	60	52
m-HPC	74[d]	34[e]
MF-PCA	57	28[e]

[a]Three samples were examined in triplicate from each of 5 sites for surface waters and 7 sites (2 wells, 3 swimming pools, and 2 whirlpools) for other waters. For each set of analyses, the method yielding the highest count was assigned a value of 100%, and the percentage yields of the other methods were calculated; finally, the percentage yields for all analyses within a treatment were averaged.

[b]Dixon and Massey *(7)*.

[c]Each bag received 100 ml of sample and 100 ml of double-strength plate count agar (PCA). MPN broth for 5-tube MPN series contained (per liter): tryptone, 5.0 g; yeast extract, 2.5 g; and glucose, 1.0 g. Double-strength MPN broth was prepared for 10-ml samples. Pour plates and spread plates were made by using PCA. The spread plates were dried overnight in a 32°C incubator before use; each plate received 0.1 or 1.0 ml of inoculum, which was spread on the surface with a sterile glass rod and allowed to absorb into the agar. For membrane filtration, the membranes were incubated on either m-HPC agar *(9)* or PCA (MF-PCA).

[d]Counts were higher than the bag method at the 10% level of significance.

[e]Counts were lower than the bag method at the 1% level of significance.

of 0.2 mM *p*-nitrophenyl glycerol *(12)* to prevent bacterial swarming also was ineffective (data not shown). The incidence of "spreaders" was decreased appreciably in 42-oz bags by reopening the bags after they were in position in the incubator to permit evaporation of excess moisture (Fig. 1). Sterile cotton balls, such as are sold in retail stores for cosmetic use, were inserted into the mouths of the bag-plates to serve as closures. Later, the cotton plugs were found unnecessary. No contamination was observed when 18 bags were incubated open and without cotton plugs in a large, walk-in incubator.

Total counts were made of water samples by using six methods: bags containing plate count agar (PCA), a 5-tube MPN procedure, pour plates of PCA, spread plates of PCA, and membrane filters incubated on two different media (Table 1). For surface waters (Table 1, column 1), the m-HPC method yielded the highest recoveries (see footnote d), followed by the spread-plate and MF-PCA methods. The MPN method and pour plates of PCA yielded recoveries equivalent to the bag method. Data for samples from wells, swimming pools, and whirlpools (Table 1, column 2) were combined for statistical analysis because the trends were similar. When these types of water samples were examined (Table 1, column 2), the m-HPC and MF-PCA methods yielded significantly less than the bag method. Thus a definite method/sample-type interaction existed. A relatively low average yield was obtained with the MPN method, but the yield was not significantly lower than the bag, pour-plate, or spread-plate methods. An example of a total count in a bag-plate is shown in Fig. 3; this sample contained only 3 viable bacteria/100 ml.

Two different media were used for coliform enumeration of 12 food samples by direct-plating in bags and in plates; MPN tests were also conducted (Table 2). The MPN method yielded significantly higher counts than bags containing full-strength VRB agar (Table 2, column 1). Counts obtained by using full-strength VRB agar in bag-plates (Bag:VRB) resulted in significantly higher yields when compared with full-strength VRB agar in plates (VRB-1). When a mixture of VRB agar and TSA was used in bags (Table 2, column 2; Bag:VRB-TSA), mean recoveries in the bag-plates were significantly higher than when VRB agar alone was used in plates (VRB-1). No significant differences in counts were obtained between bags containing full-strength VRB agar and bags containing a mixture of VRB and TSA agars

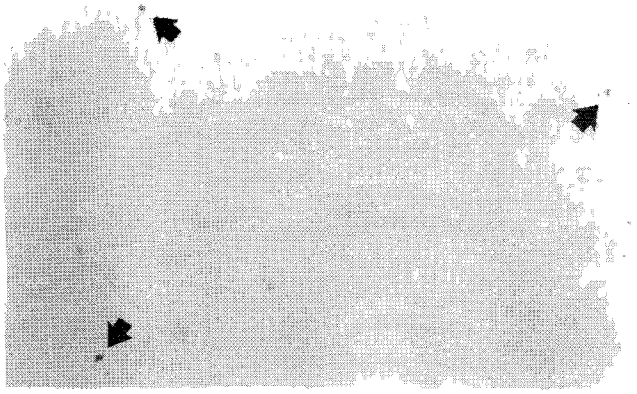

Figure 3. *Typical bag-plate after incubation; three colonies are present (arrows).*

