REACTIVATION OF STORED LACTIC STARTER CULTURES WITH A BACTERIAL PROTEOLYSATE OF MILK AND THEIR PRESERVATION WITH DIATOMACEOUS EARTH

by

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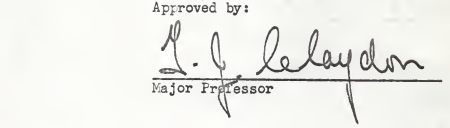

Major Professor

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INTRODUCTION

Historically, the use of lactic starter cultures is an old practice in the dairy industry. Lactic starters are essential in cheese making and in cultured dairy products manufacturing. It is well established that the length of time needed for processing such products is greatly dependent upon the rate of acid production by the starter. In addition, the quality of the finished product depends to a certain degree upon starter activity. Economic loss due to failure of lactic starters to produce enough acidity is not uncommon. Reactivation of slow lactic starters has been considered by many investigators.

In practice, as a means of facilitating propagation of lactic starters, various types of storage and preservation have been utilized. Such practices often result in reduced activity in acid production. Storage of lactic starters without losing normal activity would be of economic and technical importance for both cheese makers and for manufacturers of other cultured dairy products. From the technical standpoint, the common practice of daily transfer of cultures would be eliminated. In addition, the possibility of contamination would be greatly reduced. Moreover, desirable characteristics of a well-balanced starter culture such as aroma and flavor would more likely be maintained. From the economic point of view, savings in time, equipment, materials, and media could also be attained.

Methods used in the preservation of lactic starter cultures have involved low-temperatures storage, freezing, and freeze-drying under various conditions and with various protective additives. Although some methods are reported to maintain starter activity, a decline in rate of acid production with storage time is well known.

In recent years there has been interest in the addition of stimulatory substances to accelerate activity of lactic starters. Most of the work

reported has involved the effect of these substances on stimulating fresh lactic starters. It seems probable that such substances might be used to reactivate starters upon propagation after storage.

The work reported in this thesis was undertaken to evaluate a Pseudomonas fluorescens proteolysate of milk in maintaining and reactivating stored lactic starters. It was further directed towards determining the feasibility of preserving starter cultures with diatomaceous earth.

LITERATURE REVIEW

Investigations by many workers on the nutritional requirements of lactic starter cultures showed that certain nutrients are essential for growth. Most of the latest literature indicates that considerable attention has been devoted to the influence of protein fractions, peptides, and amino acids on growth of lactic cultures.

Amino Acids and Peptides as Growth-Promoting Factors for Lactic Starter Cultures

The need for certain amino acids or certain peptides in any media for growing lactic starter cultures has been reported by many investigators. Much of the work in this area has been reviewed by Koburger (16) in which importance of various amino acids and peptides for the growth of lactic starter cultures has been indicated. Investigation by Nivin (23) showed the need of 14 amino acids for rapid growth of lactic starters. He also pointed out that both asparagine and glutamine were required in a synthetic media for Streptococcus cremoris. The results of Wright and Skeggs (42) on the need of lactic starters for asparagine and glutamine agreed with those of Nivin (23). Wright considered that these two substances were involved in

the synthesis of more highly active compounds which function in the nutrition of the more fastidious strains of lactic starters. Pollack and Manfred (26) reported on the response of lactic starters to both glutamine and glutamic acid when added in minute amounts. The need for such substances for growth was explained as being involved in the synthesis of cell proteins. In addition, these substances might act as a catalyst in the metabolism of carbohydrates. In an investigation by Macleod and Gordon (22) it was found that four lactic starters were able to hydrolyze some commercially prepared dipeptides to their amino acid components which in turn were readily utilized by the cultures. Work by Anderson and Elliker (2) showed rapid growth in lactic starters upon addition of a combination of amino acids, while slight or depressed growth was observed when individual amino acids were added.

Woolley and Hutchings (40) reported the failure of certain strains of haemolytic streptococci to grow in the absence of glutamic acid and tryptophane.

Vitamins and Other Substances in the Nutrition of Lactic Starter Cultures

Vitamins are of no less importance than amino acids and peptides in the nutrition of lactic starter cultures. The effects of their combined action with other nutritional factors is frequently reported in the literature.

Investigation by Nivin (23) on the vitamin requirements of lactic starters showed that four vitamins were essential for efficient growth. These were biotin, pantothenic acid, nicotinic acid and thiamine. The findings of Anderson and Elliker (1) on the requirements of lactic starters for the above vitamins agreed with those of Nivin (23). In addition, it was found that riboflavin was essential for growth of some strains of lactic starters. Work by Anderson and Elliker (2) showed that certain individual vitamins gave rapid growth, while other depressed the growth of lactic

starters. Other workers, Williams et al. (39) and Kihara et al. (19), showed the need for vitamin B_6 in efficient utilization of certain dipeptides by Streptococcus faecalis. Fatty acids alone or combined with the vitamins were reported by Williams (39) to be important in the growth of several strains of lactic acid bacteria.

Relation of Proteolytic Action of Lactic Starter Cultures to Acid Production

Proteolytic activity of lactic starters has been shown to be associated with rapid acid development. For this reason, proteolytic properties of starters have been studied by a number of investigators. The literature reviewed by Koburger (15) indicated the variability of lactic starters in their proteolytic characteristics. In addition, rapid coagulation of milk was associated with proteolysis. Anderegg and Hammer (3) reported on the effect of $Ca(CO_3)_2$ in acceleration of proteolysis by certain strains of Streptococcus lactis grown in milk. They noted a reduction in the time needed for proteolysis, resulting in more rapid growth of the organisms and earlier coagulation. Associate organisms like Streptococcus citrovorus and Streptococccus paracitrovorus failed to cause any proteolysis when grown in milk.

Speck and Williamson (32) reported that less proteolysis occurred when enrichment was made with stimulatory peptides such as pancreas extract. Peterson et al. (25) considered the formation of non-protein nitrogen, (ammonia nitrogen, and amino nitrogen) to be measures of the proteolytic activity of lactic starter cultures.

Stimulation of Lactic Starter Cultures by Added Substances

The problem of slow starters is frequently encountered by cheese makers and cultured dairy products manufacturers. Investigations have shown that, experimentally, the problem can be partially overcome by adding stimulatory substances to the milk.

Kennedy (17) attributed the stimulatory properties of various extracts of plant and animal origin to certain peptides which are readily available for utilization by bacteria. Anderson and Elliker (2) reported that stimulation of lactic starter cultures by such substances as enzymatically hydrolyzed milk and liver fractions was due to a peptide or a peptide-like compound which contained glutamic acid. Work by Woolley (41) showed certain derivatives of glutamic acid were stimulatory. In addition, a certain structure of these derivatives was found essential for the stimulation effect. An investigation by Kennedy and Speck (18) showed a stimulating effect of corn steep liquor on acid-producing organisms as well as flavor and aroma-producing bacteria. An increase in numbers was obvious in case of the first group, while this effect was not detected in the second group. Morphological study showed formation of longer chains and more swollen cells than in control cultures. Work by Speck, et al. (34) showed the response of single and mixed-strains of streptococci to different stimulatory substances such as pancreas extract, liver, and yeast extracts. This stimulation was greater with slow starters than with fast starters. Leuconostoc citrovorum growth was stimulated, as measured by colony count, when pancreas extract was added.

Results of Storrs and Anderson (36) showed that the stimulatory action of yeast extract on lactic starter cultures was partially due to an increase in numbers, but more to an increase in the fermenting capacity

of the cells. Their results agreed with those of Speck et al. (34) and Koburger and Claydon (15), in that greater response to various stimulatory substances was observed with slow starters than with fast starters. However, the activity of the stimulated slow starters did not equal that of fast starters. The combined effect of yeast extract and chalk on lactic starters was investigated by Braz and Allen (4). Chalk was found to increase the viability of the cells by neutralizing the extra acidity development due to yeast extract addition. Increase in acidity development due to various stimulants was found to arise from increased fermenting capacity of the cells rather than from an increase in bacterial numbers.

