THE EFFECT OF FREEZE-DEHYDRATION ON THE SURVIVAL OF CERTAIN PSYCHROTROPHIC BACTERIA IN SKIMMILK, ICE CREAM MIX SUBSTITUTE AND COTTAGE CHEESE[1]

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SUMMARY

Studies on the effect of freeze-dehydration on the survival of psychrotrophic bacteria in milk and ice cream mix substitute showed that a species of *Achromobacter* was relatively resistant as compared to five cultures of *Pseudomonas*. The effect of freezing on the viable population varied greatly not only between cultures but also within cultures from one sampling period to another. In some instances increases in viable count were observed after freezing. The effect of freeze-dehydration on the cultures in ice cream mix substitute was in many aspects similar to that observed in skimmilk. Increases in viable count took place when freeze-dehydrated milk cultures of F11 and 54 were rehydrated and stored at 5.5 C. However, a further reduction in viable count took place in samples stored in the dehydrated form. Freeze-dehydration of milk cultures with different concentrations of bacteria did not show a definite pattern in change of viable population with cell concentration.

Freeze-dehydration of cottage cheese contaminated with cultures F11 and 54 caused an extensive reduction of the number of viable bacteria. When dehydrated samples were rehydrated and stored at 5.5 C no extensive changes in viable count occurred. In samples stored dry, however, there were further reductions in viable count. The effect of freeze-dehydration on culture 54 in cottage cheese was different from that observed in milk or ice cream mix substitute. Preliminary experiments suggest that the method used to enumerate this organism may be responsible for this phenomenon.

It is known that many species of the natural microbial flora of various foods can survive commercial freeze-dehydration *(13, 16)*. An examination of eight commercial freeze-dehydrated foods *(13)* showed that the survivors were representative of the natural flora of each product and of the contaminants introduced during handling and processing. Few studies, however, have examined the fate of microbial population in a freeze-dehydrated food during storage and the type and extent of microbial growth following rehydration and subsequent storage. May and Kelly *(8)* determined the survivors of the natural flora of chicken meat after freeze-dehydration and rehydration at room temperature for 30 minutes and at 50, 85, and 100 C for 10 minutes. Approximately 32% of the bacteria in the meat survived during dehydration and rehydration at room temperature. *Staphylococcus aureus* survived dehydration and rehydration at 60 C. Pablo et al. *(11, 12)* showed that the growth pattern of rehydrated freeze-dehydrated chicken and shrimp was greatly influenced by the level of initial population and the temperature and time of subsequent storage. After freeze-dehydration the microbial flora was essentially mesophilic. Upon storage at 4 C, there was a shift from a mesophilic to a psychrotrophic flora. *S. aureus* and fecal enterococci grew in competition with the natural flora at 20 C or above, while no increase occurred at 4 C. Lauro et al. *(7)* contaminated sterilized peas on the surface with *Serratia marcescens, Bacillus subtilis, Bacillus stearothermophilus, Saccharomyces cerevisiae* and *Lactobacillus fermenti.* The only significant change in viability due to freeze-dehydration was 1 to 2 log reductions in count with *S. cerevisiae* and *L. fermenti.* All freeze-dehydrated products showed decreases in viable population during storage at room temperature.

This study was initiated to determine the effect of freeze-dehydration and subsequent storage conditions on the survival of certain psychrotrophic bacteria in milk and certain milk products.

EXPERIMENTAL METHODS

Preparation of skimmilk, ice cream mix substitute and cottage cheese.

Skimmilk was prepared by recombination (9%) of low-heat nonfat dry milk solids with distilled water. The skimmilk was heated for 15 minutes at 121 C and 15 lb steam pressure. A sterile ice cream mix substitute containing 10% fat, 11% milk solids-not-fat, and 15% sucrose was made by mixing appropriate amounts of evaporated milk, sterile cream (30% fat), sucrose and sterile distilled water. Prior to mixing with the other ingredients, the sucrose was sterilized by flooding with diethyl ether. The ether was removed by evaporation after 24 hours. The preparation of the mix was carried out under aseptic conditions. Before use, the mix was examined for sterility by the agar plate method.

