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Method for Performing Aerobic Plate Counts of Anhydrous Cosmetics Utilizing Tween 60 and Arlacel 80 as Dispersing Agents

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An aqueous diluent containing Tween 60 and Arlacel 80 gave greater recovery of microorganisms when compared with two common diluents as determined by aerobic plate count of inoculated anhydrous cosmetics. The greater recovery was caused by better dispersion of the anhydrous cosmetics in the diluents.

The aerobic plate count as a nonspecific method for determining the microbial content of cosmetic and pharmaceutical products is well accepted (1, 2, 4, 5). Depending upon the physical nature of the product to be tested, membrane filtration or serial dilutions are usually employed to obtain a representation of the microflora present.

Although these methods are quite satisfactory for oil in water or water in oil emulsions, anhydrous products present a special problem because they are not miscible with water. With diluents presently employed, the best that can be expected from a shaken dilution of such products is an aqueous wash of a portion of the sample resulting in an inaccurate enumeration of the microbial population.

Davis (3) points out the obvious error of the "total count" performed on solid or semisolid materials. Maceration of the product breaks up colonies with unpredictable scattering, leading to large error and poor reproducibility. Buhlmann (2) suggests a 10% Tween 80 buffer solution heated to 40 C for homogenization, although he concedes that, for unstable emulsions, tests must be performed on the aqueous phase.

Tenenbaum et al. (5) suggest dispersing a sample of product in an equal volume of Tween 80. The dispersion is then brought to volume with the proper amount of 0.1% peptone-water. Although good dispersion of the product is obtained in the Tween 80, immediate clumping and separation occur when the aqueous phase is added. The problems encountered here are in accurate sampling of all phases and in adhering of the portions of the oil phase that are removed to the sides of the pipette and to the bottom of the petri dish.

Bruch (1) described a method from USP XVIII (1 November 1971) which employs isopropyl myristate (IPM) warmed to 47 C to solubilize ointment bases. But since heat-sterilized IPM is toxic to vegetative bacteria, it must be sterilized by passage through a 0.22-μm MF filter. Besides being time consuming, the toxicity of filtered IPM to gram-negative organisms may lead to erroneous results.

We investigated several methods designed specifically for anhydrous products and developed a method which yields excellent results in our laboratory. Our method, to be referred to as the AT method, utilizes the hydrophilic properties of polyoxyethylene (2) sorbitan monostearate (Tween 60; Atlas Chemical, Wilmington, Del.), and the lipophylic properties of sorbitan monooleate (Arlacel 80; Atlas Chemical, Wilmington, Del.) which, in combination with the anhydrous products tested, form a uniform emulsion.

MATERIALS AND METHODS

Ten milliliters of Arlacel 80 and 80 ml of a 10% aqueous solution of Tween 60 were autoclaved separately for 15 min at 121 C in wide-mouth milk-dilution bottles. After autoclaving, the Tween 60 solution was mixed to re-disperse the Tween 60, which tended to settle out.

Ten grams of product to be assayed was added to 10 ml of Arlacel 80 and mixed with a sterile spatula until the product was uniformly distributed in the Arlacel 80. Eighty milliliters of the Tween 60 solution was then added, and the mixture was shaken vigorously for approximately 15 s to obtain a uniform emulsion.

Once it was determined that adequate product dispersion could be obtained by this method, we proceeded to evaluate the recovery of microorganisms from anhydrous products. In particular, we were concerned that the presence of Tween 60 and Arlacel 80 might inhibit the growth of microorganisms.

Therefore, we compared the growth of five stock cultures by inoculating triplicate tubes per organism containing 10 ml of Trypticase soy broth (BBL) and triplicate tubes per organism containing Trypticase soy broth with 10% Arlacel 80 and 10% Tween 60 added. The separate inocula were 0.05 ml of a 24-h broth culture of *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11775, *Pseudomonas putida*, a laboratory isolate, and *Candida albicans* ATCC 10231.

All inoculated tubes were serially diluted, and each dilution was plated in triplicate by using Trypticase soy agar (BBL) for bacteria and Sabouraud dextrose agar for yeast. The tubes were then incubated at 35 C for 24 h, at which time they were serially diluted and plated again.

Inoculum size and subsequent 24-h growth results were determined by counting all plates containing less than 300 colonies to obtain an average inoculum for each tube. Since stock cultures are routinely serially diluted and plated at weekly intervals, acceptable ranges had already been established.

To test for the recovery of microorganisms, eight anhydrous products were inoculated with a mixed culture of bacteria and fungi and plated in duplicate weekly for 2 months, and two prevailing methods were then compared with the AT method. This represents 128 plate counts per method. Once it was established that greater recovery of the inoculated microorganisms was obtained by the AT method, a preserved and an unpreserved eyeshadow were selected and plated at more frequent intervals to obtain the data presented here.

