PRESERVATION OF MIXED STRAIN LACTIC ACID STARTER CULTURES BY FREEZING

by

GORDON LEE PARRISH

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1957

APPROVED:

Redacted for Privacy

Assistant Professor of Bacteriology

In Charge of Major

Redacted for Privacy

Chairman of Department Bacteriology

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

Date thesis is presented_____

Typed by Carol Anderson

ACKNOWLEDGMENT

The author wishes to express sincere appreciation to those who have contributed to the preparation of this thesis.

To Dr. A. W. Anderson for his patience, encouragment, and advice;

To Dr. P. R. Elliker for his helpful criticisms;

To the Agricultural Experiment Station for finanicial assistance;

To my wife for her confidence and understanding;

To all the members of the Bacteriology Department, for their helpful information and cooperation during this investigation.

TABLE OF CONTENTS

TABLE OF CONTENTS

continued

LIST OF TABLES

LIST OF TABLES

continued

LIST OF TABLES

continued

LIST OF TABLES

continued

LIST OF TABLES

continued

LIST OF TABLES

continued

LIST OF TABLES

continued

PRESERVATION OF MIXED STRAIN LACTIC ACID STARTER CULTURES BY FREEZING

INTRODUCTION

Various commercial laboratories supply lactic acid starter cultures that are more or less carefully selected for acid and flavor production depending upon their use in the dairy industries. These are supplied either in liquid or powder form. In the dairy plant these cultures are maintained in an active state by transferring them daily in a milk under aseptically controlled conditions and are employed for the preparation of bulk cultures for use in dairy products. Ordinarily a number of mother cultures are carried so that comparisons between them can be made and only those of satisfactory quality selected for use. The need for daily transfer of the mother culture increases the chance for defects to occur due to infection with bacteriophage, loss of flavor, low acid production, and contamination with undesirable bacteria. Elimination of the daily transfer of cultures, by holding for extended periods would result in a lower incidence of defective cultures.

Low temperatures have been recommended for holding of coagulated liquid or powdered cultures in plants before they are activated. Freezing also has been used with variable success, to achieve this end. The purpose of the

research outlined in this manuscript has been to increase the survival and activity of lactic acid starter cultures at temperatures.below freezing by determining some of the factors responsible for their death.

HISTORICAL

The use of sub-freezing temperatures for the preservation of bacteria has been under investigation for many years. The earlier studies were concerned with the germicidal effects of freezing. Thus many investigations were conducted on ice and frozen foods to ascertain the presence of pathogenic organisms and later to determine their resistance to the freezing and thawing process. These investigations were concerned with the resistance of the organisms and the protection afforded by the menstruum in which the organisms were frozen.

Numerous theories have been proposed to explain the freezing death of microorganisms. These have attributed the death of microorganisms on freezing to one or more of the following causes: (1) crushing action of ice crystals, (2) toxicity of solutes concentrated within and without the cell, (3) starvation, and (4) destructive metabolism.

Mechanical Destruction Theory of Freezing Death. Keith (20, p. 879) held the opinion that the immediate death of bacteria by freezing resulted from the crushing action of extracellular ice. This view was based on the results of quantitative studies of bacteria suspended in a number of different medium. The greatest mortality was observed to occur in water. He regarded the higher mortality in water as resulting from a complete

crystalization which offers no refuge from the crushing action of growing ice crystals. Hilliard and Davis (15, pp. 427-430) held views which were essentially the same as those of Keith. They were convinced that the crushing action of extracellular ice was the factor responsible for the death of bacteria by freezing. Their views were based on the observation that colloids exert a protective effect and that there was a greater mortality in suspensions frozen in water than in supercooled glucose solutions. They also noted that there was a greater mortality with repeated freezing and thawing. Weiser and Osterud (55, pp. 436-437) concluded from their studies that the death of a number of cells takes place as a result of mechanical action of extracellular ice crystals. This immediate death occurs at a brief stage in the freezing process during which extracellular ice formation is being completed. They based their conclusions on the observation that marked immediate death of Escherichia coli occurs with freezing at temperatures just below 0°C. at which intracellular ice should not form, and that most marked death occurs only during the final stages of ice formation.

Noskova (36, pp. 449-459) reported that at -30°C. death of yeast cells takes place only in the first stages of freezing when mechanical action of ice is possible. Smith and Swingles (43, pp. 481-483) conclusions that the

critical point was near 0°C., tend to add additional evidence that ice formation and hence its mechanical or crushing action is responsible for freezing death. Hollander and Nell (17, pp. 164-169) believed that immediate death is due to mechanical compression. The pressure is due to the fact that water expands 9% when it changes to ice and the damage arises from the same forces that cause bursting of enclosed vessels when water freezes into ice. They also suggested that the loss which occurs during storage of frozen bacteria may be due to the same factors that result in death above freezing, although some temperature level such as the eutectic point may be a critical temperature above which satisfactory preservation is not possible.

Recently Harrison and Cerroni (10, p. 59) compared freezing death with the death of organisms subjected to violent shaking in a Mickle tissue disentegrator and found that there was no correlation between resistance to the abrasive action of beads and survival of freezing and thawing process. He therefore suggests that mechanical crushing of the cells is not the factor responsible for freezing death.

Theory of Concentration of Solutes as Cause of Freezing Death. Other workers have attributed freezing death to the presence of toxic substances which are concentrated around the cell on freezing. Haines (6, pp.

461-462) working with several species including spores, Staphlococcus aureus, and Bacillus pyocyaneus found that B. pyocyaneus was least resistant to freezing. By freezing and storing at temperatures from -1°C. to -20°C. he was able to associate a very rapid rate of death with a higher temperature of -2°C. Further when he prepared "native protein of B. pyocyaneus he found that this protein was flocculated rapidly at -2°C., but slowly if at all at -20°C. He concluded from this that freezing death could be attributed to changes leading to flocculation of cellular protein. Weiser and Hargiss (54, p. 78) theorized that death of a number of cells may take place not only as a result of mechanical action of ice, but also as a result of concentration of solutes in the intracrystallic film. They suggest that most marked death occurs only during the final stages of ice formation when the intercrystallic films may presumably become so limited as no longer to accomodate the cells. Death probably begins in the very early stages of ice formation and increases in rate as the solutes in the intercrystallic films becomes more concentrated finally to reach a constant maximum at the completion of ice formation. Noskova (36, pp. 449-456) suggests that the destruction of yeasts on freezing at -11°C. is due to the action of a high concentration of solutes on protoplasm proteins. The concentration of solutes within the cell and in the surrounding medium

being formed as a result of the freezing out of water. Lovelock (26, pp. 423-425) concluded that damage on freezing results largely from the concentration of electrolytes within the cell. In the presence of glycerol this concentration of electrolytes is greatly reduced.

Hampil (7, pp. 190-193) suggested that repeated freezing and thawing disrupts the relationship of water and protein molecules to such an extent that death occurs more readily than when freezing temperatures are maintained constant over a long period of time. Thus she is also suggesting a flocculation or denaturation of protein as a cause of freezing death.

Richardson and Scherubel (39, pp. 1515-1564) and Maximov (32, pp. 259-291) noted that just below the freezing point of water ice formed in extracellular spaces sooner than it did within the cells. They believed that water was then extracted from the cells and death occured from a rise in concentration of certain salts which are toxic. Squires and Hartsell (44, pp. 40-45) believed that freezing kill was not entirely the result of mechanical destruction by ice crystal formation but probably due to an uneven distribution of the solutes contained in the intercrystallic film. Freezing kill then proceeds until the colloids of the bacterial cell come to equilibrium with the solutes with which it is confined. The authors also believed that freezing caused a denaturation and/or

flocculation of various cellular protein complexes during storage. That freezing effects cellular colloids is suggested by the work of Callow (3, pp. 303-323) who showed that repeated freezing irreversibly destroys the structure of gelatin gels.

Starvation and Destructive Metabolism as the Cause of Freezing Death. Keith (20, pp. 877-879) concluded that low temperature alone does not destroy bacteria, but appears to favor bacterial longivity by diminishing destructive metabolism. In other words the comparatively rapid death of bacteria at higher temperatures and their slower dying at lower temperature agrees with the theory of destructive metabolism. At temperatures where metabolism ceases altogether the cells continue to exist in a state of suspended animation. Hampil (7, pp. 190-193) in her review of low temperature work states that the preservation of bacterial cultures at low temperatures is due to cessation of metabolism.

Hilliard and Davis (15, pp. 423-431) suggest that the germicidal action of freezing temperatures is due to interference with metabolism and that cells then die by prolonged suspension of metabolic activities leading to slow death from old age or starvation. That some factor does interfer with the nutrition of frozen and thawed cultures is shown by the work of Sulzbacker (47, pp. 341-343) who found that freezing and thawing induces a long

lag phase for psychrophilic organisms, while Hartsell (12, pp. 1072-1077) showed that a highly nutritive plating medium was needed for bacteria exposed to freezing. Gunderson and Rose (5, pp. 254-263) noted that the ability of Escherichia coli and Aerobacter aerogenes to grow on violet red bile agar decreased progressively with storage in a frozen state until only 12 to 15% of the viable cells present could initiate growth.

It is obvious from a review of the work accomplished so far that no single theory completely answers the question of freezing death. It would be safer to say that freezing death can be attributed to the several inter-dependent factors which have been proposed in the various theories to date.

Theories of Protection from Freezing Death. One of the variables which may effect the survival of bacteria at sub-freezing temperatures is the physical and chemical composition of the medium in which they are frozen. Numerous workers have investigated the survival of bacteria in water, sugar, glycerol, salt solutions, and various water-bearing foods, and broth. Keith (20, pp. 877-879) obtained a large percentage of viable cells of E. coli after holding at -20°C. in 5% to 42% glycerol for six months. Smith, Pogle, and Smiles (42, pp. 186-195) studied the formation and dissolution of ice crystals in the presence of glycerol. They observed that channels

and spaces formed into which cells could lodge and thus escape the effects of ice crystallization.

Luyet and Gehenio (29, pp. 107-118) concluded that the protective action of glycerol against freezing injury depended on its penetration into the tissues, relatively low toxicity, and a low eutectic point. Lovelock (26, pp. 28-36) believed that glycerol exerted its full protective effect against hemolysis of red blood cells from freezing and thawing only when present within and without the cells. They suggested that in the presence of glycerol the concentration of electrolytes by freezing is greatly reduced and that this was sufficient to explain its protective action.