Small curd cottage cheese was manufactured from fresh skimmilk by a short-set method. High quality milk from the University dairy was separated in a sterile laboratory separator. The milk was pasteurized at 63.3 C for 30 minutes.

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The setting temperature was 32.2 C. The cheese was made in a sterile 2-gallon plastic container under aseptic conditions. The equipment which came in contact with the milk or curd was sterilized by heat. A commercial lactic culture was used. The rennet was filter-sterilized. The curd was cooked for one hour during which time the temperature was raised gradually from 32.2 to 48.9 C. Sterile distilled water (3 washes) was used as wash water. The curd was stored overnight at 5.5 C in sterile cheese cloth within a sterile glass cylinder.

Preparation of samples for freeze-dehydration.

The inoculated samples (2 ml) of skimmilk and ice cream mix substitute were placed into preweighed sterile cups made from heavy duty aluminum foil. The cups were 20 mm in height and 15 mm in diameter. The weight of the sample was determined by weighing the cup and contents. The cups then were placed at −20 C for 2 hours. While in the cold room, just prior to placing in the freeze-dryer, the aluminum was removed under aseptic conditions. The sample then was placed on a piece of sterile aluminum window screen in a weighed sterile aluminum moisture dish (height 15 mm, diameter 60 mm). This dish was placed in a sterile petri dish which had a paper clip attached to the rim of the bottom to permit the escape of vapor. The entire ensemble then was placed in the freeze-dryer. The preparation of the cottage cheese samples was essentially the same as that for skimmilk except that a larger sample (20 g) was weighed into a sterile aluminum moisture dish.

Freeze-dehydration was performed in an Industrial Dynamics pilot plant Model CPF-20 freeze-dryer. The two programs used in this study were *(a)* a platen temperature of 40 C throughout the drying cycle, and *(b)* an initial platen temperature of 30 C with a progressive increase in temperature up to about 105 C which was then successively lowered to 40 C, to prevent the product surface temperature from rising about 40 C. For this purpose thermocouples were inserted into the surface of the product and attached to the temperature controller of the dryer. The chamber pressure was 0.1 mm Hg. Dehydration rates were established by weighing the samples at various intervals during the dehydration cycle. The cycle was ended when the residual percent of initial moisture was below 3. The dehydration time for skimmilk and cottage cheese was 8 hours, for ice cream mix substitute 4 hours. Preliminary studies showed that under these conditions of freeze-dehydration, the dehydrated products retained their rigidity, rehydrated easily and did not show off-flavors when examined organoleptically. The results of this phase of the study will be reported elsewhere. The total solids content of the samples was determined according to *Standard Methods (1).*

Cultures and inoculation of samples.

Cultures F11, FLE, FE, F01, P10 and 54 were from the stock culture collection of the Department of Animal Science. They were isolated from milk and milk products and were maintained on slants of Standard Methods agar *(1).* All cultures were examined for various cultural, morphological and physiological characteristics as outlined in the *Manual of Microbiological Methods (17).* The cultures were gram-negative rods, and were polarly flagellated, except for culture 54 which had peritrichous flagella. All cultures utilized carbohydrates oxidatively *(6).* Except for culture 54, all test cultures *(a)* grew on Olson's medium *(10), (b)* were oxidase positive *(18), (c)* produced NH_3 from arginine under anaerobic conditions *(19), (d)* were resistant to 2.5 I.U. of penicillin, and *(e)* did not grow on Staphylococcus medium 110. All test cultures were sensitive to streptomycin, oxytetracycline and chloramphenicol. With respect to growth temperature, all cultures grew at 5 C. Cultures F11 and FLE produced fluorescin. On the basis of identification schemes for gram-negative organisms *(4, 5, 14, 15, 18, 19, 20)* cultures F11 and FLE were tentatively identified as *Pseudomonas* type I, cultures F01, FE and P10 as *Pseudomonas* type II, and culture 54 as an *Achromobacter* species.

