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The preservative efficacy testing method for powdered eye shadows

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Synopsis

Preservative efficacy testing is based on a sample inoculation using a microbial suspension with a determined amount of colony-forming units (CFU). After that, the number of survivors is investigated by periodic evaluations, and the results are compared with specifications. When liquid cosmetics are evaluated, it is easy to obtain homogeneity between the inoculum and the sample, but for a powder sample it cannot be guaranteed. In this context, freeze-dried microorganisms could be used to help the homogenization. In this research, the preservative efficacy is evaluated using a powdered eye shadow. The microorganisms used were *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans,* and *Aspergillus niger.* The challenge tests were performed in samples with (P) or without (NP) added preservative. The methods used to evaluate the results were the ones described in the official compendia and in the cosmetics guides of international associations, also using linear regression in calculating the D-value. The results showed that it is possible to use freeze-dried microorganisms instead of suspension to evaluate the preservative efficacy of cosmetic solids. The microorganism stability was verified by the determination of the microbial load and the minimum inhibitory concentration after freeze-drying and during the following six months.

INTRODUCTION

Preservative efficacy testing is an essential part of substantiating the safety of cosmetic products. The correct use of preservatives protects the product against contamination while it is in trade channels and in the hands of the consumer (1–4). When consumers use cosmetic products, they repeatedly challenge the cosmetic with microorganisms in saliva, on dirty hands, and in tap water (5).

In this context, preservative efficacy must be evaluated to assure the product's safety. The methodology for the evaluation of preservative adequacy is described in the official compendia for pharmaceuticals (6–9). It is also given in cosmetic guides such as those of the Cosmetic, Toiletry and Fragrance Association (CTFA) (10) and the American Society for Testing and Material (ASTM) (5). All methods are applied to liquid and semi-solid products, and they are based on the challenge test, which consists in the contamination of the product by fresh pure cultures of microorganisms, suspended in saline, followed by periodic evaluations.

The CTFA guidelines (10) describe methods for testing eye area cosmetics. The inocu-

lation method of non-aqueous eye products such as loose and pressed powders consists of spraying on or adding microbial suspensions to the cosmetic, followed by mixing. Pressed eye shadows can also be tested by swabbing or spreading an inoculum on the product surface (10–12). There has also been developed a method to evaluate the surface of pressed powders; in this method the test organisms on membrane filters are placed in direct contact with the products.

The inoculation of microbial suspensions in powders described by the CTFA (10) is a procedure similar to the official ones for pharmaceuticals and cosmetics. However, it is difficult to guarantee homogeneity when mixing a microorganism suspension with a powder, since the volume of the inoculum must be minimal (not more than 1% of the total sample amount).

The use of solid inoculum could facilitate the homogeneity between the microorganism and the sample. In this study a solid inoculum was obtained by using the process of freeze-drying.

The efficacy preservative testing period is too long, since it lasts 28 days. There is an alternative method that has been used (13,14) that involves the microorganism death curve and the determination of the decimal reduction time (D-value), which is the time required for the reduction of 90% of the microorganisms. This curve can be constructed by determining the number of surviving microorganisms after 2, 4, and 24 hours for bacteria, and 4, 8, and 24 hours for fungi.

The aim of this study was to evaluate the use of freeze-dried microorganisms to inoculate eye-shadow samples in preservative efficacy testing. In order to reach this goal, the results obtained were compared to the specifications in all the official compendia mentioned above, to those of the CTFA, and also to the determination of the D-value.

EXPERIMENTAL

TEST ORGANISMS

The test organisms were *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16 404.

STABILITY EVALUATION OF FREEZE-DRIED MICROORGANISMS

Microbial suspensions were obtained as described in the *US Pharmacopoeia 24* (6). Suspension drops of the inocula were used in order to have about 10^7 CFU/vial. They were transferred to five vials, and 1.0 ml of Molico® skim milk (Nestlé) with 5% inositol (DIFCO) was added. The samples were frozen at −70°C, and the freeze-drying process began at −55°C using a Supermodulyo 12K® (Edwards) freeze-dryer. The vials were closed with rubber stoppers. The total aerobic count was determined by the plate count technique, using the five vials (6). These determinations were made immediately and one week, 15 days, and one, two, four, five, and six months after the freeze-drying process.