Two hundred grams of the two eyeshadows were placed in 220-ml sterile plastic cups, and each cup was inoculated with 0.2 ml of a mixed culture of *P. aeruginosa*, *S. aureus*, *E. coli*, *P. putida*, *C. albicans*, *Aspergillus niger* ATCC 16404, and *Penicillium ochracium* ATCC 10112. The size of the inoculum was determined by serial dilution and plate count to be approximately 5×10^6 cells per g of product.

The inoculated products were thoroughly mixed for 1 min with a sterile 10-ml pipette. Three 1-g samples were removed immediately from each container and assayed by each of the following methods.

Method A. One gram of product was placed into 9 ml of 0.1% Tween 80 in normal saline. The tubes were shaken and Vortex-mixed, and 1.0 and 0.1 ml were plated in duplicate on Trypticase soy agar and Sabouraud dextrose agar.

Method B. One gram of product was placed into 1 ml of Tween 80. The product was thoroughly dispersed, and the volume was brought to 10 ml with 0.1% peptone-water. The tubes were shaken and Vortex-mixed and plated as above.

Method AT. One gram of product was thoroughly dispersed in 1 ml of Arlacel 80, and the volume was brought to 10 ml with 10% Tween 60 solution. Again the tubes were mixed and plated as above.

The products were plated immediately after inoculation and again after 2, 3, 6, 9, and 15 days. Duplicate 1.0- and 0.1-ml samples of each product were tested by the three methods, and the average of the four plate counts was recorded.

RESULTS

Figure 1 shows a comparison of several products that were diluted by the AT method and method B employing Tween 80 and 0.1% peptone-water. Note the heavy clumping and phase separation in the tubes containing Tween 80 and peptone-water, and the excellent dispersion by the AT method.

Table 1 shows a comparison of the total population obtained after 24 h of incubation of five microorganisms grown in Trypticase soy broth and Trypticase soy broth plus 10% Tween 60 and 10% Arlacel 80. No significant difference in the total population increase after 24 h was apparent when Tween 60 and Arlacel 80 were added to the media.

Tables 2 and 3 show the results of the preserved and unpreserved anhydrous eyeshadow inoculated with a mixed culture of bacteria and fungi. No attempt was made to differentiate the microorganisms upon counting the plates, but rather the results were reported as total organisms per gram. Sporadic results were observed with method A. Methods B and AT show a gradual decline in count, but recovery with the AT method was considerably higher in each plating.

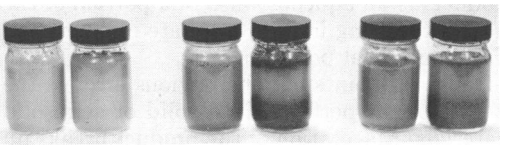

FIG. 1. *Three anhydrous products diluted by the AT method (left) and method B (right) employing Tween 80 and 0.1% peptone-water.*

TABLE 1. *Comparison of the population increase of five microorganisms incubated for 25 h in Trypticase soy broth (TSB) and Trypticase soy broth with 10% Tween 60 and 10% Arlacel 80 (TSB plus)*

Microorganism	Initial inoculum[a]/ml	Microorganisms/ml after 24 h[a]	
		TSB	TSB plus
P. aeruginosa	1.0×10^6	4.6×10^8	4.9×10^8
S. aureus	1.3×10^6	3.2×10^8	5.0×10^8
E. coli	3.0×10^6	5.7×10^8	3.4×10^8
P. putida	4.4×10^6	1.7×10^8	1.2×10^8
C. albicans	4.2×10^4	3.1×10^6	3.4×10^6

[a] Each figure is the average of at least four plate counts on each of three tubes.

TABLE 2. *Aerobic plate count of a mixed culture-inoculated preserved anhydrous eyeshadow dispersed by three methods*

Method	Aerobic plate counts[a]					
	0[b]	2	3	6	9	15
A	TNTC[c]	2,500	1,875	100	175	10
B	10,900	6,400	3,400	600	195	0
AT	TNTC	9,000	3,900	2,100	460	200

[a] Each figure is the average of four counts.
[b] Days.
[c] TNTC, Too numerous to count on a 1:100 dilution.

TABLE 3. *Aerobic plate count of a mixed culture-inoculated unpreserved anhydrous eyeshadow dispersed by three methods*

Method	Aerobic plate counts[a]					
	0[b]	2	3	6	9	15
A	TNTC[c]	8,400	9,600	820	1,195	110
B	12,700	8,400	7,600	1,950	1,050	85
AT	TNTC	11,300	10,500	3,800	2,400	500

[a] Each figure is the average of four counts.
[b] Days.
[c] TNTC, Too numerous to count on a 1:100 dilution.

DISCUSSION

We believe that the AT method will enable the cosmetic microbiologist to completely disperse water-immiscible products to form an aqueous emulsion and thereby obtain more representative results. We are satisfied that the emulsifying agents have been shown to have no toxic effect on the microorganisms listed over a 24 h period. Since plate counts on the diluent are performed within minutes and the emulsifying agents are diluted at least 15 times with agar, inhibition should be minimal if not eliminated.

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