For the individual experiments, the cultures were grown for 18 to 20 hours at 25 C either in sterile skimmilk or ice cream mix substitute. A 1% inoculum was used for all test samples. Immediately following inoculation, samples (2 ml) were prepared for freeze-dehydration. The inoculated test samples then were incubated for 10 days at 5.5 C. The sampling, freezing, freeze-dehydration and plating procedures described for 0-day were repeated after 3, 5, and 10 days of incubation.

Determination of the viable count.

In milk and ice cream mix substitute the number of viable bacteria before freezing, after freezing and after freeze-dehydration with or without subsequent holding in the dried or rehydrated state was determined by the agar plate method according to *Standard Methods (1).* The plates were incubated for 48 hours at 32 C. In cottage cheese the viable count of culture F11 was determined by surface plating of 0.1 ml aliquots of appropriate dilutions of the cheese on Olson's medium. The initial dilution was prepared by mixing 11 g of cottage cheese or rehydrated product with 99 g of sterile distilled water in a sterile blender. The same procedure was used for culture 54, except that Staphylococcus medium 110 was used as the plating medium *(5, 21).* The plates with Olson's medium were incubated at 32 C for two days, those with Staphylococcus medium 110 for 5 days. Duplicate plates were used in all experiments.

RESULTS AND DISCUSSION

Table 1 shows the effect of freezing and freeze-dehydration (platen temperature from 30 to 104.4 C) on the viable population of the six test cultures. In another series of experiments a constant platen temperature of 40 C was employed. Except for culture 54, freeze-dehydration at both platen temperature programs reduced the viable population of the cultures over 95 percent. There was little difference in the effect of the two programs on the percent reduction in viable population. Culture 54 was more resistant to freeze-dehydration at both platen temperature programs. The reduction in viable population by freezing varied greatly not only between cultures but also for the same culture from one sampling period to the other. In some cases (7 out of 24), particularly with culture P10, increases in viable count were observed after freezing. In others, for example culture FE (3-10 days) freezing had little effect on the viable count.

In a similar series of experiments, samples of ice cream mix substitute were inoculated with the test cultures and incubated for 10 days at 5.5 C. In one trial a platen temperature of 40 C was used, in the

TABLE 1. THE EFFECT OF FREEZING AND FREEZE-DEHYDRATION[a] ON THE VIABLE POPULATION OF MILK CULTURES OF F11, FO1, FLE, FE, P10, AND 54

Cultures	Plated[b]	Age of culture: 0d	% kill	3d	% kill	5d	% kill	10d	% kill
	BF	$58x10^4$		$65x10^5$		$52x10^6$		$140x10^9$	
F11	AF	$130x10^3$	78	$140x10^4$	78	$230x10^5$	56	$190x10^6$	+36[c]
	AFD	$48x10^2$	>99	$<30x10^2$	>99	$83x10^2$	>99	$97x10^4$	>99
	BF	$290x10^3$		$50x10^5$		$140x10^5$		$59x10^8$	
FO1	AF	$200x10^3$	31	$90x10^5$	+80	$98x10^5$	30	$38x10^8$	36
	AFD	$30x10^2$	99	$<30x10^2$	>99	$150x10^2$	>99	$96x10^5$	>99
	BF	$36x10^5$		$63x10^4$		$51x10^5$		$40x10^8$	
FLE	AF	$100x10^3$	97	$180x10^3$	71	$54x10^4$	89	$32x10^8$	20
	AFD	$<30x10^2$	>99	$<30x10^2$	>99	$<30x10^2$	>99	$110x10^2$	>99
	BF	$90x10^4$		$71x10^5$		$91x10^5$		$170x10^8$	
FE	AF	$49x10^4$	46	$70x10^5$	1	$91x10^5$	0	$170x10^8$	0
	AFD	$160x10^2$	98	$<30x10^2$	>99	$140x10^2$	>99	$78x10^4$	>99
	BF	$34x10^4$		$30x10^5$		$210x10^5$		$170x10^8$	
P10	AF	$45x10^4$	+32	$66x10^5$	+120	$38x10^6$	+81	$210x10^8$	+24
	AFD	$77x10^2$	98	$31x10^2$	>99	$140x10^2$	>99	$72x10^4$	>99
	BF	$160x10^3$		$100x10^4$		$210x10^4$		$210x10^5$	
54	AF	$120x10^3$	25	$83x10^4$	17	$33x10^5$	+57	$170x10^5$	19
	AFD	$69x10^3$	57	$270x10^3$	73	$67x10^4$	68	$67x10^5$	68