MICROORGANISM RESISTANCE EVALUATION BY MINIMUM INHIBITORY CONCENTRATION (MIC)

The preservatives used were methylparaben, propylparaben, and a combination of DMDM hydantoin and iodopropinyl butyl carbamate (Glydant plus®). The determination was made by using the broth dilution technique in a 96-well microtiter plate (15).

CHALLENGE TEST (10)

The sample used was a powdered eye shadow with (P) and without (NP) preservative. The amount of 15 g of the sample was added gradually to the inocula (one-vial content of freeze-dried mass) and mixed after each addition. At least six tests were performed for each microorganism, and also for each kind of sample. The bioburden was determined by the plate count technique, according to the CTFA method (10). The number of colony-forming units (CFU)/g was determined immediately at 1, 2, 7, 14, 21, and 28 days after inoculation.

DETERMINATION OF D-VALUE

The procedure of inoculation was the same as that described above. The number of CFU/g was determined after 2, 4, 24, and 48 hours for bacteria and after 4, 8, 24, and 48 hours for fungi. The death curve was constructed, and the D-value was calculated by taking the negative reciprocal of the slope of the line obtained by linear regression of the plot of the log number of surviving microorganisms as a function of time after inoculation into the test sample.

RESULTS AND DISCUSSION

Loss of viability occurred after the freeze-drying process for all tested microorganisms except *C. albicans* (Figure 1).

During six-month storage of the vials in the refrigerator, at around 5°C, oscillations occurred. Besides that, the amount of microorganisms remained at about 10^6 to 10^7 CFU/vial, the suitable load to use in the preservative efficacy test (6–10).

Freeze-dried inocula have been especially developed to contaminate products such as oils (16). Although some modifications, such as the lack of plasmids (10,17), can occur after the lyophilization process, this process is still the best one to maintain microorganism

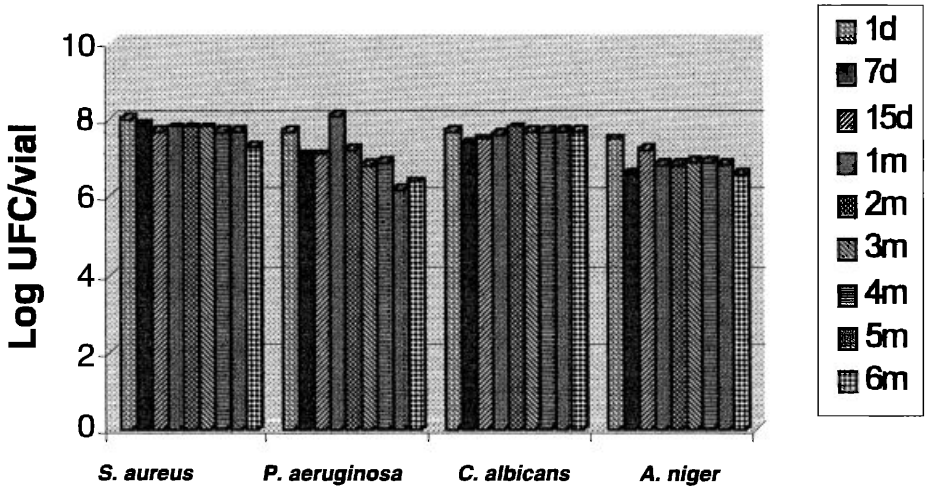

Figure 1. Freeze-dried microorganism viability during six months. d: days. m: months.

