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Effect of culture conditions and method of inoculum preparation on the kinetics of bacterial death during preservative efficacy testing

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Synopsis

The linear regression method of preservative efficacy testing was used to evaluate the effect of culture conditions and type of inoculum on the kinetics of death of *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* or *B. cereus,* and *Escherichia coli* in nonionic emulsions. The rates of death, as determined by use of D-values, were essentially unchanged when saline suspensions of bacteria grown for 24, 48, or 72 hr on agar media were used in testing.

Broth inocula performed differently in preservative efficacy testing than saline inocula prepared from surface growth on agar media. The use of 0.2% broth inocula (0.1 ml/50 mL sample) or the addition of 0.2% sterile broth to test samples produced significant decreases in the rates of inactivation of the test organisms. Broth inocula changed the kinetics of bacterial death and should not be used in routine testing. These results demonstrate the need for standardized procedures for preparation of inocula. It is recommended that preservative efficacy tests be performed using saline suspensions of bacteria grown aerobically on solid agar media.

INTRODUCTION

The development of cosmetic products includes preservative efficacy testing to demonstrate that the products are adequately preserved. Test methods include standardized procedures: the United States Pharmacopeia (USP) method (1) and the Cosmetic, Toiletry & Fragrance Association (CTFA) method (2); and rapid procedures: the linear regression method (3), the accelerated preservative test (4), and the presumptive challenge test (PCT) (5). These methods were reviewed recently by Mulberry *et al.* (6).

The preservative efficacy test methods have a number of similarities, including the test organisms, recovery systems, and the method of performing aerobic plate counts (APCs). The methods have several differences, including culture media and growth temperatures used in preparing the inocula and the times at which APCs are determined. These differences may produce variations in test results, statistical treatment of the data, and criteria used to determine whether a given product is satisfactorily preserved (1–8).

Transferring test organisms to new growth media is one of the first steps in preparing for preservative efficacy testing. Antibiotic susceptibility testing is performed with broth cultures in early log phase of growth, such as 2–5 hr growth at 35°C in Tryptic Soy Broth (TSB) (9–11). Cosmetic preservative efficacy testing routinely is performed using overnight cultures of test organisms. Thus, the USP method requires growth of bacterial cultures on a solid agar medium at 30–35°C for 18–24 hr. Lower incubation temperatures and longer incubation times are recommended for yeasts and molds (1). The testing guidelines proposed by the CTFA suggest "that no less than one million cells per milliliter or gram be used as the challenge" (2). Specific instructions are not given for growth of the test organisms or the volume of inoculum to use in testing; however, these guidelines state that harvested broth cultures or surface growth on a solid medium may be used for inoculating the product. The CTFA guideline for preservation testing of aqueous liquid and semi-liquid eye cosmetics stipulates that fresh cultures of bacteria should be used for challenging preservative systems and that broth cultures or surface growth on solid media may be used in testing (12). Different methods recommend the use of bacterial cultures grown for various combinations of 18–24 hr or 24 hr at 30–35°C, 32–35°C, 35–37°C, or 37°C (1–5,12).

It is possible that different preservative efficacy test results obtained by laboratories reporting to use the same method and test organisms may, in fact, be due to differences in the manner in which test organisms were grown prior to testing or to the type of inocula used. The quantitative data from the linear regression method enabled us to determine the effect of culture conditions, including age and growth media, and the type of inoculum on preservative efficacy test results. The studies reported here demonstrate that the culture medium affects the metabolism of bacteria and that the type of inoculum affects the results obtained in preservative efficacy testing. Specifically, the use of broth inocula decreased the rates of inactivation of the test organisms, compared to the use of saline inocula prepared from surface growth on agar media.

EXPERIMENTAL

TEST ORGANISMS

The test organisms used in this study were *Staphylococcus aureus* (FDA 209 strain), *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus cereus* (ATCC 11778), *B. subtilis* ATCC 6633 (kindly furnished by Mr. Mark Entrup, Hill Top Biolabs, Inc.), *Bacillus* sp., and *Escherichia coli* (ATCC 8739).