Garvie and Mabbit (11) stated that the increase in activity of lactic starters was found to be temporary, unless the addition of the stimulant was continued. Loss of activity in starter cultures was explained as arising from the inability of cells to utilize the protein of milk. Investigation by Claydon and Koburger (7) on a filtrate from milk cultures of Ps. fluorescens, showed that it stimulated lactic starters when 1% was added to the milk. Two out of the eight fractions prepared from the filtrate were found to stimulate lactic starters. These were a solvent precipitated fraction and the amino acids fraction. Work by Koburger and Claydon (15) showed that stimulation was partially due to amino acids, and partially due to an enzyme. A total of 17 amino acids was detected by chromatographic technique. In addition cystine and cysteine were suspected. Much of the literature reviewed by Koburger and Claydon (15) on the nature of stimulatory factors, indicated that stimulation in enzymatically hydrolyzed proteins was due to impurities that accompanied the enzyme.

Sandine et al. (30) reported that the stimulating action of the peptides depended upon certain amino acids and the arrangement of those amino acids

within the peptide molecule. Fast recovery of lyophilized starter cultures due to stimulation by enrichment with pancreas extract was reported by Koburger and Speck (14). Size of inoculum needed was 25 and 12.5% less than the recommended level, when 0.2% pancreas extract was added.

Investigation by Smith (35) showed that the stimulatory factor that is present in yeast extract appeared to be organic in nature. A recent literature review by Speck (33) showed the importance of various factors that might cause better growth of lactic starter cultures. In addition, the importance of supplementation of milk with nutrients and stimulants was indicated.

Preservation of Lactic Starter Cultures

Various methods have been used to preserve lactic starter cultures and to minimize the necessity for frequent propagation.

Preservation by freezing. Extensive work has been conducted in the past decade on freezing lactic starter cultures. Freezing liquid cultures, or liquid cultures containing additives has been investigated.

Work by Martin and Cardwell (21) showed the possibility of maintaining activity of ripened cultures by freezing at - 20 F. The practice of freezing, then thawing, transferring and refreezing again every 60 days, kept starter cultures active up to one year. Cottage cheese made with these starters scored very much the same as that made with starters that had been transferred daily. Investigation by Simmons and Graham (37) on immediate freezing at -20 F after inoculation showed that activity of the starters could be maintained up to 6 months. Thawing was conducted in a waterbath at 70 F for 2 hr. Cultures then were transferred and incubated at 70 F for 16 hr. These starters were used successfully in cottage cheese and buttermilk making.

Ruonik and Glenn (29) reported that freezing cultures at -18 ± 3 F after 15-20 hr incubation, maintained culture activity up to 150 days. Thawing was conducted by allowing the culture to stand until it became loose from the sides of the container, then the culture was dumped in the milk where thorough thawing was obtained. Investigation by Lindgren and Swartling (20) on various methods of freezing lactic cultures showed that the highest activity was maintained by immediate freezing of the cultures after inoculation. Work by Seas et al. (31) on two methods of freezing cultures, at 0 to -10 F and -68 F showed little or no advantage in using very low freezing temperatures such as -68 F. Work by Farmer and Hammer (9) showed the possibility of maintaining butter cultures frozen at -10 C up to about five months. Holding ripened cultures at various temperatures showed that bacterial cells survived for about 2 months when stored at -10 C. This compared with survival for 1, 3, 7 and 30 days when stored at 37, 30, 21 and 7 C respectively.

Preservation by freeze-drying. From the literature reviewed, this method was frequently reported in preserving lactic starter cultures.

Investigation by Lindgren and Swartling (20) showed that freeze-drying cultures maintained their activity so that it equaled that of the frozen starters. On the other hand, destruction of the bacterial cells was more in the former method. Results of Richardson and Calbert (27) on dry-ice frozen liquid cultures and lyophilized cultures stored at -28 F showed a greater drop in the activity of the frozen than the lyophilized cultures after 6 months of storage. However, higher acid production was obtained by the less active frozen cultures than by the active lyophilized cultures on prolonged incubation. Work by Richardson (28) on storing lyophilized cultures at 2 to 5 C and -23 to 28 C showed a significant loss in activity of cultures stored at higher temperatures. In addition lyophilized cultures packed under air, vacuum and

nitrogen showed no significant effect due to atmospheric conditions. Investigation by Czulak and Hammond (8), showed that lyophilized cultures stored at room temperature maintained their activity from 6 to 16 months when propagated in litmus-milk-chalk after storage.

Other methods for preserving lactic starter cultures. In addition to the previous two methods of preserving starter cultures, several modifications have been reported. Certain additives have been tested to determine their value in preserving cultures.

Glycerine addition to preserve cultures was reported by Heinemann (12), Richardson (28) and Olson (24). Heinemann (12) showed that 20% added glycerine and storage at 35 F maintained activity of the starters up to two months. Furthermore, glycerine addition and storage at 5 and -20 F maintained the activity of the starters up to 6 months. There was a sharp decline in the activity of controls when kept under the same storage conditions. Richardson (28) showed that glycerine when added at a rate of 10% to cultures before freezing them maintained their activity. Also they produced a higher acidity than controls. Investigation by Olson (24) showed that a combination of 20% glycerol, 3% salt and 30% sucrose with or without $Ca(CO_3)_2$ and storage at 45-50 F was most effective in preserving starter culture activity. Except $Ca(CO_3)_2$, none of the above compounds when used alone aided in preserving the starters. Addition of $Ca(CO_3)_2$ and storage at 45-50 F maintained the cultures activity up to 8 months.

Work by Johns (13) showed the effect of neutralizing the ripened starter with 40% sterile NaOH to 0.16% acidity on maintaining the activity of cultures on freezing. It was suggested that the neutralizing agent eliminated the increase in concentration of acid resulting from withdrawal of water in the

form of ice crystals. Plate counts on the neutralized starter after 2 months of storage were higher than the non-neutralized starters after direct freezing.

Other modifications such as starch broth, agar slopes under paraffin oil, and chalk addition were investigated by Lindgren and Swartling (20), but none of the above additives appeared to be promising.

Recent commercial literature (14) described the high water absorption capacity of a diatomaceous earth[1] product, and the possibility of utilizing this substance in the food industry. The product was reported to be mainly composed of calcium silicates, plus other mineral oxides. It was recommended as an anti-caking agent, as a grinding aid, and as a liquid carrier. Because of such properties it could be used to convert liquids to dry, free flowing powders. Although various uses of this substance were reported, nothing was mentioned about its possible use in preserving bacterial cultures. Because of the high absorption capacity, and other characteristics of the product, it might be of value in preserving lactic starter cultures.

Other Factors Influencing Reactivation of Stored Lactic Starter Cultures

Various conditions and treatments prior to and during storage have been shown to affect the activity of lactic starter cultures on subsequent propagation.