TABLE 2. *Mean percentage yields and statistical evaluation of food samples[b] as determined by using five different methods.*

Method	Bag VRB	Bag VRB-TSA
Bag:VRB	43	--
Bag:VRB-TSA	--	59
MPN	73[c]	73
VRB-1	18[d]	18[e]
VRB-2	38	38

[a]Three samples each of hamburger, mixed vegetables, broccoli, and raw milk were examined in triplicate. See footnotes a and b of Table 1 for other explanations.

[b]Each bag received 100 ml of sample and 100 ml of either double-strength VRB agar (Bag:VRB) or a 50:50 mixture of VRB and trypticase soy agars (VRB-TSA). Lauryl tryptose broth was used for the 5-tube MPN determinations. For VRB-1, both the base layer and overlay consisted of VRB agar, whereas for VRB-2, the base layer consisted of TSA agar with a VRB-agar overlay *(19)*.

[c]Yields were higher than the bag:VRB method at the 5% level of significance.

[d]Yields were lower than the bag:VRB method at the 0.1% level of significance.

[e]Yields were lower than the bag:VRB-TSA method at the 10% level of significance.

or plates containing a TSA base layer and VRB agar overlay (VRB-2 method). Although the MPN method yielded the highest counts, differences in yields between the bag and the MPN and VRB-TSA (VRB-2) methods, although substantial, were not significant.

Comparisons between results obtained by using different methods can be obscured because different dilutions and volumes were used for the bag and MF methods (100-ml), MPN (10.0, 1.0, and 0.1 ml), and pour and spread plates (1.0 and 0.1 ml). Nevertheless, results obtained by using the bag method were as reliable as methods commonly used for the analysis of samples that contain low numbers of viable bacteria.

The procedure for preparing bag-plates is simple, but some practice is necessary to prepare them skillfully and

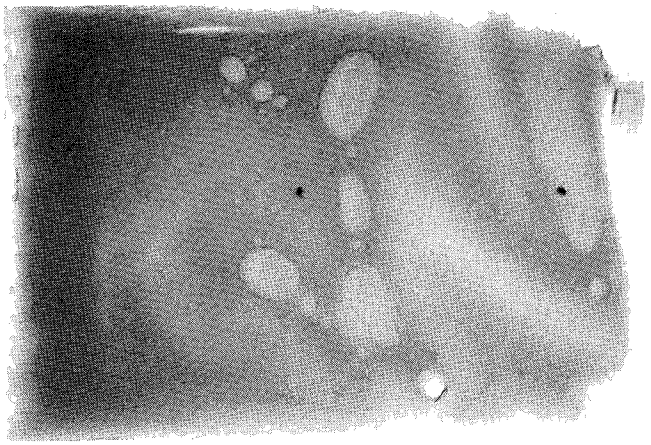

Figure 4. *Example of a poorly prepared bag-plate of VRB agar containing large bubbles. Two coliform colonies are readily detectable in a 100-ml sample.*

with few bubbles in the agar. An example of a coliform count in a bag (2 coliforms/100 ml) is shown in Fig. 4. This illustration was included to demonstrate that colony counts can be made even in bag-plates that are poorly prepared and contain large bubbles. The cost of one bag and the culture medium used therein is about $0.50, compared with $0.10 for a single plastic petri plate; however, the area of a bag-plate is about 52 in.^{2} (337 cm^2), compared with about 10 in.^{2} (62 cm^2) for a plate. Costs of a bag plate and membrane filtration using one filter would be approximately equal.

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