CULTURE CONDITIONS

The test organisms were grown on solid agar media: Tryptic Soy Agar (TSA), TSA with 0.07% (vol/vol) lecithin and 0.5% (vol/vol) Tween 80 (TSALT), or Plate Count Agar (PCA); or in broth media: TSB, Tryptic Soy Broth w/o dextrose (glucose) [TSB(-G)], and TSB(-G) with 1–5% (wt/vol) glucose. The cultures were incubated aerobically for 24, 48, or 72 hr at 37°C. All culture media were obtained from Difco Laboratories, Detroit, MI.

TEST SAMPLES

The test samples used in this study were hand and body lotions and a face cream. Lotion A contained water, glycerin, cetearyl alcohol, ceteareth-20, palm oil glyceride, glyceryl dilaurate, mineral oil, petrolatum, dimethicone, isopropyl palmitate, stearic acid, allantoin, and other ingredients. Lotion B contained water, sorbitol, stearic acid, glyceryl dilaurate, cetearyl alcohol, ceteareth-20, lard glyceride, stearamide MEA, hydrogenated vegetable oil, isopropyl palmitate, glyceryl stearate, PEG-100 stearate, dimethicone, petrolatum, allantoin, and other ingredients. The preservative system in both lotions contained methylparaben, propylparaben and Quaternium-15. Lotion C contained water, mineral oil, dimethicone, palm oil glyceride, allantoin, other ingredients, and was preserved with phenoxyethanol, methylparaben, and propylparaben. Lotion D was similar to lotion C, but contained propylene glycol. Lotion E was similar to lotion B, and lotion F was similar to lotion A, except that it contained glyceryl monolaurate in place of glyceryl dilaurate. The face cream contained mineral oil, water, beeswax, magnesium aluminum silicate, sodium borate, fragrance, isopropyl myristate, and colors.

TEST PROCEDURE

For broth inocula, 0.1 ml of each broth culture was added directly to separate 50-mL test samples. Saline inocula were prepared by suspending a loopful of TSALT surface growth of each test organism in 5 mL of saline and adding 0.1 mL of each saline suspension to separate 50-ml test samples (3). Samples were taken, APCs were performed at specified time intervals (0, 3, 5, and 24 hr) using Letheen Broth with 0.01% (vol/vol) Triton X-100 diluent and TSALT as the recovery system, and D-values were calculated as described previously (3).

pH DETERMINATIONS

The pH of the TSA, TSALT, and PCA in Petri dishes was determined after 24, 48, and 72 hr growth by the test organisms and in uninoculated (i.e., control) samples using a pH meter equipped with a flat-surface combination pH electrode. The electrode was moistened with a drop of demineralized water, and pH measurements were read directly after the electrode was placed on a sterile portion of the agar, approximately 1 cm from the region of growth.

The pH determinations of TSB and TSB(-G) cultures were made as follows: A 3-mL sample was pipetted into the barrel of a 10-mL syringe, to which was attached a sterile Sweeney-type filter with a 0.45 μm membrane. The plunger was inserted into the barrel, forcing the broth through the filter and into a small beaker. The pH measurements were taken on the filter-sterilized broth samples prepared in this manner.

EFFECT OF CULTURE BROTH ON PRESERVATIVE EFFICACY TESTING

The effect of bacteriological culture broth on preservative efficacy testing was determined by adding 0.1 mL of filter-sterilized TSB to 50-mL samples of lotion A immediately before inoculating with saline suspensions of the test organisms. The control samples were prepared by adding 0.1 mL of sterile demineralized water to samples of

lotion A immediately before inoculation with the test organisms. Samples were taken, APCs were determined, and D-values were calculated as described previously (3).

STATISTICS

Statistically significant differences between mean D-values ($\bar{X}$) of duplicate experiments were determined by the t test (13).