Investigation by Watts and Calbert (38) showed the great effect of the physiological age of the cultures at the time of lyophilization on subsequent propagation. The best age for maximum acid production was found to be between

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[1]/Micro-Cel - A product of Johns-Manville Company.

the late logarithmic phase up to the late maximum stationary phase. The practice of neutralizing the acidity of the cultures at the time of lyophilization to the normal range of milk did not increase the activity of the cultures after lyophilization. A range of 0.4 to 1.% (normal range of growth) titratable acidity at the time of lyophilization did not seem to affect the activity of the cultures upon subsequent propagation. However, when lyophilized cultures were stored at room temperature, a marked drop in their activity was detected after two weeks. On the other hand, a very slight drop in activity was detected after storage at hardening room temperatures for 12 weeks. In addition, other factors, such as individual culture characteristics, and the medium used at initial propatation were found to affect the activity of lyophilized cultures upon propagation. Foster's work (10) showed the effect of acidity and physiological age of the culture at the time of lyophilization on culture activity upon propagation. Streptococcus lactis cells from 15 to 18 hr old cultures were more active than older cells. Cultures in the late logarithmic phase or early stationary phase, when suspended in a favorable medium like milk at pH 7.0 gave maximum activity upon propagation. Work by Cardwell and Martin (5, 6) showed that there was no significant difference in the activity of the cultures when either fast or slow methods of freezing were used. Also, rate of ripening the cultures before freezing did not appreciably affect the activity of the starter. However, on subsecuent propagation less acid production was obtained when cultures were incubated for 12 hr before freezing than when cultures were incubated for 16 and 20 hr. A medium containing 14% NMS at initial propagation was found satisfactory in maintaining the activity of ripened cultures upon freezing.

Of the various methods of thawing frozen cultures, Cardwell et al. (6) reported that slow thawing at 45 F for 8 hr, or direct dumping of the frozen

culture in the milk was the most satisfactory for optimum acidity development by the cultures. Farmer and Hammer (9) showed that cultures withstood freezing and thawing from 5-7 times without a change in their activity. When this practice was repeated 7-10 times, there was a delay in coagulation on subsequent propagation, but aromas and flavors were quite satisfactory. When thawing and freezing were repeated 10-15 times, transfers failed to coagulate.

EXPERIMENTAL PROCEDURE

I. Evaluation of Pseudomonas fluorescens Milk Proteolysate in Reactivating Stored Lactic Starter Cultures

Preparation of media. Reconstituted skim milk was used throughout this study for preparation of the Ps. fluorescens proteolysate and for propagation of lactic starter cultures. Nonfat milk solids (NMS) which had been pretested to insure the absence of any inhibitory substances was reconstituted in distilled water to 9% solids. The milk was dispensed in 500 ml quantities into 1-liter Erlenmeyer flasks, and in 100 ml quantities into 250 ml dilution bottles, then autoclaved at 15 lb pressure for 17 min. After cooling, it was kept refrigerated until used.

Preparation of Pseudomonas fluorescens milk proteolysate. Stock cultures of Ps. fluorescens used in this study were propagated by weekly transfer in litmus milk. Incubation was at 25 C for two days. After development, cultures were kept refrigerated until needed for a new transfer. To prepare the proteolysate, about 0.2 ml of a fresh stock culture was transferred to 500 ml of reconstituted sterile skim milk in a 1-liter Erlenmeyer flask. After shaking, the flask was incubated at 25 C for 10 days. At this time, the milk appeared to be well proteolyzed and the pH value ranged from 7.0 to 7.2. Work by

Claydon and Koburger (7) showed that satisfactory proteolysis was obtained after such a period of incubation.

To obtain the sterile proteolysate, two different methods were used. The first and the main method was by treatment with 95% ethyl alcohol. The second was by Seitz filtration.

In the first method alcohol was added to the proteolyzed milk at rate of 25% by volume. After mixing, the preparation was centrifuged from 15-20 min at 800 g. After centrifuging, the supernatant was decanted. Most of the alcohol was removed from the supernatant by evaporation for 1 hr under reduced pressure (aspirator) in a water bath at 115-120 F. The treated proteolysate was then dispensed aseptically into dilution bottles in 100 ml quantities and stored at -5 C until used. All glassware used was sterilized by rinsing with ethyl alcohol.

The proteolysate was checked initially and periodically during storage for sterility by streaking on standard agar plates. Plates were incubated at 25 C for 3 days. After preliminary testing, the 25% alcohol treatment was found to give a satisfactorily sterile proteolysate.

Since it was possible that alcohol and evaporation treatment might affect some nutrient factor, some proteolysate was prepared by Seitz filtration for comparison. The sterile preparation was stored at -5C until used. However, since only limited amounts could be obtained and since the proteolysate prepared by the first method gave similar results to that prepared by the second method, therefore the proteolysate used in the study was prepared by treatment with ethyl alcohol.

Propagation of lactic starter cultures. Lactic starter cultures used were multiple type, mixed strain cultures obtained from commercial laboratories.

Reconstituted nonfat milk solids, described under media preparation was the medium of propigation throughout the study. In propagation from lyophilized cultures inoculations were made at rates varying from 30 mg to 1 g per 100 ml of milk. Transfers from liquid cultures were at the rate of 1% unless otherwise indicated. In all trials incubation was at 21 C.

Reactivation of lyophilized lactic starter cultures. A total of ten powdered cultures was propagated at two different levels of inoculum. The 50 mg/100 ml milk was the level recommended by the manufacturer, another level of 30 mg/100 ml milk was used for comparison and to determine whether smaller inocula could be used when proteolysate was included. Each level was inoculated in duplicate into 100 ml lots of autoclaved milk. One lot of each level constituted the control. To the other lot 1% of the Ps. fluorescens proteolysate was added.

After mixing, 9 ml quantities from each lot were dispensed into each of six sterile screw cap tubes and incubated at 21 C. Titratable acidities were measured by titrating two tubes from each lot after 12, 14 and 16 hr incubation using 0.1 N NaoH and phenolphthalein as an indicator. Results were reported as the average of two titrations.

In order to determine whether the amount of initial inoculum from powder affected the subsequent activity of cultures, two of the previous lactic cultures were propagated a second time using 1% liquid inoculum. Propagations were made from the controls and from enriched portions of each culture. Proteolysate was again added to the previously enriched cultures - but not to the controls. Tests for acid development were made in the same manner as before, except that four tubes were used for each lot instead of six. Titrations were made after 10 and 12 hr incubation.

Reactivation of liquid lactic starter cultures stored at 5 C. Three different lactic starter cultures were used in this study. Initial propagations were made from the lyophilized form using 50 mg of inocula per 100 ml milk. Duplicate lots of 100 ml were prepared from each culture. One of the duplicates served as a control (no proteolysate), and the other was enriched with 1% proteolysate. Incubation was at 21 C for 16 hr. Following incubation, the starter cultures were stored at 5 C until checked for activity at 4, 10, 14 and 21 days of storage.

In testing the effect of the proteolysate on the activity of stored cultures, both control and enriched portions were again subcultured with and without added proteolysate. This provided comparisons between cultures that were never enriched; those enriched at both propagation; those enriched only on the initial propagation; and those enriched only at the propagation following storage. Figure 1 explains the propagation and enrichment procedure.

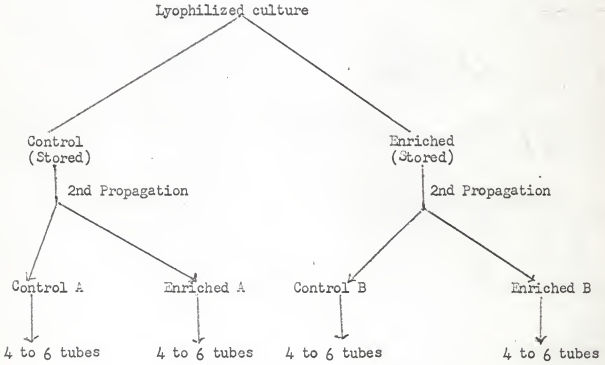

Fig. 1. Scheme of propagation and enrichment of cultures.

Tests for acidity development were conducted in the same manner as reported previously, using four tubes from each subculture and incubation for 15 and 17 hr.