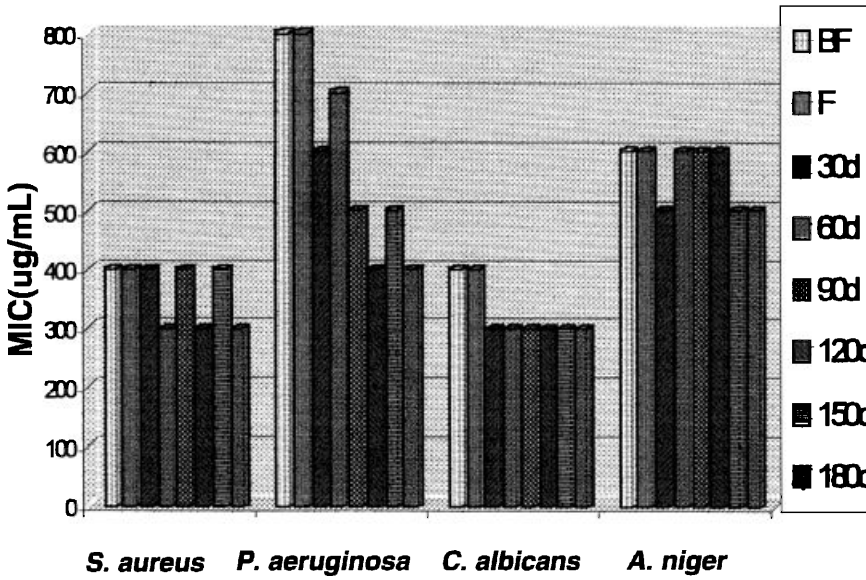

Figure 2. Minimum inhibitory concentration (μg/ml) of methyl paraben during six months. BF: Before freeze-drying. F: After freeze-drying. d: days.

culture. Moreover, the culture of microorganisms can also suffer some modification of characteristics (10,18).

The minimum inhibitory concentration (MIC) values for methyl paraben (Figure 2) and for Glydant plus® (Figures 3 and 4) were the same before and after the freeze-drying process. This performance indicated that the microorganisms did not lose resistance after the process. Propyl paraben also presented this behavior, except for the *A. niger.* Furthermore, all the results (Figures 2, 3, 4, and 5) showed variability from one month

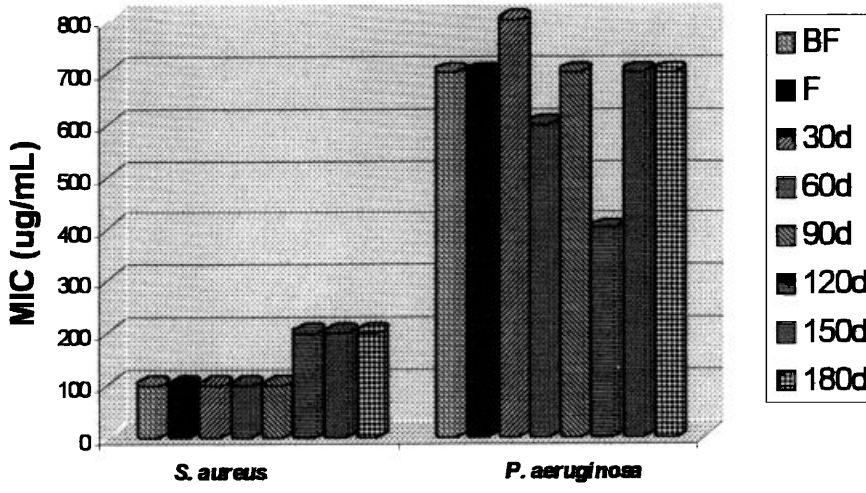

Figure 3. Minimum inhibitory concentration (μg/ml) of Glydant plus® for bacteria during six months. BF: Before freeze-drying. F: After freeze-drying. d: days.