RESULTS AND DISCUSSION

Organisms with different physiological capabilities may respond differently to changes in growth media and culture conditions. Thus, *S. aureus, P. aeruginosa, B. subtilis,* and *E. coli* may exhibit different growth rates and time in the maximum stationary phase of growth in any given culture medium. It would not be unreasonable to expect that the length of the maximum stationary phase may range from a few minutes to days or weeks, depending on the growth conditions (i.e., temperature, pH, rate of growth, etc.) and the ability of the organism to withstand the metabolic stresses imposed by the unfavorable environment (i.e., depletion of nutrients, accumulation of toxic products, low pH, etc.).

The organic acids produced during fermentation may be bacteriocidal. This antibacterial effect increases as the pH decreases (14). It is believed that metabolic stress may be reduced by the use of culture media that allow aerobic growth without causing the development of unfavorable conditions (i.e., acid pH). This may enable the population of organisms to remain in the maximum stationary phase for extended periods.

The changes in pH during aerobic growth of the test organisms on the solid media were monitored. Although the 24-hr pH determination for *S. aureus* on PCA revealed that the agar was pH 6.7, all other pH determinations for *S. aureus* and all other test organisms were alkaline at 24, 48, and 72 hr (Table I). PCA contains 0.1% glucose; TSA

Table I
pH of PCA, TSA, and TSALT After Aerobic Growth of Test Organisms on Solid Agar Media for 24, 48, and 72 hr at 37°C

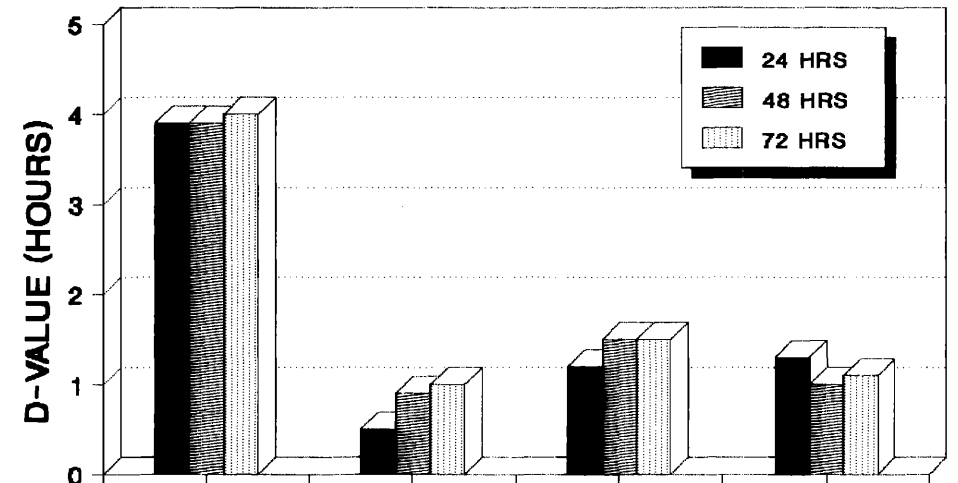
	Test culture			
Medium, time	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*E. coli*
PCA, 24 hr	6.7	8.1	7.9	7.8
PCA, 48 hr	8.2	8.4	8.4	8.2
PCA, 72 hr	8.3	8.4	8.4	8.3
TSA, 24 hr	7.8	8.2	8.0	8.3
TSA, 48 hr	8.3	8.4	8.4	8.4
TSA, 72 hr	8.4	8.5	8.4	8.4
TSALT, 24 hr	8.3	8.5	8.2	8.2
TSALT, 48 hr	8.2	8.5	8.2	8.3
TSALT, 72 hr	8.4	8.5	8.4	8.4

Table values are mean values of duplicate pH determinations on sterile agar, *ca.* 1 cm from growth. pH of uninoculated media on the day of preparation: PCA = pH 6.8, TSA = pH 7.1, TSALT = pH 7.1.

contains no added glucose. The pH values for uninoculated PCA, TSA, and TSALT were pH 6.8, 7.1, and 7.1, respectively.