Reactivation of liquid lactic starter cultures stored at -5 C. A total of 14 lactic starter cultures was used in this study in two separate trials. In the first trial, initial propagation of eight cultures was made from the lyophilized form using 50 mg. of inoculum per 100 ml milk. Duplicate lots of 100 ml were prepared from each culture. One of the duplicates served as a control (no proteolysate), and the other was enriched with 1% proteolysate. Incubation was at 21 C for 16 hr. A second transfer was made with similar treatments to the first propagation, so that control cultures were left as controls and those originally enriched cultures were enriched again, but incubation here was for 10 hr so that the cultures were not fully ripened. Following incubation, the starter cultures were stored at -5 C until checked for activity after 1 and 2 months of storage.

In testing the effect of the proteolysate on the activity of stored cultures, cultures removed from storage were allowed to stand at 5 C for about 4 hr. They were then held at room temperature for about 15-20 min, where complete thawing was obtained. Both controls and enriched cultures were subcultured at the rate of 3% inoculum with and without added proteolysate in the same manner as indicated in Figure 1. The cultures were incubated at 21 C for 12 and 14 hr. Testing for acidity development was conducted in the same manner as described previously.

To determine the effect of enrichment and freezing on subsequent propagations, a second transfer was made from the reactivated cultures in the same manner as before but using 1% inoculum. To accomplish this both the control

cultures and the originally enriched cultures were propagated with and without proteolysate. Activity of the refrozen cultures was again retested in a similar manner after 2 months storage.

The second trial was conducted on six additional lactic starter cultures. In this trial, initial propagation from lyophilized form was at rate of 1 g per 100 ml milk instead of 50 mg. After the second transfer, the cultures were stored at -5 C for 5 months. After this storage period the cultures were checked for activity by propagating the cultures at the rate of 5% inoculum. Incubation in this case was for 24 hr on the first transfer, and for 12 and 14 hr on the second. One per cent inoculum was used on the second transfer.

II - Evaluation of Diatomaceous Earth as a Potential Agent for Preserving Lactic Starter Cultures

The diatomaceous earth used in this study was a special product prepared for use as an additive to assist in drying food products.[1] Because of its high water absorption capacity and ability to convert liquids to flowing powders, it seemed desirable to test this product in preserving lactic starter cultures.

In order to insure its sterility, the diatomaceous earth was exposed in thin layers to dry heat at 170 C for 15 hr. Following heat treatment, the earth was stored in closed jars at room temperature until used. It was checked for sterility periodically by plating procedures. Glassware and other equipment used in mixing the liquid cultures with the earth were sterilized by autoclaving or rinsing in quaternary ammonium solution of 500 ppm.

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[1] Micro-Cel, Grade E. Provided by Johns-Manville, 22 East 40th St. New York, N. Y.

Blending liquid lactic starter cultures with diatomaceous earth. Ripened lactic starter cultures from fresh propagations were mixed with diatomaceous earth in a ratio of 3:1 in a Waring blender. In order to minimize air-borne contamination, the blending operation was carried out under a fume hood previously sprayed with a sodium hypochlorite solution of 500 ppm. Twenty-five g of the earth was placed in the blender jar and covered with aluminum foil. By means of a separatory funnel mounted on a ring stand 75 ml of liquid starter culture was slowly added to the diatomaceous earth through a small hole in the aluminum foil and with the blender in operation. To insure satisfactory mixing it was necessary to stop the blender occasionally and stir the mixture with a sterile spatula. Even with this procedure it was difficult to obtain uniform mixing. Different lots varied in consistency from a moderately dry powder to a granular or paste-like product. For simplicity the final product was called (DTEC) diatomaceous earth culture.

The blended product from each starter was dispensed aseptically into 8 sterile vials. Four vials were stored at each of 5 and -30 C for 21 and 60 days respectively. Portions of the liquid cultures were also stored at the same temperatures for comparison.

Testing for culture activity. Tests for activity of DTEC were made initially within 2 hr of preparation and after the storage periods indicated above. Comparisons were made with the untreated liquid cultures held under similar conditions. In a number of cases plate counts of DTEC and liquid cultures were made at the same time.

Three pairs of screw cap test tubes containing 9 ml autoclaved milk were inoculated with 1.0, 0.5, 0.1 g of DTEC. A fourth pair was inoculated with 0.1 ml of the control liquid culture for comparison. In several trials 0.5 g of

the sterile diatomaceous earth was added to the tubes containing liquid culture to test for possible inhibitory effects. The inoculated tubes were incubated at 21 C for 20 hr and tested for acidity development as previously described.

RESULTS AND DISCUSSION

I. Effect of Ps. fluorescens Milk Proteolysate in Reactivating Stored Lactic Starter Cultures

Reactivation of lyophilized cultures. The effect of Ps. fluorescens proteolysate on stimulating ten lactic starter cultures on the first transfer, from powdered form after 14 and 16 hr incubation is shown in Table 1. Titratable acidity values after 12 hr incubation were not reported because of insignificant acidity development.

Table 1. Effect of Ps. fluorescens proteolysate on activity of lactic starters, on the first propagation from lyophilized cultures as measured by titratable acidity.[a]

	Incubation at 21 C for:							
	14 hr				16 hr			
	Amount of inoculum				Amount of inoculum			
Starter	30 mg		50 mg		30 mg		50 mg	
No.	Control[b]	Enriched[c]	Control	Enriched	Control	Enriched	Control	Enriched
3	0.29	0.37	0.35	0.38	0.37	0.41[d]	0.37	0.46[d]
6	0.31	0.34	0.35	0.41	0.36	.44[d]	0.42	0.52[d]
8	0.22	0.26	0.24	0.28	0.24	0.29	0.26	0.29
9	0.23	0.28	0.30	0.32	0.40	0.53[d]	0.49	0.60[d]
21	0.28	0.31	0.30	0.34[d]	0.39	0.39	0.36	0.42
26	0.35	0.47[d]	0.44	0.58[d]	0.49	0.60[d]	0.54	0.73[d]
27	0.38	0.51[d]	0.37	0.55[d]	0.43	0.69[d]	0.46	0.70[d]
10	0.35	0.36	0.39	0.43	0.44	0.50[d]	0.50	0.56[d]
11	0.32	0.40	0.38	0.45	0.40	0.50[d]	0.45	0.57[d]
12	0.26	0.28	0.27	0.30	0.28	0.31	0.20	0.35
Avg.	0.30	0.36	0.34	0.40	0.37	0.47	0.41	0.52

a Average of 2 trials with each culture
b No proteolysate
c Proteolysate added
d Coagulation occurred

The figures in the above table represent the average of two trials with each culture with inoculum of 30 and 50 mg of powdered culture per 100 ml autoclaved milk. Acidity levels obtained on this first propagation were low even after 16 hr incubation. However in most cases enriched cultures were considerable higher than the controls. The average increases in titratable acidity of the enriched cultures over the controls after 16 hr of incubation were 0.10 and 0.11 percentage point for 30 mg and 50 mg inocula respectively, or 27% in both cases. Although the percentage rate of stimulation was equal in both levels of inoculum, high acidity values were obtained with the higher level of inoculum after 16 hr of incubation. There was considerable variation among cultures both in level of acidity and amount of stimulation exhibited. Coagulation was observed in two out of ten enriched starters at both levels of inoculum after 14 hr of incubation. At 16 hr incubation, coagulation occurred in seven out of ten enriched starters at both levels of inoculum. None of the controls showed any signs of coagulation within the experimental incubation periods.

It appeared that the recommended inoculation level of 50 mg per 100 ml milk was too low for satisfactory acid development on initial transfer. However, the Pseudomonas proteolysate stimulated acid development to nearer the desired level in most cases.