Hollander and Nell (17, pp. 164-169) theorized that the protection offered by glycerol against the effects of freezing could be explained by its physical characteristics when in solution. They suggested that when a solution of glycerol and water is cooled pure ice separates, the glycerol concentrates, and the freezing point is lowered. It is possible then that the progressive concentration of glycerol during freezing of a dilute solution may permit the ice to arrange itself during freezing with less internal stress.

Vase (51, pp. 1043-1047) in studying the influence of low temperature on soil bacteria froze Bacillus radicola at -15°C. and at -190°C. in test tubes containing 10 ml.

of a nutrient solution with dextrose added in increasing amounts from 0.01% to 15%. He found that the concentration of the medium had no effect when the temperature was lowered below the eutectic point of the sugar, but at temperatures above the eutectic point the sugar showed a marked protective action. McFarland (33, pp. 481-492) found that concentrations of 30% to 50% sucrose retarded the destruction of E. coli and some species of Saccharomyces.

Milk and milk constituents have been used to protect cells from the destructive action of freezing and thawing. Vereshchagina (52, pp. 3-6) incubated mixed starters of acid producing, and aroma producing streptococci in milk for 2 to 3 hours after coagulation and held them at 5° to 8°C. for several hours. Samples were then stored at -21°C. for 30 days, some were kept frozen while others were thawed out four times. The frozen samples retained activity during the month, coagulating milk after 16 hours and after 12 hours on the third subculture. The intermittently thawed samples were much less active, after 20 days they took 24 hours to coagulate milk. Samples stored at room temperature remained active only for 10 days. Recently Johns (18, p. 32) found that cultures neutralized to an acidity of 0.16% with 40% sodium hydroxide and frozen retained a higher degree of activity than unneutralized cultures.

SECTION I

THE EFFECT OF TEMPERATURE OF FREEZING AND THAWING ON THE RECOVERY OF LACTIC ACID STARTER CULTURES

The temperature of thawing and especially the temperature of freezing has been considered by various workers in studies of freezing death. Temperatures ranging from -1.5° to -195°C. have been shown to result in the same mortality (43, pp. 481-483) and (6, pp. 451-463). Others have reported little or no loss of organisms at the lower temperatures of -30°C. to -195°C., but considerable loss at temperatures higher than -30°C. (55, pp. 413-439) and (49, pp. 61-78). This variation in recovery at different temperatures may be attributed in part to the freezing menstruum and to the organisms. In most reports, however it is generally concluded that low temperatures give better survival than high temperatures. It has also been suggested that the better recovery at the lower temperatures is due to the elimination of the lethal effects of solute concentration by lowering the temperature below their eutectic points.

The fluctuation of temperature especially repeated freezing and thawing has been reported as being more destructive than freezing and storing at a constant temperature (55, pp. 413-439), (43, pp. 481-483), and (15, pp. 423-431). Since under normal conditions it can be expected

that the frozen starter cultures might be exposed to fluctuating freezing temperatures, the effects of fluctuation will also be considered in this section.

A final factor which will be considered is the temperature and rate of thawing, because it is an indispensable step in the use of frozen cultures.

EXPERIMENTAL

The Effect of Storage Temperature on Survival of a Lactic Acid Starter Culture. The cultures used in this study were mixed strain commercial lactic acid streptococcus starter cultures. Throughout this section and the following section these cultures will generally be refered to as lactic acid starter cultures. Activity of the cultures was maintained by daily transfer in reconstituted skim milk and incubation at 21°C. for 16 to 18 hours.

A flask of reconstituted skim milk solids which had been sterilized at 121°C. for 12 min. was inoculated with 1% of an active lactic acid starter culture (H4). The culture was incubated at 21°C. for approximately 16 hours to a pH of 4.7 and then was distributed in 10 ml. amounts into sterile screw-cap culture tubes and frozen at -10°C., -27°C., and -64°C. The tubes were removed at intervals of 1, 2, 7, 14, 28, and 42 days and thawed at 32°C. for plate counts and for a determination of activity. Plate count dilutions were made in distilled water buffered at pH 7.2. A medium (T-19) recently developed by Hanneson (8, pp. 125-126) was used for pouring duplicate plates which were then incubated at 32°C. for 2 days before counting. The activity test was performed as outlined in the following paragraph.

To duplicate tubes of 5 ml. of reconstituted skim

Table 1

Influence of Storage Temperature on Per Cent Recovery of Viable Cells of a Lactic Acid Starter Culture

Time in days	Freezing-Storage Temperature in Centigrade		
	-10 (%)[a]	-27 (%)	-64 (%)
0	100	100	100
1	55	63	93
2	7	33	60
14	4	37	60
28	3	10	70
42	1	19	86

[a] Percent recovery of viable cells

Table 2

Influence of Storage Temperature on Per Cent Recovery of Activity of a Lactic Acid Starter Culture

Time in days	Freezing-Storage Temperature in Centigrade		
	-10 (%)[a]	-27 (%)	-64 (%)
0	100	100	100
1	41	55	77
2	28	45	74
7	21	39	81
14	14	36	77
28	14	32	77
42	17	23	65

[a] Data represents the per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

milk solids which had been autoclaved for 12 min. at 121°C., 2% of the culture to be tested was added and the tubes then incubated in a water bath at 32°C. for four hours. After incubation the tubes were immediately frozen to stop further growth and were then held until a convenient time for titration. The tubes were titrated with N/20 Sodium hydroxide to a pH of 8.2 using a Cannon titrimeter. The activity of a particular culture is based on the amount of acid produced in the four hour incubation period.

The results as shown in Tables 1 and 2 would permit one to place considerable importance on the storage temperature. At -10°C. there is a very drastic reduction in cells numbers and activity after one day storage. This is followed by a gradual loss over the remaining test period until after 42 days there is a 99% loss of viable cells and an 83% loss of activity. At -27°C. the loss in activity and viable cells occurs after one day, but is not as great as that at -10°C. The loss after one day being 37% of the viable cells and 45% of the activity. Again this initial loss is followed by a gradual decrease in cells numbers and activity, but remains at a higher level than cultures frozen at -10°C. The most interesting results occur at -64°C. where the recovery of activity at the end of 42 days is 10% better than cultures frozen at -27°C., and 24% better than cultures frozen at -10°C.

after one day.

The Effect of Sucrose and Sodium Chloride on the Recovery of Activity of a Lactic Acid Starter Culture at Various Freezing and Storage Temperatures. A commercial lactic acid starter culture (H4) was incubated in reconstituted skim milk solids and reconstituted skim milk solids with 10% sucrose for 16 hours at 21°C. This culture was then divided into two equal portions. To one portion 4% sodium chloride was added. These two portions were then divided in half. One half was adjusted to pH 6.5 with neutralax, a sesquicarbonate of soda, the other portion remained untreated at pH 4.7. Ten milliliter aliquots of each of the above conditions were dispensed into sterile screw cap tubes and then frozen at -10°C., -22°C., -30°C., and -64°C. After 2 weeks the tubes were removed and thawed at 32°C. The activity was determined as previously described and compared with the activity before freezing.

The data in Table 3 indicates the very dramatic effect of temperature. The recovery of activity is increased in tubes of neutralized or unneutralized skim milk by approximately 15% to 20% as the temperature was lowered from -10°C. to -64°C. When 4% sodium chloride was added to the control cultures before freezing there was an average of 10% recovery of activity at -10°C. which was only slightly improved as the temperature was lowered.

Table 3

Effect of Various Additives and Freezing-Storage Temperature on the Per Cent Recovery of Activity of a Lactic Acid Starter Culture

Additives	Freezing-Storage Temperature in Centigrade			
	-10 (%)[c]	-22 (%)	-30 (%)	-64 (%)
Control[a] Unneut.	25	45	72	86
Control Neut.[b]	29	42	57	97
4% NaCl Neut.	12	8	17	17
4% NaCl Unneut.	8	11	17	25
10% Sucrose[d] Unneut.	25	62	80	84
10% Sucrose Neut.	23	64	78	82
10% Sucrose 4% NaCl Unneut.	9	24	34	61
10% Sucrose 4% NaCl Neut.	26	31	38	43

[a] Cultures grown and frozen in 10% reconstitued skim milk with no additives. Unneutralized pH is 4.7.

[b] Neutralized to pH 6.5.

[c] Data represents the per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[d] Sucrose added before incubation.

The addition of 10% sucrose to the control cultures does not improve the recovery at -10°C. or at -64°C., but results in approximately a 15% to 20% increase at -22°C. and -30°C. The addition of 10% sucrose to cultures containing 4% sodium chloride provides approximately a two fold increase in recovery at all temperatures when compared with tubes without 10% sucrose.

At -10°C. in the presence of 4% sodium chloride and 10% sucrose neutralization seem to improve the recovery of activity.

The Effect of Temperature Fluctuation on the Recovery of Activity of a Frozen Lactic Acid Starter Culture. A commercial lactic acid starter (H4) cultured in reconstituted skim milk at 21°C. for approximately 16 hours to a pH4.7, was distributed in 10 ml. amounts into sterile screw cap tubes and frozen at -30°C., -22°C., and -10°C. Some of the tubes were fluctuated between -30°C. and -22°C., between -30°C. and -10°C., and between -22°C. and -10°C. The remainder of the tubes were held at -30°C., -22°C., and -10°C. After each fluctuation, tubes were removed along with tubes from the three freezing temperatures which had not been fluctuated and the activity determined. The tubes were subjected to this fluctuation proceedure four times, with a 24 hr. storage period at each temperature.

Table 4

Effect of Fluctuating the Storage Temperature of a Frozen Lactic Acid Starter Culture on the Per Cent Recovery of Activity

Treatment	Number of Temperature Fluctuations				
	0	1	2	3	4
	(%)[a]	(%)	(%)	(%)	(%)
Held at -30°C.	100	84	83	72	72
Held at -22°C.	100	67	57	45	37
Held at -10°C.	100	44	39	34	29
Fluctuated from -30°C. to-22°C.	100	77	64	51	38
Fluctuated from -30°C. to -10°C.	100	61	46	28	19
Fluctuated from -22°C. to -10°C.	100	48	39	23	18

[a] Data represents the per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

The results are summarized in Table 4. It can be seen that the least loss in activity occured in those tubes held at -30°C. The second best recovery was obtained for tubes which had been held at -22°C. and those fluctuated between -30°C. and -22°C. The recovery of activity for these conditions averaged about 35% less than tubes held at -30°C. The lowest recovery was obtained for tubes fluctuated between -30°C. and -10°C., between -22°C. and -10°C., and those held at -10°C.