These pH determinations suggested that extended incubation on solid agar media did not cause these bacteria to produce acid that could cause metabolic stress; consequently, the effect of incubating the test organisms on TSALT for 24, 48, and 72 hr on preservative efficacy testing was studied. Excellent reproducibility of test data was obtained when these cultures were used in preservative efficacy testing of lotion A. Thus, D-values obtained with 24-, 48-, and 72-hr cultures were within 0.1 hr for *S. aureus,* 0.5 hr for *P. aeruginosa,* 0.3 hr for *B. subtilis* (ATCC 6633), and 0.3 hr for *E. coli* (Figure 1). These differences of <0.5 hr for repetitive analyses of lotion A samples using the same test organism are considered to be within the precision limits of the linear regression method (7). This study was repeated two times with essentially the same results. Similar results were obtained when this experiment was repeated, in triplicate, with lotion B. This demonstrates that saline inocula from surface growth from 24-, 48-, or 72-hr TSALT cultures provide essentially the same D-values in preservative efficacy testing of these lotions with these test organisms.

Although surface growth of the test organisms on solid media produced negligible acidity during the 72-hr incubation period, growth in TSB, which contains 0.25% glucose, caused an appreciable drop in pH of some of the test cultures after growth for 24 hr (Table II). The data illustrate that *S. aureus* and *E. coli* reduced the pH of TSB from the initial pH of 7.2 to pH 5.3 and 5.7, respectively. These organisms are facultative aerobes, which means that they can grow either aerobically or anaerobically. Although the static broth tubes were incubated aerobically, it is believed that rapid



	S.aureus	P.aeruginosa	Bacillus	E.coli
24 HRS	3.9	0.5	1.2	1.3
48 HRS	3.9	0.9	1.5	1
72 HRS	4	1	1.5	1.1

Figure 1. D-values obtained in lotion A using saline suspensions from 24-, 48-, and 72-hr TSALT cultures of *S. aureus* and *E. coli*.

Table II
pH of Culture Media After Inoculation With the Test Organisms and Incubation for 24 hr at 37°C

Medium	Test organism			
	S. aureus	*P. aeruginosa*	*B. subtilis*	*E. coli*
TSALT	8.2	8.1	8.1	8.3
TSB	5.3	7.4	6.4	5.7
TSB(-G)	7.1	7.6	7.1	7.0
TSB (uninoculated control) = pH 7.2				

Table values are pH readings made on the surface of TSALT and in filter-sterilized broth media.

growth of these organisms reduced the oxidation/reduction potential, thereby causing the organisms to ferment the glucose with the production of organic acids that reduced the pH. Members of the genus *Bacillus* are generally considered to be aerobic; however, *B. subtilis* is capable of anaerobic growth and weak fermentation in complex media containing glucose (15). The data in Table II show that *B. subtilis* decreased the pH of TSB to pH 6.4 after growth for 24 hr. The genus *Pseudomonas* is "respiratory, never fermentative" (16). Thus, pseudomonads dissimilate carbohydrates without production of sufficient organic acids to decrease the pH of the culture medium.

The data in Table II also show that growth of the test organisms for 24 hr in TSB(-G) did not produce sufficient acid to decrease the pH of the medium, because all cultures had pH values $\geq$7.0 at 24 hr. Thus, the presence of a readily utilizable carbohydrate, such as glucose, has an effect on metabolism and acid production in broth cultures of some test organisms. The composition of these culture media is given in Table III.