In order to determine the effect of the proteolysate and rate of initial inoculation on subsequent propagation, two of the ten cultures were repropagated after 4 days of storage at 5 C. Inoculation was at the rate of 1% liquid culture with or without added proteolysate. The results are presented in Table 2.

Table 2. Effect of rate of initial inoculum and proteolysate addition on acid production by lactic starter cultures on the second transfer as measured by titratable acidity.

Starter No.	Size of initial inoc. and treatment		Incubation at 21 C for 10 hr	12 hr
K-8	30 mg	Control[a]	0.75	0.77
	30 mg	Enriched[b]	0.76	0.81
	50 mg	Control	0.73	0.77
	50 mg	Enriched	0.77	0.80
K-9	30 mg	Control	0.71	0.80
	30 mg	Enriched	0.73	0.82
	50 mg	Control	0.72	0.79
	50 mg	Enriched	0.76	0.81

[a]No proteolysate
[b]Proteolysate added again at 2nd transfer

From the figures in the above table, it is evident that the size of the initial inoculum made little difference in starter activity on the 2nd transfer. Slight stimulation was obtained due to addition of the proteolysate in both starters especially after 12 hr incubation. Both cultures were very active and this may have limited the stimulatory effect of the proteolysate.

Reactivation of liquid cultures stored at 5 C. Average titratable acidity values from four trials with each of three lactic cultures after storage periods up to 21 days are presented in Table 3. In the initial propagations from commercial lyophilized cultures, titratable acidities were low, but higher in cultures containing added proteolysate. Following the first transfer the starters were stored at 5 C until checked for activity after 4, 10, 14 and 21 days.

When cultures were propagated after four days of storage, they developed considerably higher acidities than on the first propagation from

Table 3. Effect of Ps. fluorescens milk proteolysate on restoring activity of lactic starter cultures stored at 5 C, as measured by titratable acidity.1/

Starter No.	Initial propagation titratable acidity before storage		Storage period in days	Incubation at 21 C: Propagation after storage Incubation for 15 hr			
				Control		Enriched B	
	Control	Enriched		Control A	Enriched A	Control B	Enriched B
1	0.37	0.43	4	0.67	0.80	0.55	0.57
			10	0.78	0.84	0.56	0.86
			14	0.57	0.63	0.34	0.62
			21	0.51	0.57	0.25	0.27
2	0.30	0.35	4	0.82	0.84	0.62	0.85
			10	0.60	0.65	0.38	0.66
			14	0.53	0.62	0.36	0.51
			21	0.27	0.31	0.25	0.26
3	0.25	0.26	4	0.78	0.84	0.56	0.86
			10	0.58	0.63	0.41	0.63
			14	0.42	0.52	0.38	0.50
			21	0.30	0.38	0.26	0.27

Control A, No proteolysate was added.
Enriched A, proteolysate was added (1st time).
Control B, proteolysate was discontinued after storage.
Enriched B, proteolysate addition was continued.

1/ Avg of 4 trials with each starter.

powder. When stored cultures containing no proteolysate (Control) were propagated with or without proteolysate (Enriched A and Control A), a definite increase in titratable acidity was obtained in all cases from the added proteolysate. The amount of stimulation varied with the different cultures, but averaged 0.09 percentage point or 11%.

When stored cultures containing proteolysate (Enriched) were propagated with or without added proteolysate (Enriched B and Control B), a striking difference was obtained in titratable acidity. The cultures from which the proteolysate was withheld dropped in activity below those which had never been enriched (Control A). This is clear upon comparison of the two columns (Control B) and (Control A) in Table 3. However, cultures that continued to receive proteolysate (Enriched B) developed the highest acidities of all and averaged 0.22 percentage point or 40% above cultures from which the proteolysate was withheld (Control B). The amount of stimulation again varied with the different cultures.

After 10 and 14 days of storage the effect of the proteolysate was generally the same as after four days storage. A marked drop in activity was obtained when cultures that contained proteolysate on initial transfer before storage were deprived of this stimulant on propagation after storage (Control B). Activity of the starter cultures decreased with storage and after 21 days cultures were too inactive for practical use. After this period of storage the proteolysate generally was incapable of restoring activity.

Reactivation of liquid cultures stored at -5 C for 1 month. The results on the effect of Ps. fluorescens milk proteolysate in restoring activity of eight frozen cultures after 1 month of storage at -5 C are shown in Table 4.

On the second transfer and before freezing the cultures, titratable acidities were low, but were higher in cultures containing added proteolysate.

When the cultures were propagated after 1 month of storage, there were striking differences in acidity levels among the different cultures, but stimulation was observed in all the enriched cultures. When stored cultures containing no proteolysate (Control) were propagated with or without added proteolysate (Enriched A and Control A), a definite increase in titratable acidity was obtained in all cases from the added proteolysate. The amount of stimulation varied with the different cultures but averaged 0.07 and 0.13 percentage points or 23 and 29.5% after 14 and 16 hr incubation, respectively.

When stored cultures containing proteolysate (Enriched) were propagated with and without added proteolysate (Enriched B and Control B), the cultures from which the proteolysate was withheld developed much less acid than those that continued to receive proteolysate, the difference being quite striking, with some cultures (Control B). However, six out of the eight cultures from which the proteolysate was withheld developed acidities higher than those which had never been enriched (Control A) after 14 hr of incubation, while five cultures showed such a characteristic after 16 hr incubation.

The overall average for the eight cultures that continued to receive the proteolysate (Enriched B) was the highest of all and averaged 0.23 and 0.16 percentage point or 66 and 36% above cultures never receiving proteolysate (Enriched A) after 14 and 16 hr of incubation respectively. They also developed higher acidity than those cultures receiving proteolysate for the first time (Enriched A) when propagated after storage, but the rate of stimulation was higher after 14 hr than after 16 hr incubation. The amount of stimulation varied with the different cultures.

Table 4. Effect of Ps. fluorescens milk proteolysate on restoring activity of lactic starter cultures stored at −5 C for 1 month as measured by titratable acidity.

Starter No.	Incubation for 10 hr Titratable acidity at freezing		Propagation after storage: Incubation at 21 C for 14 hr				16 hr			
			Control		Enriched		Control		Enriched	
	Control	Enriched	Control A	Enriched A	Control B	Enriched B	Control A	Enriched A	Control B	Enriched B
3	0.30	0.30	0.37	0.42	0.39	0.42	0.47	0.55	0.45	0.53
6	0.55	0.73	0.43	0.53	0.34	0.37	0.58	0.73	0.45	0.53
21	0.29	0.35	0.34	0.49	0.44	0.74	0.37	0.66	0.48	0.81
26	0.41	0.66	0.40	0.50	0.49	0.60	0.49	0.64	0.63	0.75
27	0.37	0.55	0.38	0.43	0.45	0.59	0.46	0.58	0.56	0.74
10	0.38	0.41	0.39	0.46	0.34	0.37	0.53	0.64	0.44	0.50
11	0.31	0.42	0.27	0.32	0.33	0.38	0.32	0.43	0.44	0.53
12	0.31	0.34	0.26	0.28	0.31	0.28	0.28	0.32	0.33	0.40
Avg.	0.36	0.47	0.35	0.43	0.38	0.58	0.44	0.57	0.47	0.60

Control A - No proteolysate.
Enriched A - Enriched with 1% proteolysate for the first time after storage.
Control B - Enrichment was discontinued after storage.
Enriched B - Enrichment was continued after storage.

Although three of the eight cultures gave normal acid development on the first transfer when proteolysate was used, a second propagation was made to further evaluate the recovery rate of the frozen starters. Inoculation was at the rate of 1% liquid culture with and without added proteolysate. The results of this second propagation are presented in Table 5.