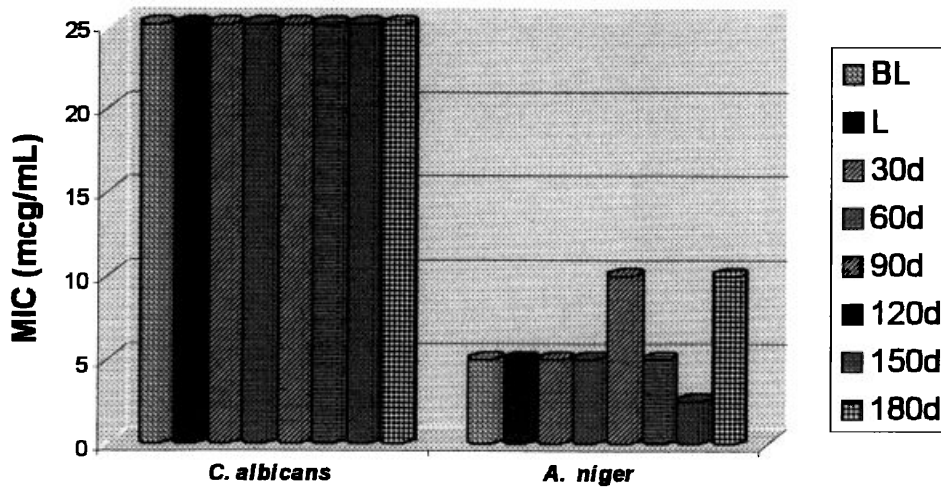

Figure 4. Minimum inhibitory concentration (μg/ml) of Glydant plus® for fungi during six months. BF: Before freeze-drying. F: After freeze-drying. d: days.

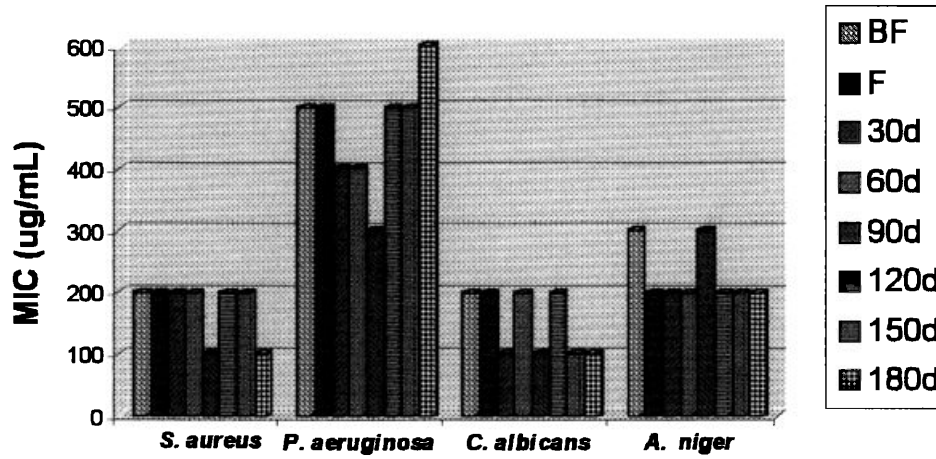

Figure 5. Minimum inhibitory concentration (μg/ml) of propyl paraben during six months. BF: Before freeze-drying. F: After freeze-drying. d: days.

to another, which could be attributed to the method, since the oscillations were unimportant.

The adequate support agent must be used to increase the survival level, and to protect the microorganisms against damage that could occur during the freeze-drying process (19). In this research, skim milk added to inositol was used since it is known to be a good agent to preserve the viability of microorganisms (20).

It would be possible to maintain a better viability for the freeze-dried microorganisms. Some authors demonstrated that the use of ampoules instead of vials could show better results (17,21). The option of using vials was adopted in order to make the process easier, but the study of freeze-dried microorganisms in ampoules is being performed. The microorganisms were tested by the linear regression method (13) and by the official ones (6–10).

Table I
D-Values and Correlation Coefficients of the Preservative Efficacy Test for Bacteria

	S. aureus				*P. aeruginosa*			
	Preservative		No preservative		Preservative		No preservative	
Test	D-value	R	D-value	R	D-value	R	D-value	R
1	12.0	0.9938	57.8	0.9603	13.1	0.9999	16.3	0.9751
2	14.9	0.9963	—	0.7309	9.8	0.9892	13.1	0.9805
3	16.1	0.9997	133.3	0.9924	13.2	0.9472	14.8	0.9897
4	14.5	0.9993	—	0.6423	22.1	0.9570	13.9	0.9927
5	13.7	0.9635	294.1	0.9896	14.3	0.9978	14.1	0.9813
6	10.1	0.9990	—	0.4672	13.9	0.9921	18.2	0.9912
M ± SD	13.6 ± 1.97		161.7 ± 98.54		14.4 ± 9.15		15.1 ± 4.2	

M ± SD: Mean ± standard deviation.