[a]The platen temperature ranged from 30 to 104.4 C.

[b]The samples were plated before freezing (BF), after freezing (AF) and after freeze-dehydration (AFD).

[c]Increase in viable population.

other the platen temperature ranged from 30 to 101.7 C. The effect of freezing and freeze-dehydration on the cultures in ice cream mix substitute was in many aspects similar to that observed in skimmilk. Culture 54 was more resistant to freeze-dehydration than the other test cultures. The reduction in viable count of culture 54 in ice cream mix substitute was in most instances somewhat less than in milk. In ice cream mix substitute the viable count increased after freezing in 13 out of 24 cases, in milk in only 7 out of 24 cases. Except for culture 54, freeze-dehydration of the cultures in ice cream mix substitute usually caused large reductions (over 90 percent) in viable count. Exceptions were *(a)* 10-day old cultures of FE and F11 and *(b)* a 5-day old culture of FO1.

Numerous studies have shown that different microbial species or different strains of the same species can vary greatly in their sensitivity to freezing. In the present study too, the sensitivity of freezing of cultures of *Pseudomonas* differed greatly in many cases. The same was true for cultures of the same species but of different ages. Differences in the phase of growth and cell concentration may be responsible in part for this observation. Increases in viable count after freezing may be caused by a disruption of clusters which would increase the viable count by the plating technique. With respect to the observed reductions in viable count (percent kill), it should be pointed out that it is not certain that either freezing or freeze-dehydration has destroyed the multiplication mechanisms of the cell. It is possible that, under a different set of conditions with respect to nutrients, temperature and time of incubation, an increased number of survivors could have been observed *(2, 3)*.

Table 2 shows the effect on the viable population of storing freeze-dehydrated milk cultures F11 and 54 in the dry and rehydrated state. Cultures F11 and 54 were selected to represent two types of cultures namely sensitive and relatively resistant to freeze-dehydration. Following inoculation, the skimmilks were stored at 5.5 C for 5 and 10 days. In this manner, studies could be made on cultures at two age levels. Immediately after freeze-dehydration, some of the samples were rehydrated by adding sterile distilled water. The amount added was the same as that removed during freeze-dehydration. The rehydrated samples were stored in separate sterile containers at 5.5 C. The viable count was determined after 1, 3, 5, and 7 days of storage. The remainder of the freeze-dehydrated samples were stored at 25.5 C in separate sterile containers for up to 4 weeks. The viable count of these samples was determined after 3, 7, 14, 21, and 28 days. Just before plating, the

TABLE 2. THE EFFECT OF HOLDING ON THE VIABLE POPULATION OF FREEZE-DEHYDRATED[a] MILK CULTURES OF F11 AND 54 WHEN STORED IN REHYDRATED AND DRIED FORMS

Culture and age	Plated[b]		Rehydrated and stored (5.5 C)				Stored dry at 25.5 C and then rehydrated				
	BF	AFD	1d	3d	5d	7d	3d	7d	2wk	3wk	4wk
F11 5d	110×10^7	160×10^5	180×10^5	79×10^7	38×10^8	140×10^8	220×10^2	140×10^2	$<30\times10^2$	$<30\times10^2$	
	% kill	99					>99	>99	>99	>99	
F11 10d	300×10^7	82×10^6	68×10^6	46×10^7	170×10^8	39×10^8	c				
	% kill	97									
54 5d	45×10^8	150×10^5	210×10^5	55×10^6	56×10^7	43×10^7	86×10^4	33×10^3	$<30\times10^2$		
	% kill	67					98	>99	>99		
54 10d	66×10^6	290×10^5	47×10^6	42×10^7	89×10^7	32×10^7	240×10^4	120×10^3	65×10^4	270×10^4	260×10^3
	% kill	56					96	>99	>99	96	>99

[a]The platen temperature ranged from 30 to 104.4 C.