From the titratable acidity values it is obvious that all the cultures were brought up to normal activity when the proteolysate was added, (Enriched A and Enriched B) after 14 and 16 hr incubation. In addition, all the cultures from which the proteolysate was withheld (Control B) showed higher activity than those which were never enriched (Control A) after both incubation periods. Two of the eight cultures (21 and 11) were very slow when they were never enriched with the proteolysate (Control A), and where the proteolysate was later withheld from them (Control B). On the other hand these two slow starters were stimulated from the added proteolysate but their activity still did not equal that of the fast starters. The average increase in acidity due to added proteolysate (Enriched B) above cultures which had never been enriched (Control A), was 0.24 and 0.21 percentage point or 41% and 31% after 14 and 16 hr incubation respectively. However, the cultures that continued to receive proteolysate (Enriched B) developed the highest acidities of all. The second highest acidity developed with cultures which were enriched for the first time on the second propagation (Enriched A), and the lowest with the cultures which had never been enriched (Control A).

Reactivation of liquid cultures stored at -5 C for 2 months. After 2 months of storage the effect of the proteolysate on restoring activity of frozen cultures was generally the same as after one month (Table 6), but the rate of stimulation appeared to be slightly low. Moreover, a marked drop was

Table 5. Effect of Ps. fluorescens milk proteolysate on acid production by frozen lactic starter cultures on the second transfer as measured by titratable acidity.
(1 month storage)

Starter No.	Incubation at 21 C for 14 hr: Control A	Enriched A	Control B	Enriched B	16 hr: Control A	Enriched A	Control B	Enriched B
3	0.74	0.77	.80	.83	0.83	0.89	0.89	0.89
6	0.89	0.89	0.85	0.88	0.88	0.91	0.88	0.90
21	0.32	0.74	0.37	0.76	0.36	0.77	0.38	0.85
26	0.70	0.80	0.81	0.87	0.83	0.87	0.87	0.90
27	0.50	0.67	0.66	0.77	0.58	0.81	0.72	0.83
10	0.78	0.83	0.79	0.84	0.84	0.87	0.86	0.88
11	0.43	0.71	0.83	0.84	0.54	0.82	0.85	0.88
12	0.36	0.53	0.42	0.78	0.42	0.68	0.47	0.83
Avg.	0.58	0.74	0.69	0.82	0.66	0.83	0.74	0.87

Control A – No proteolysate.
Enriched A – Enriched with 1% proteolysate for the first time.
Control B – Enrichment was discontinued after storage.
Enriched B – Enrichment was continued on propagation.

observed in the activity of all cultures. It is likely that part of the loss of activity was due to the thawing and refreezing of cultures at the 1 month period. The average increases in acid production by the cultures which continued to receive the proteolysate (Enriched B) were 0.03 and 0.05 percentage point or 11 and 17% above cultures which had never received proteolysate (Control A) after 14 and 16 hr incubation respectively. The amount of stimulation varied with the different cultures.

To evaluate further the recovery rate of the frozen starters, a second transfer was made at the rate of 1% liquid culture with or without proteolysate. The results are presented in Table 7.

It is clear from the titratable acidity values that enriched cultures (Enriched A and B) gave higher acidities due to added proteolysate. In addition, stimulation in both cases was higher than on the first propagation. The average increases in acid production by cultures enriched for the first time (Enriched A) above non-enriched cultures (Control A) were 0.10 and 0.16 percentage point or 24 and 31% after 14 and 16 hr incubation respectively. On the other hand, the average increases in acid production by the cultures which continued to receive the proteolysate (Enriched B) over their respective controls were 0.10 and 0.20 percentage point or 24% and 39% after 14 and 16 hr incubation respectively. The average level of acid production by the cultures from which the proteolysate was withheld (Control B), however, was equal to that of the cultures which had never received proteolysate (Control A), after both incubation periods. However, striking differences were observed among the different cultures. Generally, addition of the proteolysate to the cultures for the first time (Enriched A) increased the activity of seven out of eight cultures to the normal range after 14 and 16 hr incubation. The

Table 6. Effect of Ps. fluorescens milk proteolysate on restoring activity of lactic starter stored at -5 C for 2 months as measured by titratable acidity.

Starter No.	Incubation at 21 C for 14 hr				16 hr			
	Control		Enriched		Control		Enriched	
	Control A	Enriched A	Control B	Enriched B	Control	Enriched	Control	Enriched
3	0.25	0.27	0.28	0.31	0.28	0.31	0.30	0.37
6	0.24	0.26	0.25	0.26	0.24	0.26	0.25	0.26
21	0.28	0.36	0.32	0.42	0.31	0.51	0.38	0.58
26	0.31	0.32	0.24	0.26	0.34	0.40	0.26	0.26
27	0.31	0.31	0.26	0.28	0.33	0.40	0.29	0.31
10	0.30	0.31	0.27	0.29	0.31	0.38	0.29	0.31
11	0.25	0.27	0.27	0.30	0.25	0.29	0.28	0.34
12	0.25	0.26	0.24	0.26	0.26	0.28	0.25	0.27
Avg.	0.27	0.29	0.27	0.30	0.29	0.35	0.29	0.34

Control A - No proteolysate.
Enriched A - Enriched with 1% proteolysate for the first time after storage.
Control B - Enrichment was discontinued after storage.
Enriched B - Enrichment was continued after storage.

Table 7. Effect of Ps. fluorescens milk proteolysate on acid production by frozen lactic starter on the second transfer as measured by titratable acidity.
(2 months storage)

Starter No.	Incubation at 21 C for 14 hr				16 hr			
	Control		Enriched		Control		Enriched	
	Control A	Enriched A	Control B	Enriched B	Control A	Enriched A	Control B	Enriched B
3	0.56	0.66	0.66	0.76	0.71	0.81	0.80	0.86
6	0.40	0.50	0.34	0.40	0.50	0.68	0.45	0.62
21	0.30	0.47	0.35	0.60	0.33	0.65	0.37	0.71
26	0.59	0.68	0.45	0.53	0.75	0.86	0.68	0.66
27	0.33	0.41	0.35	0.40	0.42	0.52	0.41	0.49
10	0.54	0.60	0.40	0.49	0.70	0.82	0.52	0.64
11	0.36	0.51	0.44	0.52	0.44	0.66	0.60	0.70
12	0.28	0.32	0.34	0.46	0.32	0.35	0.36	0.64
Avg.	0.42	0.52	0.42	0.52	0.52	0.68	0.51	0.71

Control A – No proteolysate.
Enriched A – Enriched with 1% proteolysate for the first time.
Control B – Enrichment was discontinued after storage.
Enriched B – Enrichment was continued after storage.

proteolysate increased the activity of all cultures when enrichment was continued (Enriched B) but the amount of stimulation varied with the different cultures. The greatest stimulation was obtained in the slow cultures.

Reactivation of liquid cultures stored at -5C for 5 months. Titratable acidity values for one trial with each of six lactic starter cultures after 5 months of storage at -5 C are shown in Table 8.

When cultures were propagated after 5 months of storage, they developed rather lower acidities than those developed on the second transfer before freezing. When stored cultures containing no proteolysate (Control) were propagated with or without added proteolysate (Enriched A and Control A), a definite increase in titratable acidity was obtained in all cases from the added proteolysate after 24 hr incubation. The amount of stimulation varied with the different cultures, but averaged 0.05 percentage point or 17%.

When stored cultures containing proteolysate (Enriched) were propagated with or without added proteolysate (Enriched B and Control B), again a definite increase in titratable acidity was obtained in all the cases from the added proteolysate. The average increase in titratable acidity in this case was 0.13 percentage point or 30% after 24 hr incubation.