Table II
D-Values and Correlation Coefficients of the Preservative Efficacy Test for Fungi

	C. albicans				*A. niger*			
	Preservative		No preservative		Preservative		No preservative	
Test	D-value	R	D-value	R	D-value	R	D-value	R
1	4.6	0.9420	—	0.0458	—	0.000	—	0.1726
2	4.3	0.9706	—	0.2421	—	0.000	—	0.4079
3	4.4	0.9886	—	0.0400	—	0.8891	—	0.8807
4	4.0	0.9888	—	0.1095	—	0.2121	—	0.3375
5	7.3	0.9011	21.6	0.9199	—	0.4064	—	0.1300
6	6.8	0.9846	—	0.8323	—	0.8755	—	0.5704
7	3.8	0.9946	19.9	0.9942	—	0.0922	NE	NE
8	4.4	0.9960	25.2	0.9700	NE	NE	NE	NE
M ± SD	4.95 ± 3.51		22.23 ± 3.83		—		—	

NE: Not executed.
M ± SD: Mean ± standard deviation.

It was possible to determine the average D-values for *S. aureus* and *C. albicans* in samples with preservative, but samples without preservative did not have good correlation coefficients (Tables I and II). Mean D-values were 13.6 hours and 4.95 hours, respectively. Nevertheless the D-values showing good correlation, for both cases presented lower rates when using the preservative compared with the others.

The analysis of variance for the two different treatments (with and without preservative) for *S. aureus* confirms that samples with preservative presented different behavior compared to samples without preservative, providing an F-value of 10.54 and a critical F-value of 5.11. For *C. albicans,* the F-value was 217.51 and the critical value was 5.12, showing again that the two different groups are different (Table III).

For *P. aeruginosa* (Table I), the average D-values were almost the same for the tests with and without preservative: 14.4 hours and 15.1 hours, respectively. The comparison between the two treatments showed no difference, providing an F-value of 0.131 and a

Table III
Statistics Data Comparing D-Values in Preserved and Unpreserved Powders

Microorganism	F-value	Critical F-value
Staphylococcus aureus	10.54417	5.59146
Pseudomonas aeruginosa	0.131475	4.964591
Candida albicans	217.5148	5.117357

critical F-value of 4.96 (Table III). However, in Figure 6, one can observe that the curves constructed for the tests with preservative presented a steeper slope (i.e., faster die-off) than the ones for the tests without preservative. Such behavior can also be seen for *S. aureus* and *C. albicans* (Figures 6 and 7).