The Effect of Repeated Freezing and Thawing on the Recovery of Activity of Several Lactic Acid Starter Cultures. Commercial starter cultures were grown in reconstituted skim milk solids at 21°C. for approximately 16 hours to a pH of 4.7. The cultures were then distributed in 5 ml. portions in screw cap tubes and repeatedly frozen in a brine bath at -22°C. and thawed in a 32°C. water bath. The tubes were held at -22°C. for 1 hour before thawing. The activity was determined before freezing and after each freezing-thawing cycle.

The harmful effect of repeated freezing and thawing is shown by the results summarized in Table 5. The average loss for the six cultures after the second freezing and thawing was 28% or almost 3 times greater than that which occured after the first freezing and thawing. The recovery of two of the cultures, H4 and H18, was approximately 10% to 20% greater than for the other four cultures.

Table 5

Effect of Repeated Freezing and Thawing on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures

Cultures	Number of Freezing-thawings				
	0 (%)[a]	1 (%)	2 (%)	3 (%)	4 (%)
H4	100	96	74	59	38
H18	100	101	71	59	38
M	100	89	59	45	21
FB	100	82	50	38	22
FC	100	93	62	46	22
C4	100	83	61	46	28
Average	100	91	63	49	28

[a] Data represents the per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Table 6

Effect of Thawing Temperature on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures Frozen at -27°C. for Two Weeks

Culture	Thawing Temperature in Centigrade			
	21 (%)[a]	32 (%)	37 (%)	50 (%)
H4	58	46	46	29
FB	46	39	39	27
FC	49	43	47	35
M	54	42	42	29
Average	52	42	43	30

[a] Data represents the per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

The Effect of Thawing Temperature on the Recovery of Activity of Frozen Lactic Acid Starter Cultures. Reconstituted skim milk solids were inoculated with 1% of each of four lactic acid starter cultures commonly employed in the dairy industry. The cultures were incubated at 21°C. for approximately 16 hours to pH 4.7. Ten milliliter portions were distributed into culture tubes, the activity determined and the tubes then frozen at -27°C. After a week they were thawed at temperatures of 21°C., 32°C., 37°C., and 50°C. and the activity again determined.

From the results in Table 6 it would appear that thawing temperature has little effect on the recovery of activity, although at 50°C. the activity is consistently lower. The recovery for tubes thawed at 21°C. is about 10% higher than that at 32°C. and 37°C., but the most significant difference in recovery is between 50°C. and the lower temperatures.

DISCUSSION

From the results it is apparent that the critical or dangerous temperature zone presented by Luyet and Gehenio (27, p. 253) which extends from 0°C. to -40°C. applies also to commercial lactic starter cultures frozen in skim milk. In this range 0°C. to -40°C. storage death is rapid but decreasing as the temperature is lowered toward -40°C.

Several factors may be involved in the improved recovery of activity at the lower freezing temperatures. At lower temperatures the rate of freezing is increased due to the greater temperature differential and thus the cells should pass through the critical zone more rapidly with a consequent improvement in the initial recovery. Further the decrease in rate of storage death of the cultures at lower freezing temperature may be attributed to the effect of temperature on the reaction rate of the destructive changes in the medium and the cell as a result of lowering the temperature.

The toxic effect of concentrating salts can be considered one of the important causes of death at freezing temperatures within the critical zone 0°C. to -40°C. At temperatures below the eutectic points of the salts, the toxic action is eliminated through crystallization of the salt, assuming of course that the toxic effect of salts depends on their being in solution.

The addition of 4% sodium chloride (Table 3) was made in an attempt to demonstrate the toxic action of salts on freezing of commercial lactic acid starter cultures. It was felt that the addition of more salt to the culture prior to freezing would result in a greater mortality than would be obtained for cultures without the added sodium chloride. This assumption is not entirely correct since the addition of 4% sodium chloride to the culture may have altered or damaged the cell prior to freezing rendering them more susceptible to freezing death. If the cells have not been altered by the 4% added sodium chloride then its toxicity should be limited by lowering the temperature. Observations indicate only a slight increase in per cent recovery of activity for cultures containing 4% sodium chloride at -64°C., thus it is possible that the cells were damaged prior to freezing. That the cells were not damaged by the high salt concentration prior to freezing is suggested by the fact that the activity for cultures containing both 4% sodium chloride and 10% sucrose were considerably improved by lowering the temperature.

The lack of a protection action for sucrose at -10°C. leads one to believe that destructive forces are so rapidly acting or so great as to be impossible to eliminate by the addition of 10% sucrose. At -64°C. the protective action of sucrose is either eliminated as a result of the

temperature being below the eutectic point of the sugar, or the maximum survival of a culture at any temperature below a certain point, -40°C., may have been obtained. This has been suggested by Turner (49, pp. 61-78) who found that even at -78°C. there was a slight loss of viable organisms of Treponema due to the freezing process, but that on storage there was no further loss. Vass (51, pp. 1043-1047) also demonstrated that sucrose did not improve recovery when the temperature was below the eutectic point of sugar.

It is also quite apparent that a fluctuation in temperature of 8°C. to 20°C., but still below the freezing point is undesirable since each fluctuation of the culture will result in a loss of activity comparable to the loss observed for tubes held at the higher temperature for the same period of time. Weiser and Oesterud (55, pp. 425-428) found that fluctuation between temperatures in the range -1.5°C. to -195°C. resulted in a mortality intermediate between that obtained for controls stored at the two temperatures. These observations were also noted for lactic acid starter cultures fluctuated between -30°C. and -22°C., however after two fluctuations between -30°C. and -10°C., and between -22°C. and -10°C. the results obtained show a poorer recovery of activity than do the cultures stored at -10°C. This indicates that for cultures frozen in milk some lethal factors associated with fluctuation

exists at the higher temperatures.

When the temperature fluctuation of cultures involves repeated freezing and thawing the destruction of cells and the loss of activity is considerable and it appears that the greatest loss in activity occurs after the second freezing and thawing. This is consistent with other published data (15, pp. 423-431), (55, pp. 431-439), (46, pp. 293-300), and (9, pp. 711-715). It is conceivable that the loss of organisms after the first freezing is due to the death of those cells which are damaged or other wise more susceptible to freezing. Following the first freezing, a number of the viable cells remaining may have been damaged and are therefore more susceptible to the effects of the second freezing. Thereafter the ratio of damaged to undamaged cells appears to remain somewhat constant for suceeding freezing-thawings.

The lethal factor(s) for fluctuations in temperature may be the same as those involved in the death of cells on repeated freezing and thawing. This is considered a possibility although the culture appears to be completely frozen at the highest temperature of fluctuation. Actually the higher temperature probably resulted in some melting of the extracellular ice, thus diluting the medium directly around the cell causing the cell to attempt to adjust to the new osmotic pressure. When the temperature is again lowered the water freezes resulting in a concentration

of solutes and a consequent increase in the osmotic pressure to which the cell must again attempt to adjust. Repeated exposure of the cells to drastic changes in osmotic pressure may result in destruction of the cell membrane. The permeability of the membrane may be altered and growth initiation after thawing would be slow if the cells were not already dead.

The thawing of cultures at temperatures between 21°C. and 37°C. in a water bath has little effect on recovery when the quantity of frozen culture is small and thawing can be accomplished in a short time (5 to 10 min.). Dubos (2, pp. 101-112) working with Pneumococci found that thawing at a high temperature resulted in disruption of the cell. He attributed this to the action of autolytic enzymes liberated by the process of freezing and to the distruction of some structure of the cell which normally prevents cellular enzymes from attacking a substrate of cell architecture. Pneumococci, however are notoriously delicate organisms and autolyze much more readily than most other bacteria. Therefore it is not possible to say with any degree of certainty that this concept applies to the lactic acid starter bacteria, although observations show less recovery when the cells were thawed at 50°C.

SECTION II

THE EFFECT OF VARIOUS ADDITIVES AND HYDROGEN ION CONCENTRATION ON THE RECOVERY OF ACTIVITY OF FROZEN LACTIC ACID STARTER CULTURES

Milk, cream, and milk constituents have been shown by several early workers to provide a greater recovery of viable organisms than freezing in other menstrua such as broth, water, and various buffers. Since milk is the usual medium used for culturing commercial lactic acid starter bacteria, the organisms are already present in a medium which offers good protection from freezing death. Nevertheless, some loss of activity does occur on freezing. The incorporation of additional milk solids in the form of skim milk solids, whey solids and sugars including sugars other than lactose was considered a possible means of improving the recovery of activity. The effect of hydrogen ion concentration has also been considered as a factor in freezing death and will be studied in this section.

EXPERIMENTAL

The Effect of Added Skim Milk Solids and Whey Solids on Recovery of Activity of Frozen Lactic Acid Starter Cultures. Reconstituted skim milk, to which 4, 6, and 10% skim milk or whey solids were added was steamed in an Arnold sterilizer for 30 min. Flowing steam was used rather than steam under pressure, since on autoclaving the milk containing the added whey solids coagulated. After steaming the milk was cooled to 21°C. and inoculated with 1% of a 16 to 18 hour culture. The inoculated milk was then incubated at 21°C. to a pH of 4.7 to 4.8, usually 12 to 14 hours for 10% solids milk and slightly longer for higher solids milk (16 to 18 hours for 20% solids milk). The culture was then dispensed in 5 ml. quantities and alternately frozen and thawed as described in Section I page 22.

The average recovery of activity for the four cultures in 10% reconstituted skim milk (control) was 49% (Table 7). The addition of 4% skim milk solids provided approximately 13% increase in recovery of activity, while 6% added skim milk resulted in approximately a 26% increase in recovery of activity. In 20% skim milk solids the increase in recovery of activity was approximately 35% although one of the cultures (FB) in two out of the three replicates had an activity which was slightly less than the activity

Table 7

Effect of Added Skim Milk Solids and Whey Solids On Per Cent Recovery of Activity of Frozen Lactic Acid Starter Cultures

Conc. of solids added	Culture				
	H4 (%)[b]	FB (%)	FC (%)	M (%)	Average (%)
Control[a]	53	42	50	50	49
Skim milk					
4%	61	55	72	60	62
6%	68	70	87	76	75
10%	75	63	115	82	84
Whey					
4%	74	72	67	67	70
6%	79	72	73	80	76
10%	84	80	96	74	84

[a] Cultures grown and frozen in 10% reconstituted skim milk solids with no additives.