The cultures from which the proteolysate was withheld (Control B) maintained their activity above those which had never been enriched (Control A). Also they developed slightly higher acidities than cultures enriched for the first time (Enriched A). However, cultures that continued to receive proteolysate (Enriched B), developed the highest acidities of all and developed 0.21 percentage point or 66% above cultures never receiving proteolysate (Control A). They also developed considerably greater acidities than those cultures receiving proteolysate for the first time (Enriched A). The amount of stimulation

Table 8. Effect of Ps. fluorescens milk proteolysate on restoring activity of frozen lactic starter cultures at -5 C for 5 months as measured by titratable acidity.

Starter No.	Incubation for 16 hr titratable acidity at freezing		Incubation at 21 C for 24 hr Propagation[a]/after Storage			
			Control		Enriched	
	Control	Enriched	Control A	Enriched A	Control B	Enriched B
1	.72	.80	0.44	0.54	0.52	0.65
2	.72	.74	0.33	0.42	0.63	0.73
3	.72	.87	0.54	0.74	0.56	0.82
4	.85	.88	0.24	0.30	0.25	0.35
5	.88	.90	0.21	0.30	0.37	0.54
6	.83	.87	0.21	0.26	0.24	0.26
Avg.	.79	.84	0.35	0.41	0.43	0.56

Control A - No proteolysate.
Enriched A - Enriched with 1% proteolysate for the first time after storage.
Control B - Enrichment was discontinued after storage.
Enriched B - Enrichment was continued after storage.

a/ Size of inoculum was 5%.

growth varied with the different cultures.

Although half of the starters were brought back to normal growth range on the first propagation after freezing, especially in case of proteolysate addition, a second propagation was made to further evaluate recovery of activity of the cultures. The results are presented in Table 9.

The titratable acidities were much higher than those of the first transfer. The effect of the proteolysate was generally the same as on the first transfer after 14 and 16 hr incubation. Moreover, all the cultures were brought up to normal activity after 14 and 16 hr incubation, with the exception of one culture which was slow even when enriched for the first time. However, it produced normal activity upon continued enrichment after 14 and 16 hr incubation. Average increase in acid production by the cultures enriched for the first time (Enriched A) was 0.17 percentage point or 33% and 28% above cultures which had never been enriched (Control A) at both incubation periods respectively. While the average increases in acid production by the cultures which continued to receive the proteolysate (Enriched B) above those from which the proteolysate was withheld (Control B) was 0.11 and 0.07 percentage point or 16% and 9%, after 14 and 16 hr incubation respectively.

It is clear that the average titratable acidities for the cultures enriched for the first time (Enriched A) were equal to that of those form which the proteolysate was withheld (Control B) at each incubation period. This showed that the cultures form which the proteolysate was withheld (Control B) still carried the effect of previous proteolysate enrichment, so they equalled in activity those enriched for the first time (Enriched A).

Although the effect of the proteolysate on recovery of lactic starter cultures activity was generally the same after 1, 2 and 5 months of storage, some differences were detected. On the first propagation after 5 months of

Table 9. Effect of Ps. fluorescens milk proteolysate on acid production by frozen lactic starter cultures upon the second transfer as measured by titratable acidity. (5 months storage)

Starter No.	Incubation at 21 C							
	14 hr				16 hr			
	Control		Enriched		Control		Enriched	
	Control A	Enriched A	Control B	Enriched B	Control	Enriched	Control	Enriched
1	0.45	0.70	0.75	0.75	0.57	0.78	0.81	0.83
2	0.44	0.68	0.48	0.63	0.57	0.79	0.70	0.76
3	0.45	0.79	0.51	0.82	0.55	0.85	0.62	0.89
4	0.32	0.38	0.77	0.82	0.41	0.57	0.83	0.85
5	0.63	0.70	0.72	0.78	0.74	0.77	0.78	0.81
6	0.69	0.65	0.71	0.76	0.71	0.75	0.77	0.82
Avg.	0.48	0.65	0.65	0.76	0.59	0.76	0.76	0.82

Control A - No proteolysate.
Enriched A - Enriched with 1% proteolysate for the first time after storage.
Control B - Enrichment was discontinued after storage.
Enriched B - Enrichment was continued after storage.

storage, half of the cultures gave low acidity values similar to those after 1 and 2 months storage. Although the other half gave high acidity values, they were very much below their respective values at initial propagation. In addition, when proteolysate was withheld from the cultures (Control B) upon propagation after 5 months storage, they gave higher acidities than those which were never enriched (Control A). They also gave slightly higher acidities after 24 hr incubation than those enriched for the first time. The latter condition was not observed after 1 and 2 months storage.

II. Effect of Diatomaceous Earth in Preserving Lactic Starter Cultures

Activity after storage at 5 C. Activity of the preserved cultures as measured by acid production on propagation after storage up to 21 days is indicated in Table 10.

Comparisons were made with controls inoculated from liquid cultures stored under the same conditions. For the treated cultures, only the titratable acidity values for those having the smallest inoculum (0.1 g) were reported since this amount was more nearly equivalent to the 0.1 ml of inoculum of the control samples.

In the first trial the initial propagations from DTEC (within 2 hr of mixing) gave lower acidities than the controls. This would be expected since propagations from fresh liquid cultures should give maximum activity. However, after 14 days storage, results from DTEC were almost as good as the controls and near their initial level. No explanation for these results can be offered. By the 21st day both DTEC and control cultures had lost most of their activity.

In the second trial titratable acidity on the initial propagation from DTEC cultures was much less than from the controls. In subsequent transfers

Table 10. Effect of diatomaceous earth on preserving lactic starter cultures stored at 5 C as measured by titratable acidity[a] developed on subsequent propagation.

Trial No.	Starter No.	Initial Propagation		Propagation after storage for					
				7 days		14 days		21 days	
		DTEC[b]	Control[c]	DTEC	Control	DTEC	Control	DTEC	Control
1	21	0.68	0.83	0.25	0.47	0.65	0.64	0.22	0.24
	27	0.75	0.87	0.20	0.84	0.70	0.81	0.19	0.26
	Avg.	0.71	0.85	0.22	0.65	0.67	0.72	0.20	0.25
2	6	0.29	0.77	0.26	0.56	0.25	0.77	0.23	0.38
	26	0.30	0.78	0.22	0.39	0.20	0.40	0.18	0.29
	32	0.34	0.78	0.22	0.83	0.20	0.77	0.19	0.46
	Avg.	0.38	0.78	0.23	0.89	0.27	0.58	0.20	0.37

a/ Incubation at 21 C for 20 hr.
b/ Liquid culture blended with diatomaceous earth, (DTEC).
c/ Liquid culture.

after 7, 14 and 21 days of storage, DTEC continued to show much less activity than controls. By the 21st day even the controls showed considerably reduced activity.

Activity after storage at -30 C. Results on the activity of preserved DTEC after storage up to 8 weeks are presented in Table 11.

In the first trial both DTEC were maintained up to 6 weeks of storage without a significant loss in activity. Moreover, except for the 4 week period culture 21 showed higher activity than its respective control up to 8 weeks of storage, while culture 27 was more active than its control after 4 and 6 weeks of storage. After 8 weeks storage, both controls and DTEC became inactive. Average increase in acid production by DTEC above their respective controls was 0.27 and 0.23 percentage point or 71% and 62% after 4 and 6 weeks storage respectively.

In the second trial the DTEC continued to show a low level of activity after storage. However, titratable acidities were slightly higher than on initial propagation after 2 and 4 weeks storage (Table 10), but were much below the controls.

An important point should be considered here; namely, that titratable acidity values of DTEC are not directly comparable with those from the controls because the inoculum of 0.1 g of the DTEC form contains only 75% of liquid starter culture. To give more comparable values the readings for DTEC should be increased 33% which would bring them closer to values for controls.