[b]The samples were plated before freezing (BF) and after freeze-dehydration (AFD).

[c]Sample was completely proteolyzed.

TABLE 3. THE EFFECT OF CELL CONCENTRATION ON THE SURVIVAL OF CULTURES F11 AND 54 AFTER FREEZE-DEHYDRATION[a]

Culture	Plated[b]	Concentration of cells				
		A	B	C	D	E
F11	BF	51×10^7	52×10^6	43×10^5	46×10^4	37×10^3
	AFD	170×10^5	170×10^4	36×10^3	260×10^2	34×10^2
	% kill	97	97	>99	94	91
54	BF	81×10^6	110×10^5	100×10^4	140×10^3	110×10^2
	AFD	37×10^6	40×10^5	45×10^4	37×10^3	35×10^2
	% kill	54	64	55	74	68

[a]The platen temperature ranged from 30 to 104.4 C.

[b]The samples were plated before freezing (BF) and after freeze-dehydration (AFD).

samples were rehydrated as described before. As observed previously, culture 54 was more resistant to freeze-dehydration than culture F11. An increase in viable count was observed during storage of the rehydrated samples. A further reduction in viable count took place in the samples stored in the dehydrated form. A similar observation was made by Lauro et al. *(7)* with various microbial species on peas and by Baird-Parker and Davenport *(3)* with S. *aureus* in freeze-dehydrated milk. Extensive proteolysis took place in culture F11 after incubation for 10 days at 5.5 C. There was little material left after freeze-dehydration which made recovery of these samples for experiments on the effect of storage in the dehydrated form impractical.

Table 3 shows the effect of freeze-dehydration on the viable count of cultures F11 and 54 at different levels of cell concentration. A 20-hour milk culture of each was employed. Five 10-fold dilutions (A-E) were prepared with sterile skimmilk. No definite pattern could be detected in the reduction of the viable count with respect to cell concentration.

A study was made of the effect of freeze-dehydration on psychrotrophic bacteria in cottage cheese. For this purpose, cottage cheese was prepared under aseptic conditions as described in the experimental methods section. Cultures F11 and 54 were grown on slants of plate count agar at 5.5 C for 7 days. The growth was removed from the slants with cold sterile distilled water. Contamination of the curd was achieved either by *(a)* inoculation of the milk immediately after addition of the starter and rennet, or *(b)* by adding the bacteria to the sterile wash water. Contamination with the wash water was carried out at a high (Experiment 1) and a low level (Experiment 2).

In each series of experiments a control batch of cottage cheese was prepared from the same milk but

TABLE 4. THE EFFECT OF REHYDRATION AND STORAGE ON THE VIABLE COUNT[a] OF COTTAGE CHEESE CONTAMINATED WITH CULTURE F11

Exp.	BF	AFD[b]	Rehydrated and stored (5.5 C) for 1d	3d	5d	7d	Stored dry (25.5 C) and rehydrated after 3d	7d	2wk	3wk	4wk
					F11 in washing water						
1	60×10^4	30×10^2	230×10^2	160×10^2	190×10^2	180×10^2	14×10^2	18×10^2	<10	–	–
	% kill	>99							>99		
2	130×10^2	3×10^2	2×10^2	22×10^2	29×10^2	17×10^2	19×10^2	38×10^2	<10	–	–
	% kill	98							>99		
					F11 in the milk						
1	70×10^3	59×10^2	33×10^2	18×10^2	22×10^2	35×10^2	49×10^2	26×10^2	14×10^2	<10	<10
	% kill	92								>99	>99

[a]The count per g of cottage cheese was determined before freezing (BF) and after freeze-dehydration (AFD).
[b]The platen temperature ranged from 30 to 104.4 C.