[b] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

in 16% skim milk solids.

When whey solids are used as an additive the addition of 4% solids resulted in approximately 17 to 30% increase in recovery. The addition of 6% whey solids does not greatly improve the recovery of activity for most of the cultures (Table 7). The addition of 10% whey solids results in an average increase of 35% for the four cultures tested. Equal concentrations of skim milk or whey solids resulted in approximately the same percentage increase in recovery of activity.

The Effect of Added Glucose, Sucrose, and Lactose on the Recovery of Activity of Lactic Acid Starter Cultures. This experiment has been carried out to show that sugars can protect lactic acid starter cultures from freezing death. The sugars were added during reconstitution of the skim milk solids. The milk was then sterilized, cooled to 21°C., and inoculated with 1% of an active lactic acid starter culture. After incubation at 21°C. for approximately 16 to 18 hours to a pH of 4.7 the cultures were distributed in 5 ml. amounts in sterile screw-cap tubes and subjected to repeated freezing and thawing. The effect of adding the sugars to the cultures just before freezing was also determined by adding a slurry of the various concentrations of sugar. Although this resulted in a slight dilution of the culture it was not sufficient to show a lower initial activity.

Table 8

Effect of Sugars Added Just Before Freezing on Per Cent Recovery of Activity of Lactic Acid Starter Cultures Exposed to Repeated Freezing and Thawing

Conc. of sugar added	Culture				
	H4 (%)[b]	FB (%)	FC (%)	M (%)	Average (%)
Control[a]	25	28	33	31	29
Glucose					
2.5%	37	57	58	67	55
5.0%	46	71	49	60	56
7.5%	41	78	71	72	66
Sucrose					
5.0%	47	57	49	85	60
10.0%	48	72	64	79	66
15.0%	52	79	82	84	74
Lactose					
5.0%	48	58	66	55	57
10.0%	50	70	61	87	67
15.0%	52	45	70	106	68

[a] Cultures grown and frozen in 10% reconstituted skim milk solids with no additives.

[b] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Table 9

Effect of Sugars Added Before Incubation on Per Cent Recovery of Activity of Cultures Exposed to Repeated Freezing and Thawing

Conc. of Sugar Added	Culture				
	H4 (%)[b]	FB (%)	FC (%)	M (%)	Average (%)
Control[a]	25	28	33	31	29
Glucose					
2.5%	45	68	43	59	54
5.0%	59	73	57	77	67
7.5%	63	82	61	111	79
Sucrose					
5.0%	44	89	47	69	62
10.0%	58	60	57	97	68
15.0%	58	87	67	117	82
Lactose					
5.0%	47	57	67	89	65
10.0%	56	81	57	135	82
15.0%	46	101	81	101	82

[a] See Table 8

[b] See Table 8

Table 10

Effect of Glycerol Added Just Before Freezing On Per Cent Recovery of Activity of Lactic Acid Starter Cultures Exposed to Repeated Freezing and Thawing

Conc. of Glycerol added	Culture				
	H4 (%)[b]	FB (%)	FC (%)	M (%)	Average (%)
Control[a]	25	28	33	31	29
Glycerol					
5.0%	48	58	70	81	64
10.0%	50	70	70	75	66
15.0%	52	45	52	77	55

[a] See Table 8

[b] See Table 8

The addition of 2.5% glucose, 5% sucrose or lactose resulted in almost twice the recovery obtained for the control culture (Tables 8 and 9). The maximum protection based on the average values for the four cultures is obtained when 7.5% glucose, 15% sucrose, and 10% lactose are added. This average maximum protection results in an increase in activity of approximately 40% over the control. The variation observed for the individual cultures do not indicate the use of more than 5% to 7.5% glucose, or 5% to 10% sucrose or lactose. For glycerol the maximum recovery was obtained when 5% glycerol was added just before freezing and there is an indication that lower concentrations could be used (Table 10). The results do not indicate that the addition of sugars to the culture just before freezing is any more effective than addition before incubation.

A Comparison of the Effect of Additives, Neutralization Quantity of Frozen Culture and Time on the Recovery of Activity of Lactic Acid Starter Cultures. The purpose of this experiment was to study the effects of various conditions on the recovery of cultures frozen in larger quantities. Preliminary experiments indicated about a 10% to 15% lower recovery in quarts than in tubes. The difference in the treatments of tube samples and quart samples appeared to be the slow rate of freezing and thawing. Therefore to minimize the affect of slow

freezing, the cultures stored in quarts were frozen in a continuous ice cream freezer and then stored under the same conditions as tube samples.

Six cultures of mixed strain commercial lactic acid starters were chosen as representative of those most commonly used in the dairy industry. These cultures were maintained in an active condition by transferring daily in 10% reconstituted skim milk. The containers used for storing the cultures were quart paper cartons and screw cap tubes. The additives were spray process, low heat, non-fat, skim milk solids; whey solids; and sucrose. Neutralization of the cultures to pH 6.5 was accomplished by using a 25% solution of a sesquicarbonate of soda (neutralax).

The skim milk solids were reconstituted to 10% on a weight/weight basis. Additives were incorporated at the time of reconstituting. Ten gallon culture cans containing approximately 75 lbs. of the milk or milk plus additives were sterilized at 93°C. to 100°C. for one hour. The cans were then immediately cooled to 21°C. Incubation was carried out in a water bath at 21°C. until cultures reached approximately a pH of 4.7. The incubation time for 10% skim milk solids cultures and 10% skim milk plus 10% sucrose cultures was between 12 to 16 hours. A longer incubation time was required for the 10% whey solids cultures and the 20% skim milk culture due to the increased

buffering effect of the added solids.

After incubation cultures were cooled to prevent further growth and were dispensed and frozen as soon as possible. Each can of culture was divided into two equal aliquots. One portion was run through the ice cream freezer which was operated at a temperature of -15°C. to -18°C. When the temperature of the culture had reached -2°C. to -3°C. the semi-solid culture was withdrawn from the freezer into the quart paper cartons. The tubes cultures were taken from the same portion of the culture before it was frozen in the ice cream freezer. A sample of the culture also was taken at this time for the initial activity determination.

The second half of the culture was adjusted to pH 6.5 with the 25% solution of neutralax during the time the first half of the culture was being frozen in the continuous ice cream freezer. As soon as the machine had been flushed clean of the previous culture this neutralized culture was then frozen. Samples of the culture were taken just prior to freezing for tube freezing and for the initial activity determination. The continuous freezer is always flushed clean with water and sanitized before the next batch is frozen.

All cultures were stored in an ice cream hardening room at -23°C. and samples taken for determination of

activity at 2, 4, 12, and 14 weeks. The samples were thawed in a 32°C. water bath and the activity determined and compared with the initial activity. The results are summarized in the tables as per cent recovery of activity. All the data was statistically analyzed by the analysis of varience and the least significant difference. The least significant difference was determined at the 5% level.

When 10% sucrose was added a significant increase in recovery was obtained for 4 of the 6 cultures frozen in tubes and for 3 of the 6 cultures frozen in quart cartons. The additions of whey solids provided significant improvement for 3 of the cultures in both tubes and quart cartons, while 20% reconstituted skim milk solids provided protection for 3 cultures in tubes and 4 of the 6 cultures tested in cartons. (Tables 12-48) There was no significant difference between the additives in their ability to protect the cultures from freezing death.

A comparison of neutralized and unneutralized cultures shows a significant loss of 20% to 30% for all 6 cultures in tubes and a 11% to 24% loss for all cultures in quart cartons.

There appears to be no significant loss over the time from 2 to 14 weeks except for 2 cultures, H4 and M. The loss for cultures H4 and M occured between 4 and 12 weeks. There was an initial loss for all cultures during the first two weeks. One cannot determine if the various

Table 11

Analysis of Variance of Lactic Acid Starter Cultures
Frozen in Tubes

Source of Variation	Degrees of freedom	F values[b] for different cultures					
		H4	H18	FB	FC	C4	M
Total	63						
Replicates	1	1.04	2.87	1.04	1.15	1.21	0.08
Additives	3	5.35**	1.98	3.04	4.93*	5.02*	3.30*
Error a	18[a]						
Neutralization	1	14.70**	11.45**	10.60**	13.46**	6.85*	6.30*
Neutralization x Additives	3	1.10	1.90	1.32	2.74	0.76	1.62
Error b	24						
Time	3	4.11*	1.36	0.51	2.35	1.49	9.69*
Error c	18						
Time x Additive	9	2.68*	1.10	0.45	0.90	1.16	3.29*
Error d	54						
Time x Neutralization	3	0.30	0.86	1.32	1.06	1.22	0.97
Time x Additive x Neutralization	9	0.37	0.73	0.31	0.84	0.16	0.60
Error e	70						

[a] All error terms are pooled errors of the 6 cultures used in the experiment
[b] Variance ratios
* Significant at the 5% level of probability
** Significant at the 1% level of probability

Table 12

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FC Frozen in Tubes

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	24	27	26
10% sucrose	63	45	54
10% whey	98	47	72
10% skim milk	83	36	60
Neutralization means	67	39	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.
Least significant difference (LSD) for neutralization at the 5% level is 16%.
LSD for additives at the 5% level is 26%.

Table 13

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FC Frozen in Tubes

Additive	Time in weeks				Additive means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Control[b]	33	31	20	21	26
10% sucrose	68	61	44	45	54
10% whey	85	89	61	55	72
10% skim milk	69	62	46	57	60
Time means	64	62	43	44	

[a] See Table 12
[b] See Table 12
LSD for additives (see Table 12).
Time not significant.

Table 14

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FC Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Unneutralized	72	77	60	61	68
Neutralized	55	48	25	28	39
Time means	64	62	43	44	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) for neutralization at the 5% level is 16%.

Time is not significant.

Table 15

Influence of additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in Tubes

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	28	21	24
10% sucrose	87	45	66
10% whey	89	48	68
10% skim milk	60	30	45
Neutralization	66	36	

[a] See Table 14

[b] Cultures grown and frozen in 10% skim milk without additives.