The substantial drop in pH produced by *S. aureus* and *E. coli* in TSB suggested that the effect of glucose concentration on acid production by these organisms should be determined. The data presented in Figures 2 and 3 show the pH values obtained after 24 and 48 hr growth of these organisms in TSB(-G) with 0–5% glucose. Both organisms produced the minimum pH at 24 hr when grown in TSB(-G) containing 3% glucose [TSB(+3% G)]. Continued incubation for another 24 hr resulted in further decreases

Table III
Composition of TSA, TSALT, PCA, TSB, and TSB(-G)

Ingredients	TSA	TSALT	PCA	TSB	TSB(-G)
Pancreatic digest of casein (Tryptone; Trypticase)	15	15	5	17	17
Papaic digest of soybean meal (Soytone)	5	5	—	3	3
Sodium chloride	5	5	—	5	5
Agar	15	15	15	—	—
Yeast extract	—	—	2.5	—	—
Dextrose (glucose)	—	—	1	2.5	—
Dipotassium phosphate	—	—	—	2.5	2.5
Tween 80 (polysorbate 80)	—	0.5*	—	—	—
Lecithin	—	0.07*	—	—	—

Table values in g per liter of medium.
* Supplements to dehydrated TSA.

in pH when the broth contained ≥4% glucose and slight increases in pH when the broth contained ≤2% glucose.

TSB(+3% G) was selected for further study because both *S. aureus* and *E. coli* produced the lowest pH values in this broth at 24 hr. The effect of culture media on preservative efficacy testing was determined using *S. aureus* and *E. coli* grown on TSALT and in TSB(-G) and TSB(+3% G). No consistent pattern was observed between the pH of the medium at 24 hr and the D-values obtained in challenge testing with these organisms. It is possible that the buffering effect of the dipotassium phosphate in the broth helped prevent the pH from decreasing sufficiently to stress these organisms and alter their performance in preservative efficacy testing.

The results obtained using saline and TSB(-G) inocula in preservative efficacy testing of a cream and lotions C–F are presented in Table IV. The mean D-values obtained with TSB(-G) inocula of *S. aureus* were significantly larger ($p \leq 0.05$) than the mean D-values obtained with saline inocula in all test samples. These results are illustrated in Figure 4. The mean D-values obtained with TSB(-G) inocula of *E. coli* were significantly larger ($p \leq 0.10$) than the mean D-values obtained with saline inocula in the cream and in lotions C and F, directionally larger in lotion D, and essentially the same in lotion E. TSB(-G) was used to preclude the possibility of glucose fermentation that could result in the production of organic acids (and possibly other inhibitory metabolites) that could stress the test organisms.

The data in Table V show the effect of TSB on the D-values obtained in preservative efficacy tests of lotion A with *S. aureus, P. aeruginosa, B. cereus,* and *E. coli*. The mean D-values obtained in samples containing TSB were significantly larger ($p = 0.05$) when challenged with *S. aureus* or *E. coli* and directionally larger when challenged with *P. aeruginosa* or *B. cereus,* compared to the controls. This corroborates a previous report