TABLE 5. THE EFFECT OF REHYDRATION AND STORAGE ON THE VIABLE COUNT[a] OF COTTAGE CHEESE CONTAMINATED WITH CULTURE 54

BF	AFD[b]	Rehydrated and stored (5.5 C) for 1d	3d	5d	7d	Stored dry (25.5 C) and rehydrated after 3d	7d
			Culture 54 in washing water				
150×10^4	12×10^2	14×10^2	23×10^2	25×10^2	<10	2×10^2	<10
% kill	>99					>99	>99
			Culture 54 in the milk				
110×10^2	2×10^2	1×10^2	5×10^2	<10	<10	1×10^2	<10
% kill	98					>99	>99

[a]The count per g of cottage cheese was determined before freezing (BF) and after freeze-dehydration (AFD).
[b]The platen temperature ranged from 30 to 104.4 C.

without addition of a test culture. Samples of the control cheese were plated in the same manner as the contaminated cheese. Few if any gram-negative bacteria were detected. In addition, the characteristics of colonies on the plates containing contaminated cheese were checked and compared with those of the contaminant, either culture F11 or 54. Immediately after freeze-dehydration, some of the samples were rehydrated with sterile distilled water and stored at 5.5. C in separate sterile dishes for 1, 3, 5, and 7 days. The remaining dehydrated samples were stored at 25.5 C for 3, 7, 14, 21, and 28 days. The viable count was determined after each storage interval. Freeze-dehydration caused an extensive reduction in the viable count of F11 (Table 4). In some cases increases in viable count occurred during holding of the rehydrated samples. In milk, however, the increases in count during storage of the rehydrated samples were more extensive. It is possible that the conditions in rehydrated cottage cheese, for example pH, did not support growth of bacteria "damaged" during freezing and dehydration. No viable bacteria could be detected after 2 to 3 weeks in the dehydrated samples stored in the dry form.

Table 5 shows the effect of freeze-dehydration on culture 54 in cottage cheese. In this case, freeze-dehydration caused an extensive reduction in the viable count of culture 54. Rehydration and storage of the dehydrated samples at 5.5 C for 1 to 3 days did not change the viable count extensively. No viable *Achromobacter* could be detected in the dehydrated samples stored for 7 days at 25.5 C. The effect of freeze-dehydration on culture 54 in cottage cheese was different from that in milk or ice cream mix substitute. The effect of freeze-dehydration on culture 54 seemed much more extensive in cottage

cheese than in milk or ice cream mix substitute. Preliminary experiments suggest that the method used to enumerate may be responsible for this phenomenon. In order to enumerate *Achromobacter* (culture 54) in cottage cheese, Staphylococcus medium 110 was employed. This medium although excellent for the recovery of non-treated *Achromobacter* species may be somewhat deficient or inhibitory to treated (heat, cold, dehydration) organisms. This seems to be indicated by the fact that the colonies of *Achromobacter* on Staphylococcus medium 110 from freeze-dehydrated samples were smaller than those which did not receive freeze-dehydration treatments. In a study on the isolation of S. *aureus* on various recovery media, Baird-Parker and Davenport *(3)* reported that surface plating on laboratory media did not support the growth of all viable cells after freeze-dehydration. Recovery in these media could be improved by adding blood or catalase as well as pyruvate. Nelson *(9)* showed that 5% NaCl in Plate Count Agar had no effect upon counts of unheated enterococci, but this NaCl concentration reduced markedly the apparent survival of sublethally heated organisms. It is possible that similar factors are responsible for the poor recovery of *Achromobacter* from freeze-dehydrated foods on Staphylococcus medium 110. In view of these results, further studies on the conditions required for the recovery of microorganisms from freeze-dehydrated foods seem highly desirable.

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