LSD for Neutralization (see Table 14).

LSD for Additives at the 5% level is 26%.

Table 16

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in Tubes

Additive	Time in weeks				Additive means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Control[b]	36	28	16	18	24
10% sucrose	94	65	48	56	66
10% whey	94	84	42	53	68
10% skim milk	50	52	35	42	45
Time means	69	57	35	42	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least Significant difference (LSD) for additives at the 5% level is 26%.
LSD for time at the 5% level is 22%.
LSD for time x additives at the 5% level is 16%.

Table 17

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Un-Neutralized	81	74	50	58	66
Neutralized	56	40	21	26	36
Time means	69	57	35	42	

[a] See Table 16.

LSD for neutralization at the 5% level is 16%.
LSD for time (see Table 16).

Table 18

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in Tubes

Additive	Unneutralized (%)[a]	Neutralized (%)	Additive means (%)
Control[b]	36	30	33
10% sucrose	69	51	60
10% whey	54	48	51
10% skim milk	95	47	71
Neutralization means	64	44	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) for additives at the 5% level is 26%.
LSD for neutralization at the 5% level is 16%.

Table 19

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in Tubes

Additive	Time in weeks 2 (%)[a]	4 (%)	12 (%)	14 (%)	Additive means (%)
Control[b]	47	38	24	23	33
10% sucrose	83	82	37	38	60
10% whey	79	64	29	32	51
10% skim milk	88	108	41	47	71
Time means	74	73	33	35	51

[a] See Table 18.
[b] See Table 18.

LSD for additives (see Table 18).
LSD for Time at the 5% level is 22%.
LSD for time x additives at the 5% level is 16%.

Table 20

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Unneutralized	83	88	38	45	64
Neutralized	65	58	27	26	44
Time means	74	73	33	35	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) for neutralization at the 5% level is 16%.

LSD for time at the 5% level is 22%.

Table 21

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in Tubes

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	30	22	26
10% sucrose	86	55	71
10% whey	69	60	64
10% skim milk	76	43	59
Neutralization means	65	45	

[a] See Table 20.

[b] Cultures grown and frozen in 10% skim milk without additives.

LSD for additives at 5% level is 26%.

LSD for neutralization (see Table 20).

Table 22

Influence of Additives and Time on The Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in Tubes

Additive	Time in weeks				Additive means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Control[b]	32	29	25	19	26
10% sucrose	82	70	77	55	71
10% whey	71	77	66	44	64
10% skim milk	59	71	57	51	59
Time means	61	62	56	42	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) for additives at the 5% level is 26%.
Time is not significant.

Table 23

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Unneutralized	72	65	70	54	65
Neutralized	49	58	42	31	45
Time means	61	62	56	42	

[a] See Table 22.

LSD for neutralization at the 5% level is 16%.
Time is not significant.

Table 24

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H18 Frozen in Tubes

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	33	31	32
10% sucrose	66	51	59
10% whey	72	34	53
10% skim milk	82	32	57
Neutralization means	63	37	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.
Least significant difference (LSD) at the 5% level is 16%.
Additives not significant.

Table 25

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H18 Frozen in Tubes

Additive	Time in weeks				Additive means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Control[b]	45	31	28	24	32
10% sucrose	73	70	44	49	59
10% whey	66	56	45	45	53
10% skim milk	60	55	55	57	57
Time means	61	53	43	44	

[a] See Table 24.
[b] See Table 24
Additives and time not significant.

Table 26

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H18 Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[b]	(%)	(%)	(%)	(%)
Unneutralized	78	81	56	58	63
Neutralized	44	45	30	29	37
Time means	61	53	43	44	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) for neutralization at the 5% level is 16%.
Time is not significant.

Table 27

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in Tubes

Additive	Unneutralized	Neutralized	Additive Means
	(%)[a]	(%)	(%)
Control[b]	29	19	24
10% sucrose	72	42	57
10% whey	81	32	57
10% skim milk	52	39	46
Neutralization means	58	33	

[a] See Table 26.
[b] Cultures grown and frozen in 10% skim milk without additives.

LSD for neutralization (see Table 26).
Additives not significant.

Table 28

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in Tubes

Additive	Time in weeks				Additive means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Control[b]	31	23	20	22	24
10% sucrose	61	69	47	50	57
10% whey	59	61	52	55	57
10% skim milk	49	49	39	46	45
Time means	50	50	39	43	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Time and Additives not significant.

Table 29

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in Tubes

Neutralization	Time in weeks				Neutralization means
	2	4	12	14	
	(%)[a]	(%)	(%)	(%)	(%)
Unneutralized	56	67	53	58	58
Neutralized	44	34	26	29	43
Time means	50	50	39	43	

[a] See Table 28.

Least significant difference for neutralization at the 5% level is 16%.

Time is not significant.

Table 30

Analysis of Variance of Lactic Acid Starter Culture Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Source of variation	Degrees of freedom	F values[a] for different cultures					
		H4	H18	FB	FC	C4	M
Total	31						
Replicates	1	0.04	0.20	9.03	0.17	4.10	4.36
Additives	3	7.06**	5.59**	1.64	3.67*	5.37*	1.63
Error[b] a	18						
Neutralization	1	25.78**	7.25*	17.70**	9.49**	21.61**	4.95*
Neutralization x additive	3	2.28	3.28	0.74	1.89	1.22	0.96
Error b	24						
Time	1	2.58	2.28	0.79	1.31	5.31	0.45
Error c	6						
Time x additive	3	0.80	0.58	0.99	1.22	1.93	0.40
Error d	18						
Time x neutralization	1	2.47	1.42	2.59	4.80*	0.66	0.04
Time x additive x neutralization	3	0.27	1.22	3.80*	1.24	3.21*	1.77
Error e	24						

[a] Variance ratio.
[b] All error terms are pooled errors of the 6 cultures used in the experiment.
* Significant at the 5% level of probability.
** Significant at the 1% level of probability.

Table 31

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized (%)[a]	Neutralized (%)	Additive means (%)
Control[b]	13	7	10
10% sucrose	27	20	23
10% whey	19	14	17
10% skim milk	35	11	23
Neutralization means	23	13	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.
Least significant difference (LSD) at 5% for neutralization is 10%.
Additives not significant.

Table 32

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks 12 (%)[a]	14 (%)	Additive means (%)
Control[b]	13	7	10
10% sucrose	23	23	23
10% whey	18	16	17
10% skim milk	25	22	23
Time means	19	17	

[a] See Table 31.
[b] See Table 31.
Additives and time not significant.

Table 33

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Neutralization	Time in weeks		Neutralization means
	12	14	
	(%)[a]	(%)	(%)
Unneutralized	25	22	23
Neutralized	14	12	13
Time means	19	17	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) at 5% for neutralization is 10%.

Time is not significant.

Table 34

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H18 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	13	7	10
10% sucrose	24	26	25
10% Whey	25	16	20
10% skim milk	56	19	38
Neutralization means	29	17	

[a] See Table 33.

[b] Cultures grown and frozen in 10% skim milk without additives.

LSD at 5% for additives is 15%.

LSD at 5% for neutralization is 10%.

Table 35

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H18 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks		Additive means
	12 (%)[a]	14 (%)	(%)
Control[b]	12	7	10
10% sucrose	27	23	25
10% whey	23	18	20
10% skim milk	42	33	38
Time means	26	20	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) at 5% level for additives is 15%.
Time is not significant.

Table 36

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Starter Culture H18 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Neutralized	Time in weeks		Neutralization means
	12 (%)[a]	14 (%)	(%)
Unneutralized	32	27	29
Neutralized	21	13	17
Time means	26	20	

[a] See Table 35

LSD at 5% for neutralization is 10%.
Time not significant.

Table 37

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	15	8	11
10% sucrose	48	26	37
10% whey	44	20	32
10% skim milk	49	15	32
Neutralization means	39	17	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.
Least significant difference (LSD) at 5% level for additives is 15%.
LSD at 5% for neutralization is 10%.

Table 38

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks		Additive means
	12	14	
	(%)[a]	(%)	(%)
Control[b]	16	7	11
10% sucrose	42	31	37
10% whey	39	26	32
10% skim milk	33	31	32
Time means	33	23	

[a] See Table 37
[b] See Table 37
LSD at 5% for Additives is 15%.
Time is not significant.

Table 39

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture C4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Neutralization	Time in weeks		Neutralization means
	12 (%)[a]	14 (%)	(%)
Unneutralized	44	34	39
Neutralized	21	13	17
Time means	33	23	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) at 5% level for neutralization is 10%.

Time not significant.

Table 40

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	9	7	8
10% sucrose	49	21	35
10% whey	52	20	36
10% skim milk	46	13	29
Neutralization means	39	15	

[a] See Table 39.

[b] Culture grown and frozen in 10% skim milk without additives.

LSD at 5% for additives is 15%.

LSD at 5% for neutralization is 10%.

Table 41

Influence of Additive and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks		Additive means
	12 (%)[a]	14 (%)	(%)
Control[b]	6	10	8
10% sucrose	30	40	35
10% whey	33	39	36
10% skim milk	27	32	29
Time means	24	30	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) at 5% level for additives is 15%.
Time not significant.

Table 42

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Neutralization	Time in weeks		Neutralization means
	12 (%)[a]	14 (%)	(%)
Unneutralized	35	43	39
Neutralized	13	17	15
Time means	24	30	

[a] See Table 41.

LSD at 5% for neutralization is 10%.
Time not significant.

Table 43

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	23	9	16
10% sucrose	27	15	21
10% whey	44	17	31
10% skim milk	38	12	25
Neutralization means	33	13	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) at 5% level for neutralization is 10%.
Additives not significant.

Table 44

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks		Additive means
	12	14	
	(%)[a]	(%)	(%)
Control[b]	16	15	16
10% sucrose	22	21	21
10% whey	32	29	31
10% skim milk	29	21	25
Time means	25	21	

[a] See Table 43.

[b] See Table 43.

Additives not significant.
Time not significant.

Table 45

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Neutralization	Time in weeks		Neutralization means
	12	14	
	(%)[a]	(%)	(%)
Unneutralized	36	30	33
Neutralized	14	12	13
Time means	25	21	23

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) at 5% level for neutralization is 10%.

Time not significant.