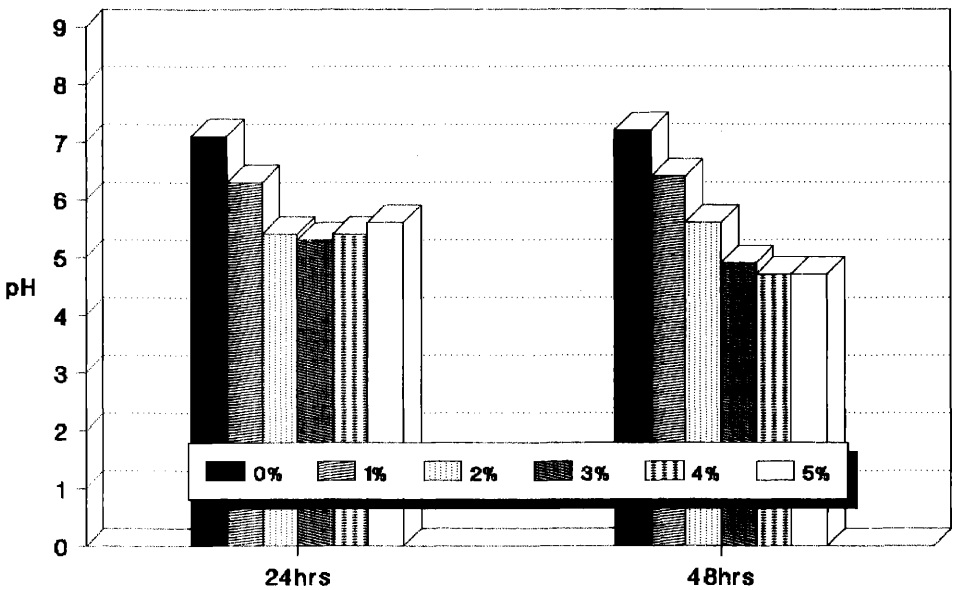


Figure 2. pH of *S. aureus* cultures after growth for 24 and 48 hr in TSB(-G) supplemented with 0–5% glucose.

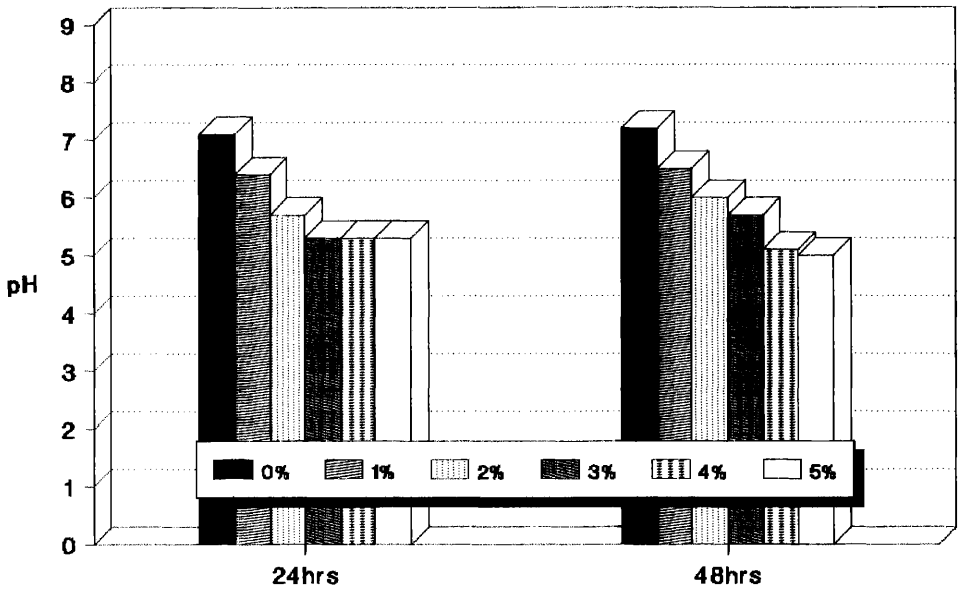


Figure 3. pH of *E. coli* cultures after growth for 24 and 48 hr in TSB(-G) supplemented with 0–5% glucose.

from our laboratory, in which it was observed that the addition of Brain Heart Infusion Broth (BHI) to test samples increased the D-values over those obtained with saline inocula (3).

Table IV
Effect of Growth Medium and Suspending Liquid on D-Values Obtained Using *S. aureus* and *E. coli* in Preservative Efficacy Testing of a Cream and Several Test Lotions

Test organisms	Growth medium/ suspending liquid	Cream		Lotion C		Lotion D		Lotion E		Lotion F	
		D-value	$\bar{X}$	D-value	$\bar{X}$	D-value	$\bar{X}$	D-value	$\bar{X}$		
S. aureus	TSALT/saline	15		20		14		5.4		9.2	
			15*		21**		14*		5.8*		7.9**
		15		22		14		6.2		6.5	
S. aureus	TSB(−G)	60		94		49		10		20	
			63*		86**		52*		10*		20**
		65		78		55		10		19	
E. coli	TSALT/saline	22		55		5.1		11		45	
			21**		58**		5.3		12		44***
		20		60		5.4		12		42	
E. coli	TSB(−G)	47		86		9.0		12		60	
			44**		84**		7.6		11		57***
		40		80		6.2		10		53	