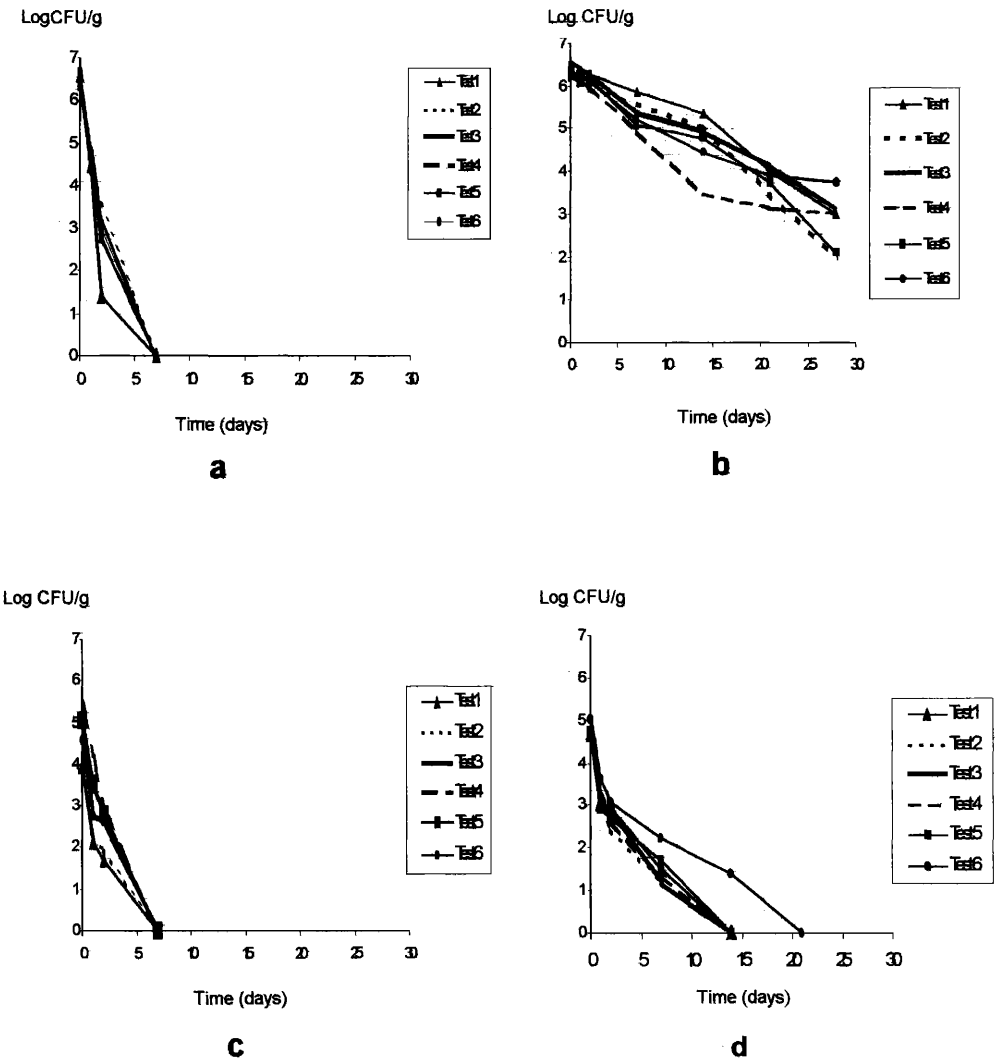

Figure 6. Survival curve of bacteria in the sample with preservative (P) and without preservative (NP). (a) *S. aureus,* sample P. (b) *S. aureus,* sample NP. (c) *P. aeruginosa,* sample P. (d) *P. aeruginosa,* sample NP.