Table 46

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures FC Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Unneutralized	Neutralized	Additive means
	(%)[a]	(%)	(%)
Control[b]	11	4	8
10% sucrose	16	15	16
10% whey	44	14	29
10% skim milk	35	15	25
Neutralization means	26	12	

[a] See Table 45.

[b] Cultures grown and frozen in 10% skim milk without additives.

LSD at 5% level for additives is 15%.

LSD at 5% level for neutralization is 10%.

Table 47

Influence of Additives and Time on the Per Cent Recovery of Activity of Lactic Starter Culture FC Frozen in an Ice Cream Freezer and Stored in Quart Cartons

Additive	Time in weeks		Additive means
	12	14	
	(%)[a]	(%)	(%)
Control[b]	9	7	8
10% sucrose	17	15	16
10% whey	31	27	29
10% skim milk	30	20	25
Time means	22	17	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Cultures grown and frozen in 10% skim milk without additives.

Least significant difference (LSD) at 5% level for additives is 15%.
Time not significant.

Table 48

Influence of Neutralization and Time on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FC Frozen in an Ice Cream Freezer in Quart Cartons

Neutralization	Time in weeks		Neutralization means
	12	14	
	(%)[a]	(%)	(%)
Unneutralized	30	13	22
Neutralized	23	11	17
Time means	26	12	

[a] See Table 47.

LSD at 5% level for neutralization is 10%.
Time not significant.

Table 49

Analysis of Variance for Cultures Frozen in Tubes vs. Cultures Frozen in Cartons

Source of variation	Degrees of freedom	F values[a] of different cultures					
		H4	H18	FB	FC	C4	M
Total	95						
Tubes vs. cartons	1	19.96	31.17	22.99	39.20	32.45	36.92
Error	94						

[a] Variance ratio. All values significant at the 0.1% level of probability.

additives have an affect on storage death, since time between 2 and 14 weeks does not significantly influence recovery of activity for all but 2 of the cultures frozen in tubes.

The additives were found to significantly influence storage death for the two instances mentioned above. All of the additives for culture H4 result in a significantly higher recovery than the control at each time interval, while 10% added sucrose or whey solids provided a significantly higher recovery than 20% solids after 2 weeks but not thereafter (Tables 15-17). All of the Additives for culture M resulted in a significantly higher recovery than the control after 2 and 4 weeks, however after 12 and 14 weeks only 10% added sucrose and the 20% solids showed a significantly higher recovery (Tables 18-20).

Neutralized cultures of C4 in cartons show a significantly lower recovery than unneutralized cultures after 12 weeks but no significant difference exists between these cultures after 14 weeks (Tables 37-39). At the 2 and 4 week intervals an apparent improvement in recovery of neutralized quart cultures was observed. The quart cultures had been allowed to thaw in a 32°C. water bath for 2½ to 3 hours. It was later found that by agitation, thawing could be accomplished in 1 to 1½ hours. When the activity of the cultures thawed rapidly and those thawed

slowly were compared it was found that slow thawing resulted in a higher activity than fast thawing. Further observation showed that the pH of the slowly thawed quarts was lower than the pH of the rapidly thawed quarts. From this it was concluded that a portion of the slowly thawed cultures had reached a temperature where further growth of the organisms could take place thus increasing the number of organisms present and resulting in a higher activity. It was for this reason that data for the 2 and 4 week time intervals was not included in the statistcal analysis of cultures frozen in quart cartons.

An analysis of the recovery of activity in tubes with the recovery in quart cartons shows that there is a significant difference at the 0.1% level. (Table 49) This difference appears to be one of lower recovery of activity for quart cartons cultures compared at the 12 and 14 week intervals.

The Effect of Hydrogen Ion Concentration and Type of Neutralizing Agent on the Recovery of Activity of Lactic Acid Starter Cultures. A recent report by Johns (18, p. 32) stated that neutralization of cultures prior to freezing gave a better recovery. When cultures were neutralized with neutralax to pH 6.5 prior to freezing, it was found that such cultures always resulted in a poorer recovery of activity. In order to clarify the confusion which seems to exist, several lactic acid starter cultures which

had been incubated to pH 4.7 in reconstituted skim milk were adjusted to pH levels from 4.5 to 7.5 at 0.5 pH unit intervals. The cultures were neutralized with a 25% solution of neutralax and were then dispensed in 5 ml. aliquots into screw-cap tubes and frozen at -22°C. for two weeks. At this time they were removed and thawed at 32°C. The activity was determined before and after freezing as previously described.

The results summarized in Table 50 shows that at pH levels of 4.5 to 7.0 no greater improvement can be observed for any one pH level. At pH 7.5 an average of approximately 20% increase over the other pH levels was obtained, however the initial activity was considerably lower than at the other levels.

Since Johns (18, p. 32) used sodium hydroxide, it was thought necessary to compare this and a similar neutralizing agent with neutralax. Several lactic acid starter cultures were adjusted to pH 6.5 with a 25% solution of neutralax and a 40% solution of sodium hydroxide or potassium hydroxide. The neutralized cultures were then subjected to repeated freezing and thawing at -22°C. and 32°C. An unneutralized control of each culture was also frozen. It was found that this resulted in a definite improvement in recovery for the neutralized cultures (Table 51). Cultures neutralized with sodium hydroxide or potassium hydroxide showed an increase in immediate

Table 50

Effect of Hydrogen Ion Concentration on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures Frozen for Two Weeks at -22°C.

pH	Culture			
	H4 (%)[a]	FB (%)	M (%)	Average (%)
4.5	53	36	35	41
5.0	63	34	36	44
5.5	53	46	42	47
6.0	41	38	41	40
6.5	37	34	35	35
7.0	50	40	44	45
7.5	65	71	62	66

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Cultures neutralized with a 25% solution of neutralax.

recovery when compared to cultures neutralized with neutralax.

A third experiment was performed comparing cultures neutralized by neutralax and sodium hydroxide. This time the cultures were held at -22°C. for two weeks before thawing and determining the activity. The results of this experiment conflict with the previous experiment, since neutralization does not provide added protection on storage. (Table 52)

The effects of neutralization with neutralax and sodium hydroxide on storage recovery were compared by freezing in quart cartons. Active lactic acid starter cultures were incubated in reconstituted skim milk, and reconstituted skim milk with 10% sucrose to a pH of 4.7. The milk was reconstituted and sterilized in the same manner as for the previous experiment involving the use of the continuous ice cream freezer. The cultures were neutralized just before freezing by using a 25% solution of neutralax or 40% sodium hydroxide. The portion of the culture to be frozen slowly was distributed into tubes and cartons at the time the remainder was being frozen in the continuous ice cream freezer at -15°C. to -18°C. Tubes and quarts for slow freezing were placed immediately in a storage room at -23°C. The quarts filled from the ice cream freezer were placed in the same storage room.

Table 51

Effect of Neutralizing with Various Agents on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures Subjected to Repeated Freezing and Thawing

Neutralizing agent	Culture						
	H4 (%)[a]	FB (%)	FC (%)	C4 (%)	H18 (%)	M (%)	Average (%)
Control[b]	43	21	23	27	39	24	30
Neutralax	59	34	29	53	55	32	44
Sodium hydroxide	76	63	50	64	70	61	64
Potassium hydroxide	82	74	52	59	82	65	69

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Unneutralized (pH 4.7).

Table 52

The Effect of Various Neutralizing Agents on the Per Cent Recovery of Activity of Lactic Acid Starter Cultures Frozen for Two Weeks at -22°C.

Neutralizing agent	Culture			
	H4 (%)[a]	FB (%)	M (%)	Average (%)
Control[b]	38	30	25	31
Neutralax	37	31	37	36
Sodium hydroxide	45	44	39	43

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

[b] Unneutralized (pH 4.7).

The temperature of these frozen cultures was -2°C. to -3°C. at the time of storage at -23°C. The activity of each of the cultures was determined before freezing and again at two week and four week intervals. At these time intervals single samples of the tubes and cartons for each condition were removed for determination of activity. The data obtained at the four week interval was statistically analysed by the analysis of variance and the least significant difference was determined at the 5% level for convenience in comparison.

That 10% added sucrose provides a significant improvement in recovery of activity is shown by the summarized in Tables 53-62. No significant difference was observed between the control and 10% sucrose in the slow frozen quarts. A comparison of tubes with slow freezing and fast freezing of quarts shows a significant increase in recovery for cultures frozen in tubes over cultures frozen in the ice cream freezer. Tube frozen samples of culture FB showed a significantly better recovery than slow frozen quart cultures, but for all other cultures no significant difference was observed. Slow frozen quart cultures of H4 resulted in a significant improvement in recovery of activity over cultures frozen in the ice cream freezer.

Culture M showed a significant increase in recovery

Table 53

Analysis of Variance for Cultures Neutralized with Neutralax or NaOH and Frozen in Tubes and Cartons

Source of variation	Degrees of freedom	F values[a] for different cultures		
		H4	FB	M
Total	35			
Replicates	1	9.71	2.62	25.48*
Additives	1	910.08***	932.78***	1997.48***
Error[b] a	3			
Neutralization	2	2.93	2.58	9.75**
Neutralization x additive	2	1.69	1.32	2.64
Error b	12			
Container	2	12.64**	24.04**	6.29*
Error c	6			
Container x additive	2	0.18	9.56*	0.15
Error d	12			
Container x neutralization	4	0.39	1.16	1.37
Container x additive x neutralization	4	1.22	0.74	1.14
Error e	24			

[a] Variance ratio.
[b] All error terms are pooled errors for the 3 cultures used in the experiment.
* Significant at the 5% level of probability.
** Significant at the 1% level of probability.
***Significant at the 0.1% level of probability.

Table 54

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture H4 Frozen in Tubes and Cartons

Neutralizing agent	Additive		Neutralization means
	Control[a] (%)[b]	10% sucrose (%)	(%)
Unneutralized	18	40	29
Neutralax	17	50	34
NaOH	24	51	37
Additive means	20	47	

[a] Cultures grown and frozen in 10% skim milk without additives.
[b] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
Least significant difference (LSD) at 5% level for additives is 1%.
Neutralization not significant.

Table 55

Influence of Additive and Container on the Per Cent Recovery of Activity of Frozen Lactic Acid Starter Culture H4

Container	Additive		Container means
	Control[a] (%)[b]	10% sucrose (%)	(%)
Tubes	28	57	43
Freezer cartons	9	33	21
Static Cartons	23	52	37
Additive means	20	47	

[a] See Table 54
[b] See Table 54
LSD at 5% level for additives is 1%.
LSD at 5% level for container is 11%.