Table values are D-values in hours.
* Mean D-values obtained in TSALT/saline and TSB(-G) were significantly different ($p = 0.01$).
** Mean D-values obtained in TSALT/saline and TSB(-G) were significantly different ($p = 0.05$).
*** Mean D-values obtained in TSALT/saline and TSB(-G) were significantly different ($p = 0.10$).

It is believed that decreases in the rates of inactivation of test organisms during preservative efficacy testing of samples containing TSB or TSB(-G), as reflected by increases in the D-values for these organisms, are due to the inactivation of a portion of the preservative system or to a protective effect of nutrients in the culture medium (3). Moss and Speck (17) reported that peptides present in trypticase, which is a component of TSB and TSB(-G), were responsible for improved recovery of freeze-injured *E. coli*. The effect of components of these broths on repair of sublethally injured bacteria or on D-values was not determined in the current work. These data demonstrate that the addition of broth inocula or sterile broth media to test samples undergoing preservative efficacy testing produces errors in the test results, namely, an increase in D-values, in most cases.

Use of broth inocula constitutes a form of abuse testing (i.e., determining tolerance to the addition of extraneous materials, dilution, or adverse physical conditions). Significant decreases in the rates of inactivation of bacteria were produced with 0.2% broth (0.1 mL/50 mL sample) in these studies. Thus, broth inocula should not be used in routine preservative efficacy testing. A similar recommendation was made in an earlier report from our laboratory (3).

The data presented here illustrate that the culture medium affects the metabolism of bacteria and that the type of inocula (i.e., saline vs. broth) affects the kinetics of microbial death during preservative efficacy testing. The kinetics of microbial death in preservative efficacy testing were discussed by Bean (18) and in reports from this laboratory (3,7,8,19). We are unaware of reports in recent literature that attempt to address the difference in rates of death obtained in preservative efficacy testing of cosmetic or pharmaceutical products using broth-vs.-saline inocula. It is believed that the use of nonstandardized procedures, such as different culture media, growth conditions, and in-

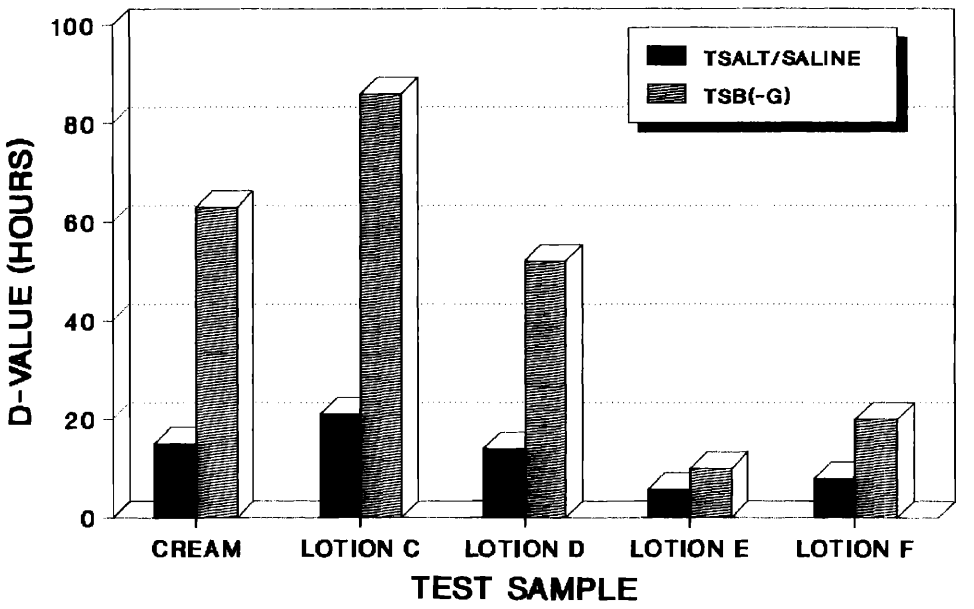


Figure 4. Comparison of D-values obtained in preservative efficacy testing of a cream and four lotions using saline and TSB(-G) inocula of *S. aureus*.