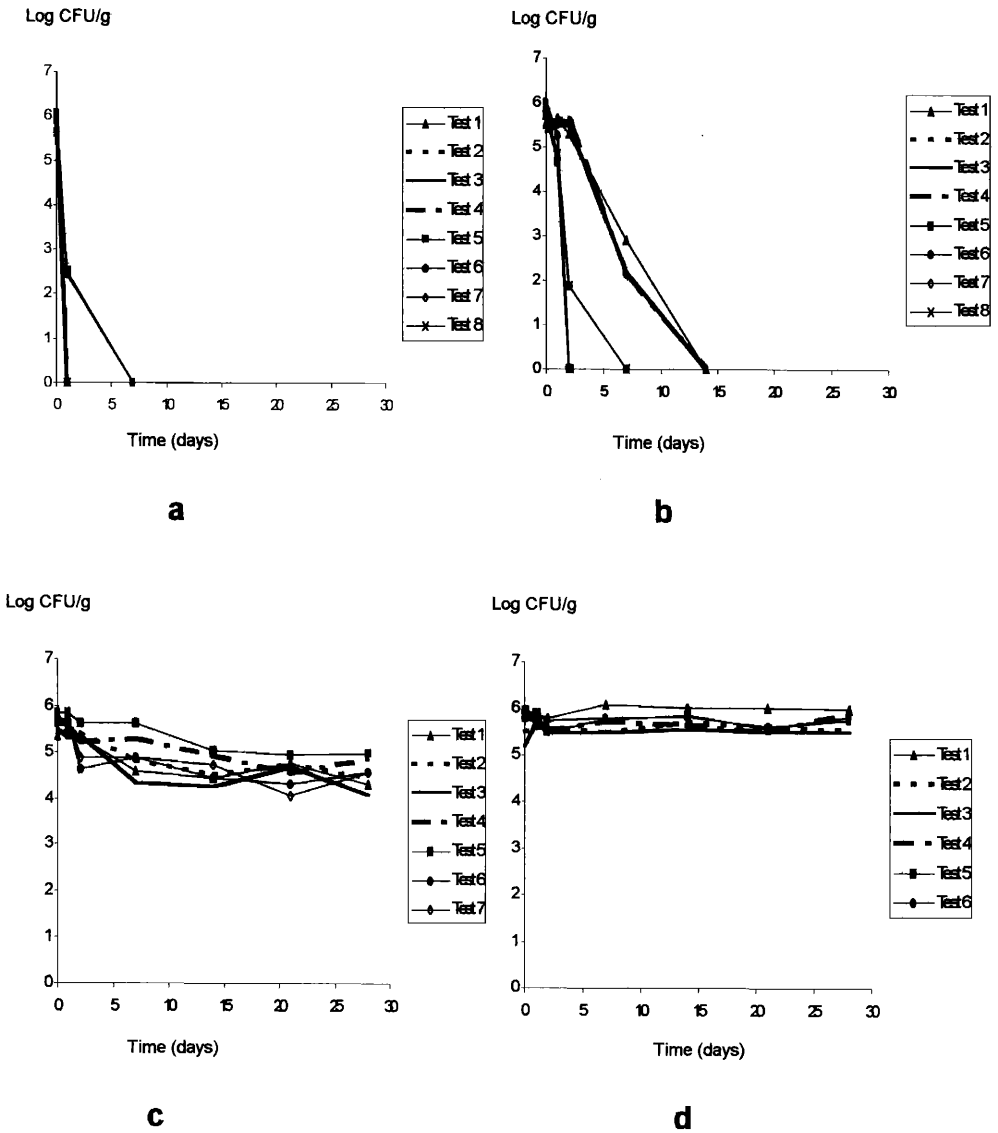

Figure 7. Survival curve of fungi in the sample with preservative (P) and without preservative (NP). (a) *C. albicans,* sample P. (b) *C. albicans,* sample NP. (c) *A. niger,* sample P. (d) *A. niger,* sample NP.

One exception was noted in the curves for *A. niger* (Figure 7) that presented similar behavior for both treatments. These data indicate that the preservative in the powder was not effective against this organism. The same behavior can be noticed in Table II. The D-value average and the analysis of variance could not be calculated because of the low correlation coefficients.

The results obtained against *S. aureus* in both samples (with and without preservative) indicated that the antimicrobial activity was effective according to the US and the Japanese pharmacopoeia requirements (6,9). However compared to the British and European pharmacopoeias (7,8) and the CTFA (10) requirements, just the preserved sample

Table IV
Regression Analysis for All Tested Microorganisms Using Sample With Preservative (P) and Without Preservative (NP) (y = a + bx)

Microorganism	a	p (a)	t (a)	b	p (b)	t (b)	R (%)
S. aureus (P)	6.98	0.000	73.71	(0.082)	0.000	(20.94)	96.95
S. aureus (NP)	6.96	0.000	70.10	(0.005)	0.000	(16.48)	91.60
P. aeruginosa (P)	6.64	0.000	45.06	(0.072)	0.000	(11.82)	91.13
P. aeruginosa (NP)	5.98	0.000	31.71	(0.002)	0.000	(9.48)	84.85
C. albicans (P)	6.43	0.000	26.43	(0.191)	0.000	(6.55)	80.01
C. albicans (NP)	7.07	0.000	39.28	(0.025)	0.000	(7.89)	78.04
A. niger (P)	6.88	0.000	117.75	(0.002)	0.000	(10.36)	79.81
A. niger (NP)	7.02	0.000	153.88	(0.000)	0.775	(0.29)	4.47

was effective. Homogeneous results were again observed with *P. aeruginosa* and *C. albicans.* The samples with or without preservative were approved by all tests according to these specifications.

When the sample with preservative was challenged with *A. niger,* the tests were accepted by the US and Japanese pharmacopoeias (6,9) and not approved by the other compendia previously mentioned. The sample without preservative did not pass the criteria of the British and European pharmacopoeias and the CTFA (7,8,10).

All the results discussed above indicated that the tests for each kind of sample, for each kind of microorganism, presented similar behavior. Table IV shows a regression using all the tests for each kind of sample (P and NP), and also for each microorganism. The *t* values for "a" and "b" indicated that good models, describing the regression, were obtained. Added to that, one can observe different values of "a" and "b," mainly "b," between the P and NP samples. These results are in agreement with the ones shown in Figures 6 and 7.

CONCLUSIONS

It can be concluded that it is possible to use freeze-dried microorganisms as the inoculum in efficacy preservative tests for solid cosmetics. Added to that, all the microorganisms tested can be used to inoculate the sample during the first six months, once they maintain viability between 10^5 and 10^6 UFC/vial and their resistance.

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