Table 56

Influence of Neutralization and Container on the Per Cent Recovery of Activity of Frozen Lactic Acid Starter Culture H4

Neutralizing agent	Container			Neutralization means
	Tubes	Freezer cartons	Static cartons	
	(%)[a]	(%)	(%)	(%)
Unneutralized	39	19	31	29
Neutralax	41	21	39	34
NaOH	47	22	43	37
Container means	43	21	37	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
Least significant difference (LSD) at 5% level for containers is 11%.
Neutralization not significant.

Table 57

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture FB Frozen in Tubes and Cartons

Neutralizing agent	Additive		Neutralization means
	Control[b]	10% sucrose	
	(%)[a]	(%)	(%)
Unneutralized	17	41	29
Neutralax	25	49	37
NaOH	17	51	34
Additive means	20	47	

[a] See Table 56.
[b] Cultures grown and frozen in 10% skim milk without additives.
LSD at 5% level for additives is 1%.
Neutralization not significant.

Table 58

Influence of Additive and Container on the Per Cent Recovery of Activity of Frozen Lactic Acid Starter Culture FB

Container	Additive		Container means
	Control[b]	10% sucrose	
	(%)[a]	(%)	(%)
Tubes	28	75	51
Freezer cartons	8	35	22
Static cartons	23	31	27
Additive means	20	47	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
[b] Cultures grown and frozen in 10% skim milk without additives.
Least significant difference (LSD) at 5% level for additives is 1%.
LSD at 5% level for container is 11%.

Table 59

Influence of Neutralization and Container on the Per Cent Recovery of Activity of Frozen Lactic Acid Starter Culture FB

Neutralizing agent	Container			Neutralization means
	Tubes	Freezer cartons	Static cartons	
	(%)[a]	(%)	(%)	(%)
Unneutralized	47	21	20	29
Neutralax	54	20	35	37
NaOH	53	24	26	34
Container means	51	22	27	

[a] See Table 58.
LSD at 5% level for container is 11%.
Neutralization not significant.

Table 60

Influence of Additives and Neutralization on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen in Tubes and Cartons

Neutralization agent	Additive		Neutralization means
	Control[a]	10% sucrose	
	(%)[b]	(%)	(%)
Unneutralized	23	55	39
Neutralax	30	70	50
NaOH	28	76	52
Additive means	27	67	

[a] Cultures grown and frozen in 10% skim milk without additives.
[b] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.
Least significant difference (LSD) at 5% level for additives is 1%.
LSD at 5% level for neutralization is 7%.

Table 61

Influence of Additive and Container on the Per Cent Recovery of Activity of Lactic Acid Starter Culture M Frozen

Container	Additive		Container means
	Control[a]	10% sucrose	
	(%)[b]	(%)	(%)
Tubes	35	75	55
Freezer cartons	20	58	39
Static cartons	25	68	46
Additive means	27	67	

[a] See Table 60
[b] See Table 60
LSD at 5% level for additives is 1%.
LSD at 5% level for containers is 11%.

Table 62

Influence of Neutralization and Container on the Per Cent Recovery of Activity of Frozen Lactic Acid Starter Culture M

Neutralizing agent	Container			Neutralization means
	Tubes	Freezer cartons	Static cartons	
	(%)[a]	(%)	(%)	(%)
Unneutralized	49	33	35	39
Neutralax	59	37	55	50
NaOH	58	48	50	52
Container means	55	39	46	

[a] Values represent per cent recovery of activity. The activity is based on the amount of acid produced by cultures incubated at 32°C. for 4 hours.

Least significant difference (LSD) at 5% level for containers is 11%.

LSD at 5% level for neutralization is 7%.

of activity for neutralized cultures, however no significant difference was observed between the two neutralizing agents. A slight improvement in recovery of activity was observed for neutralized control cultures which had been slowly frozen in quart cartons. This improvement may be attributed to further growth of the culture during the interval necessary for freezing to take place. The growth is evidenced by a lower pH for neutralized cultures after thawing.

DISCUSSION

The protective effect of added milk solids can be attributed principally to the sugar present as lactose. In as much as each gram of milk solids contains approximately 0.5 grams of lactose and each gram of whey solids contains approximately 0.7 grams of lactose, the addition of these substances involves primarily an addition of lactose. The milk solids also provide some milk salts and in the case of skim milk solids, casein. These added milk salts and casein however do not appear to contribute to the protection of the cells from freezing death, since a comparison of the recoveries obtained by the addition of sugars and by the addition of a comparable amount of lactose in the form of milk solids results in essentially the same degree of protection (Tables 7, 8, and 9).

From the results obtained for the sugars and glycerol it appears that the molar concentration of the additive may be involved in the mechanisms of protection. This suggests something more than just providing a refuge for the cells from the mechanical effect of forming ice crystals as proposed by Keith (20, pp. 877-879). Further the presence of casein in milk should provide more than enough space for the cells to escape these mechanical effects of ice crystals.

Other factors, such as osmotic pressure and freezing

point depression are based on the molar concentration of solutes. A lowering of the freezing point of the medium may protect the cells by limiting the concentration of toxic electrolytes at any one temperature.

The addition of sugar before freezing increases the osmotic pressure which should result in a slight dehydration of the cell prior to freezing. Although some dehydration of the cell is necessary to protect the cell from intracellular freezing there should be sufficient loss of water from the cell on freezing in milk without the addition of sugars.

In Section I page 20, it was noted that addition of 10% sucrose protected the cell from damage by 4% sodium chloride. It is not certain whether this protection was exerted before or during freezing, but in either case the addition of sucrose limited the toxic action of the high concentration of sodium chloride. Since high concentrations of salt are capable of denaturing protein, it is conceivable that the action of sodium chloride and other salts was on the cell membrane and that the protective action of the sucrose was in limiting the access of the sodium chloride to the cell membrane.

The presence of sugar or glycerol also limits the amount of ice formed through their water binding properties and therefore on freezing the per cent of unfrozen water

will increase and eventually toxic salts will concentrate sufficiently to cause some damage to the cells. If this toxic action is temperature dependent it might be expected that the rate of the reaction will be slower at the lower temperature which has been achieved.

The use of a continuous ice cream freezer has not been effective in preventing the greater loss in quarts as compared to freezing in tubes. The recovery for quarts still seems to be approximately 15% lower than cultures frozen in 10 ml. amounts. It would appear at first that the rate of freezing does not effect the recovery of frozen culture, however the cultures as they are obtained from the ice cream freezer are at a temperature of -2°C. to -3°C. and are not a solid frozen mass. Therefore it is probable that there is still a lot of unfrozen water and that complete freezing of quarts will take longer than for tube cultures. Thus the time required to pass through the most dangerous zone of freezing is still sufficiently long to result in a greater mortality in quarts than in tubes. Repeated freezing and thawing may also be responsible for the lower recovery obtained for cultures frozen in an ice cream freezer. This occurs as a result of the frozen culture being scraped from the wall of the freezer into the unfrozen portion of the culture.

The results of experiments involving neutralization are somewhat confusing. For the most part these

experiments indicate that the reaction of the frozen culture has no effect on recovery. However, one experiment which was analyzed statistically showed a significantly lower recovery for neutralized cultures, while another experiment which was performed in a similar manner showed that there was no significant difference between these cultures.

An improvement in recovery of activity was shown when neutralized cultures are subjected to repeated freezing and thawing. It is assumed that repeated freezing and thawing only enhance the effects of factors involved in the immediate death, and that little or no storage death occurs. It might be concluded from this that pH effects only immediate death of microorganisms. This effect is negligible on one freezing and thawing, but becomes more apparent with repeated freezing and thawing. One factor which has not been considered is that the lactic acid bacteria may be more acid tolerant than species previously tested and therefore less affected by a low pH. Of course this does not resolve the conflict between the results of these experiments and that of Johns (18, p. 32) who showed that neutralized lactic acid cultures always survived freezing better than unneutralized cultures.

SUMMARY AND CONCLUSIONS

Commercial lactic acid starter cultures were frozen in reconstituted skim milk solids at different temperatures. From the results of these experiments we must conclude that the lowest temperature available is the best temperature for freezing and storing these cultures for maximum recovery of activity.

Freezing temperatures between -10°C. and -30°C. result in an increase in recovery of activity when 10% sucrose is added, although at higher or lower temperatures the recovery of activity was not improved.

The recovery of activity was low at all freezing temperatures when 4% sodium chloride was added to the culture before freezing, although there was a slight improvement at -64°C. This suggests that either the salt damaged the cells prior to freezing so that recovery at any temperature would be poor or that the action of this high concentration of salt is so rapid that even at -64°C. satisfactory recovery of activity can not be obtained.

The addition of 10% sucrose to the cultures containing 4% sodium chloride seems to improve the recovery of activity and therefore sucrose must protect the cells in some manner from the toxic action of the salt. Other additives were also found to increase the recovery of activity of cultures frozen at -22°C. The addition of

skim milk and whey solids was found to provide protection for the cells from freezing death. This protection was attributed to the presence of lactose in the milk solids, since a comparison of the recovery obtained by adding approximately equal quantities of lactose in the form of milk solids resulted in essentially the same protection as added glucose, sucrose, or lactose.

Cultures which were fluctuated between different storage temperatures were found to show a recovery of activity intermedate between the recovery obtained for cultures stored at the extremes of temperature fluctuation. It is obvious then that for maximum recovery of activity the cultures should be held at a constant storage temperature. In the case of repeated freezing and thawing the loss in activity after the second freezing-thawing was greater than after the first freezing-thawing. Thus cultures once frozen should not be refrozen.

A comparison of neutralized cultures with unneutralized cultures indicated that for the most part neutralization does not improve the recovery of frozen starter cultures. In one experiment in which the neutralized and unneutralized cultures were subjected to repeated freezing and thawing the neutralized cultures always showed a better recovery. It was also found that sodium hydroxide and postassium hydroxide were better neutralizing agents than neutralax, a sesquicarbonate of soda. In other experiments,

when the neutralized and unneutralized cultures were frozen and stored at a constant temperature, no improvement could be detected at any pH between 5.0 and 7.0. It was thought that pH may have a slight effect on immediate recovery which can be detected by repeated freezing and thawing but not by storage.