Table V
Effect of TSB on D-values of Test Organisms in Lotion A

Test organism	Addition of 0.1 ml of TSB or sterile deionized water	D-value	$\bar{X}$
S. aureus	Water	6.8	
			6.2*
	Water	5.6	
S. aureus	TSB	12	
			14*
	TSB	15	
P. aeruginosa	Water	0.7	
			0.9
	Water	1.1	
P. aeruginosa	TSB	1.1	
			1.2
	TSB	1.2	
B. cereus	Water	1.5	
			1.3
	Water	1.0	
B. cereus	TSB	1.6	
			1.5
	TSB	1.4	
E. coli	Water	14	
			15*
	Water	16	
E. coli	TSB	24	
			24*
	TSB	24	

Table values are D-values in hours.
* Mean D-values obtained with TSB addition were significantly larger than with water addition ($p = 0.05$).

ocula may help explain the different preservative efficacy test results obtained by different laboratories.

The need for standardized test conditions in antimicrobial evaluation is recognized. Thus, the influence of growth phase of a number of organisms, including *S. aureus* and *P. aeruginosa,* on microdilution susceptibility tests was examined by Barry *et al.* (20). They observed that 18–24-hr surface growth on Blood Agar produced similar results as broth cultures (5–6-hr BHI and 2–4-hr TSB) when the turbidity of the inocula were standardized by adjusting turbidity to match that of a McFarland 0.5 standard. Wicks *et al.* noted the need for controlling the bacterial population of the inoculum within defined limits when conducting antimicrobial susceptibility tests (21). Mayhall and Apollo studied the effect of *S. aureus* growth phase on antibiotic resistance and found that log phase organisms were killed much more rapidly than stationary phase inocula (22).

Although the current studies demonstrated that the growth medium affects the results

of preservative efficacy testing, the rate of inactivation of test organisms is independent of the concentration of organisms introduced in the sample (up to the point at which the preservative system is overwhelmed by the inoculum). This was substantiated by demonstrating that different concentrations of *S. aureus* (from 1.5×10^3 to 1.8×10^6 *S. aureus*/mL) gave similar rates of inactivation in preservative efficacy tests of lotion (3). Thus, use of inocula adjusted to a standard turbidity is not needed for preservative efficacy testing performed by the linear regression method.

Although the studies on the effects of incubation of cultures up to 72 hr used emulsions, it is believed that similar results would be observed with different test organisms in preservative efficacy tests of different samples. Nevertheless, the suitability of using cultures incubated for extended periods should be determined using cosmetic and/or pharmaceutical products of interest in each laboratory. The effect of extended incubation of yeasts and molds was not studied because these organisms typically have slower growth rates than the test bacteria used in this work and because preservative testing generally is performed with cultures that have been incubated for 2–7 days at 20–25°C (1).

The quantitative data on the kinetics of bacterial inactivation obtained by use of the linear regression method often provide investigators with a better understanding of preservative testing than is possible by use of non-quantitative methods. This work demonstrated that culture conditions and the type of inoculum affect the kinetics of bacterial death during preservative efficacy testing. It is evident that the acceptance criteria used in determining the suitability of preservative systems are dependent on the results of test methods used (1–3,8) and that changing methods may necessitate reevaluation of acceptance criteria. It is recommended that test methods be modified to use saline suspensions of test organisms grown on solid agar media rather than broth inocula.

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