A lower recovery of activity was obtained for cultures stored in quart lots than for cultures frozen in 5 or 10 ml. amounts. The difference in rate of freezing was considered to be the factor responsible, however, when the culture is frozen in an ice cream freezer and then dispensed into quart cartons the same difference in recovery was observed. This suggests that the rate of freezing is not responsible and that we must look elsewhere for the answer perhaps to the thawing process. It has been shown that the temperature of thawing has little effect on recovery when the quantity of frozen culture is small and thawing can be accomplished in a short time. Only at a thawing temperature of 50°C. is the recovery for tube cultures significantly reduced. In the case of quarts however where thawing requires approximately 1½ hours it is possible that the rate of thawing may be more important. This suggests that some means must be found to thaw the quart cartons more rapidly other than by using high temperature.

The following recomendations are suggested for

maximum recovery of activity of frozen lactic acid starter cultures:

1. Freeze and store the cultures at the lowest temperature available, preferably -30°C. or lower.

2. If the cultures are to be stored at temperatures of -30°C. or higher, then 2.5% to 5% glucose, 5% to 10% sucrose, 5% to 10% lactose or 2.5% to 5% glycerol should be added at the time the milk is reconstituted.

3. Or, add comparable amounts of lactose in the form of milk solids.

4. Maintain the frozen cultures at a constant storage temperature.

5. Thaw the cultures as rapidly as possible without subjecting them to temperatures higher than 37°C. preferably 21°C.

6. For freezing cultures in quart cartons or larger quantities, a continuous ice cream freezer can be used, however there appears to be no greater recovery of activity than when cultures are distributed unfrozen and allowed to freeze slowly. In fact, if slowly frozen cultures are neutralized an apparent increase in recovery of activity can be obtained do to the additional growth which can take place before freezing occurs.

BIBLIOGRAPHY

1. Appelman, M. D. and Saul S. Hahn. Factors limiting survival of enteric organisms in frozen orange concentrate. Bacteriological proceedings 1:35. 1951.

2. Dubos, R. J. Mechanism of the lysis of pneumococci by freezing and thawing, bile, and other agents. Journal of experimental medicine 66:101-112. 1937.

3. Callow, E. H. The velocity of ice crystallization through supercooled gelatine gels. Proceedings of the Royal Society (London) 108A:307-323. 1925.

4. Flosdorf, E. W. and S. Mudd. Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. Journal of immunology 29:389-425. 1935.

5. Gunderson, M. J. and K. D. Rose. Survival of bacteria in a precooked, fresh frozen food. Food research 13:254-263. 1948.

6. Haines, R. B. The effect of freezing on bacteria. Proceedings of the Royal Society (London) 124B:451-463. 1937-38.

7. Hampil, Bettylee. The influence of temperature on the life processes and death of bacteria.

Quarterly review of biology 7:172-196. 1932

8. Hannesson, Gudlaugur. Factors related to growth stimulation and inhibition of lactic streptococci. Master's thesis. Corvallis, Oregon state college, 1955. 180 numb. leaves.

9. Harrison, Arthur P. Survival of bacteria upon repeated freezing and thawing. Journal of bacteriology 70:711-715. 1956

10. Harrison, Arthur P., and Rose E. Cerroni. The fallacy of "crushing death" in frozen bacterial suspensions. Bacteriological proceedings, 1956, p. 59.

11. Harrison, Arthur P., et. al. Survival of bacteria under various conditions of storage. Bacteriological proceedings, 1952, p. 20.

12. Hartsell, S. E. The longevity and behavior of pathogenic bacteria in frozen foods. The influence of the plating medium. American journal of public health 41:1072-1077. 1951

13. _____. The growth initiation of bacteria in defrosted eggs. Food research 16:96-106. 1951.

14. Hess, E. The effects of freezing on marine bacteria. I. Quantitative studies. Journal of the biological board of Canada 1:95-108. 1934.

15. Hilliard, C. M. and M. A. Davis. The germicidal action of freezing temperatures upon bacteria. Journal of bacteriology 3:423-431. 1918.

16. Hilliard, C. Thorosson, and R. Stone. Notes on the factors involved in the germicidal effect of freezing and low temperatures. Science 42:770-771. 1915.

17. Hollander, D. H. and E. E. Nell. Improved preservation of Treponema pallidum and other bacteria by freezing with glycerol. Applied microbiology 2:164-169. 1954.

18. Johns, C. K. Preserving the activity of frozen cheese starter culture. The Canadian dairy and ice cream journal 35:32. 1956.

19. Keane, K. J. Comparative efficiency of some compounds containing the amino acid group in protecting tissues against freezing injury. Biodynamica 7:137-169. 1953.

20. Keith, S. C. Factors influencing the survival of bacteria at temperatures in the vicinity of the freezing point of water. Science 37:877-879. 1913.

21. Kyes, P. and T. Potter. The resistance of avian tubercule bacilli to low temperatures with especial reference to multiple changes in

temperature. The journal of infectious diseases 64:123-134. 1939

22. Levitt, J. Frost killing and hardiness of plants. Minneapolis, Burgess, 1941. 211p.

23. Levitt J. and G. W. Scarth. Frost hardening studies with plant cells. I. Osmotic and bound water changes in relation to frost resistance with the seasonal cycle. Canadian journal of research 14:257-284. 1936.

24. _____. Frost hardening studies with plant cells. II. Permeability in relation to frost resistance with the seasonal cycle. Canadian journal of research 14:285-305. 1936

25. Lockhead, A. G. and A. H. Jones. Studies of the numbers and types of microorganisms in frozen vegetables and fruits. Food research 1:29-39. 1936.

26. Lovelock, J. E. The mechanisms of the protective action of glycerol against haemolysis by freezing and thawing. Biochemica et biophysica acta 11:28-36. 1953.

27. Luyet, B. J. and P. M. Gehenio. Life and death at low temperatures. Normandy, Mo., Biodynamica, 1940. 341p.

28. _____. The mechanisms of injury and death

by low temperature. Biodynamica 3:33-99. 1940

29. _____. The effect of glycerol in limiting ice formation in tissues sugjected to low temperatures. Biodynamica 7:107-118. 1952.

30. Luyet, B. J. and F. K. Keane. Comparative efficiency of ethylene glycol, glucose and sodium chloride in protecting tissues against freezing injury. Biodynamica 7:119-131. 1952.

31. Mattick, A. T. R. and H. J. Rogers. Preservation of starter cultures. Proceedings of the society of agricultural bacteriology, 1936, p. 40. (Abstracted in Dairy Science abstracts 2:54. 1940.)

32. Maximov, N. A. Internal factors of frost and drought resistance in plants. Protoplasma 7: 259-291. 1929.

33. McFarland, V. H. Behavior of microorganisms at subfreezing temperatures. I. Freezing distribution studies. Food research 5:43-57. 1940.

34. _____. Behavior of microorganisms at subfreezing temperatures. III. Influence of sucrose and hydrogen ion concentration. Food research 6:481-492. 1941.

35. Moran, T. The freezing of gelatine gel. Proceedings

of the Royal Society (London) 112A:30-46. 1926.

36. Noskova, G. L. The causes of death of yeast on freezing. Mikrobiologiya 16:449-459. 1947. (Abstracted in Chemical abstracts 43(3): 8437c. 1949.)

37. Park, W. H. Duration of life of typhoid bacilli derived from 20 different sources in ice; effect of intense cold on bacteria. Science 13:323. 1901.

38. Prudden, J. M. On bacteria in ice and their relation to disease, with special reference to the ice supply of New York City. Medical record 31:341-350, 369-378. 1887.

39. Richardson, W. D. and E. J. Scherubel. The deterioration and commercial preservation of flesh foods. I. General introduction and experiments on frozen beef. Journal of the American chemical society 30:1515-1564. 1908.

40. Record, B. R. and R. Taylor. Some factors influencing the survival of Bacterium coli on freeze-drying. Journal of general microbiology 9:475-484. 1953.

41. Smart, H. F. Growth and survival of microorganisms at sub-freezing temperatures. Science 82:525. 1935.

42. Smith, A. U., C. Pogle, and J. Smiles. Microscopic

observations of living cells during freezing and thawing. Journal of the Royal microscopic society 71:186-195. 1951.

43. Smith, E. F. and D. B. Swingle. The effect of freezing on bacteria. Science 21:481-483. 1905.

44. Squires, R. W. and S. E. Hartsell. Survival and growth initiation of defrosted Escherichia coli as affected by frozen storage menstrua. Applied microbiology 3:40-45. 1955.

45. Stille, B. Uber den verlauf des absterbens von mikroorganismen bei wiederholtem gefrieren. Archiv fur mikrobiologie 13:293-300. 1943.

46. Sulzbacher, J. Effect of freezing and thawing on the growth rate of bacteria stored in ground meat. Food technology 6:341-343. 1952.

47. Tanner, F. W. and G. I. Wallace. Effect of freezing on microorganisms in various menstrua. Proceedings of the society of experimental biology and medicine 29:32-34. 1931.

48. Turner, T. B. The preservation of virulent Treponena pallidum and Treponema pertenue in the frozen state with a note on the preservation of filterable viruses. Journal of experimental medicine 67:61-78. 1938.

49. Turner, T. B. and N. L. Brayton. Factors influencing the survival of spirochetes in the frozen state. Journal of experimental medicine 70:639-650. 1939.

50. Vass, A. F. The influence of low temperature on soil bacteria. Cornell university agriculture experiment station memorandum 27:1043-1047. 1919.

51. Vereshchagina, V. Freezing butter starters (Zamorazivanie zakvaski dlya mosla). Molochnaya promyshlennost 7:3-6. 1940. (Abstracted in Dairy science abstracts 10:125. 1948-49.)

52. Wallace, G. I. and F. W. Tanner. Microbiology of frozen foods. I. Historical revue and summary of results. Fruit products journal 13:52-54. 1933.

53. Weiser, R. S. and C. O. Hargiss. Studies on the death of bacteria at low temperatures. II. The comparative effects of crystallization, vitromelting, and devitrification on the mortality of Escherichia coli. Journal of bacteriology 52:71-79. 1946.

54. Weiser, R. S. and C. M. Osterud. Studies on the death of bacteria at low temperature. I. The freezing temperature, repeated fluctuation of

temperature, and the period of exposure to freezing temperature on the mortality of Escherichia coli. Journal of bacteriology 50:413-439. 1945.