The large difference in results between trials 1 and 2 at both storage temperatures may be partly due to differences between cultures. However, observation suggests that much of the difference may have arisen from the physical consistency of the product obtained on blending liquid cultures

Table 11. Effect of diatomaceous earth on preserving lactic starter cultures stored at -30 C as measured by titratable acidity[a] developed on subsequent propagation.

Trial No.	Starter No.	Propagation after storage for 2 weeks DTEC[b]	2 weeks Control[c]	4 weeks DTEC	4 weeks Control	6 weeks DTEC	6 weeks Control	8 weeks DTEC	8 weeks Control
1	21	0.65	0.64	0.25	0.60	0.60	0.25	0.33	0.25
	27	0.70	0.81	0.67	0.61	0.58	0.48	0.28	0.45
	Avg.	0.67	0.72	0.65	0.38	0.59	0.36	0.30	0.35
2	6	0.33	0.74	0.34	0.71	---	---	0.21	0.35
	26	0.26	0.71	0.26	0.64	---	---	0.17	0.30
	32	0.35	0.83	0.38	0.81	---	---	0.21	0.75
	Avg.	0.31	0.76	0.32	0.72			0.20	0.46

[a] Incubation at 21 C for 20 hr.
[b] Liquid culture blended with diatomaceous earth, (DTEC).
[c] Liquid culture.

with diatomaceous earth. In the first trial the blended product (DTEC) had the consistency of a heavy dough, while in the second trial it was more granular in texture. This difference may have been a factor in culture viability during storage. More information is needed on the technique of mixing and its effect on culture preservation.

Relationship of bacterial plate counts to acidity developed on propagation. Titratable acidity values and plate counts on diatomaceous earth (DTEC) are given in Table 12.

It is clear from these data that a sharp drop in bacterial counts occurred when the cultures were blended with the diatomaceous earth, since counts on the control cultures were many times higher. However, there was considerable variations among cultures, also counts on DTEC varied inconsistently with storage. Highest counts were obtained after storage for 2 weeks at -30 C. Also, culture 6 gave higher counts after 7 days at 5 C than on initial plating. On the other hand, control cultures showed the highest counts on initial plating, and a gradual decline in numbers occurred after 7 days storage at 5 C and after 14 days at -30 C.

There was a close relationship between the numbers and the acidity developed on propagation. The marked reduction in plate counts of the cultures after blending may be partly due to trapping of cells between the particles of diatomaceous earth and in the small granules formed. Other physical or physical chemical factors may be involved. Lack of uniformity in mixing may also be a cause of inequalities in counts. In addition a relatively high pH of 8.6 of the diatomaceous earth may have had a toxic effect on the cells.

On the other hand, during inoculation of cultures, the diatomaceous earth did not have an inhibitory effect because when liquid cultures were propagated

Table 12. Relationship of bacterial plate counts of (DTEC)[a] and liquid cultures to acidity developed on propagation.

Starter No.	Initial propagation (within 2 hr of blending)			
	Colony plate count (000)		Titratable acidity[b]	
	DTEC	Control	DTEC	Control
6	1,700	68,000	0.29	0.77
26	150	9,000	0.30	0.78
32	1,450	128,000	0.34	0.80
	After 7 days storage at 5 C			
6	3,500	40,000	0.26	0.56
26	100	7,000	0.22	0.39
32	800	100,000	0.22	0.83
	After 14 days storage at -30 C			
6	3,600	28,000	0.33	0.74
26	150	4,600	0.26	0.71
32	4,000	TNC	0.35	0.83

[a] Diatomaceous earth blended with liquid cultures.
[b] Incubation at 21 C for 20 hr.

with 5 to 10% added earth, the titratable acidity developed was actually slightly higher than with controls. (Data not shown)

Although the foregoing preliminary studies on the possibility of using diatomaceous earth as a lactic culture preservative were not consistently satisfactory, trials with some cultures showed promise in this respect. Considerably more information is needed on fundemental effects of such treatments on bacterial preservation. Improved techniques and equipment are

needed for obtaining a product of optimum physical properties. Finally greater attention should be given to aseptic techniques to minimize contamination.

Since the lyophilization method of preserving lactic starter cultures is long and expensive, a simpler procedure of preservation would have considerable merit.

SUMMARY AND CONCLUSIONS

Work was undertaken to determine the effect of Ps. fluorescens proteolysate of milk in reactivating stored lactic starter cultures. It was further directed towards evaluating diatomaceous earth (Micro-Cel) in preserving cultures.

Reconstituted skim milk was inoculated with a strain of Ps. fluorescens and incubated 10 days at 25 C. Sterile proteolysate was prepared by treatment of the proteolyzed milk with 25% ethyl alcohol, with subsequent evaporation of the alcohol under vacuum.

Ten lyophilized lactic starter cultures obtained from commercial laboratories were propagated with and without 1% added proteolysate. The average increase in titratable acidity resulting from addition of proteolysate was 27% after 16 hr incubation at 21 C. Variation was observed among cultures. Coagulation was obtained in seven of the enriched cultures during the 16 hr period.

Ripened liquid cultures enriched with 1% proteolysate on initial propagation and control cultures without proteolysate were stored at 5 C and -5 C for 21 and 150 days respectively. After storage the control and enriched cultures were each subcultured with and without added proteolysate and incubated at 21 C.

With cultures stored at 5 C for 4 days those continuing to receive proteolysate on subculture, produced only slightly more acid than controls that had never been enriched, but produced higher acidities over those from which the proteolysate was withheld. The withholding of the proteolysate from cultures that were enriched prior to storage resulted in lower acid development than with non-enriched controls. However, cultures enriched for the first time after storage produced 11% more acid than non-enriched controls. After 14 and 21 days of storage relationships were generally similar but titratable acidity levels were lower.

With cultures stored at -5 C after 1 month storage, the stimulatory effect of added proteolysate was evident, but titratable acidity values obtained were relatively low. Cultures again receiving proteolysate on subculture produced 35% more acid than controls that had never been enriched. Cultures enriched for the first time after storage produced 29.5% more acid than non-enriched controls after 16 hr incubation. A second propagation of all cultures brought acidities up to the normal range, with the enriched cultures being highest. Results after 2 months storage were generally similar.

The effect of the proteolysate on restoring activity of cultures stored at -5 C for 5 months was also generally similar to that after 1 and 2 months of storage. Cultures that continued to receive proteolysate on subculture produced 60% more acid after 24 hr incubation than controls that had never been enriched. Cultures enriched for the first time after storage produced 17% more acid than non-enriched controls. However, cultures from which the proteolysate was withheld gave higher titratable acidity values than those never enriched, and slightly higher than those enriched for the first time. A second propagation of all cultures brought acidity levels up to the normal range with the enriched cultures being highest.

Generally, slow cultures were stimulated the most due to added proteolysate, but in many cases their activity still did not equal that of the fast cultures.

In this study the activity of frozen liquid cultures on the first transfer after storage was generally low. This was true after 1 and 2 months storage even with cultures frozen at low acidities. While after 5 months storage at higher acidities half of the cultures gave relatively high acidities on propagation. Also, the limited number of propagations before freezing might be a factor.

Ripened lactic starter cultures were mixed with diatomaceous earth at a ratio of 3:1. The DTEC were stored at 5 and -30 C for 21 and 60 days respectively. Liquid portions of the same cultures were stored under the same conditions and served as controls.

With cultures stored at 5 C there were striking differences among the different cultures. However, activity of DTEC were much below their respective controls. Moreover, some cultures dropped in activity on initial propagations, and continued to drop in activity up to 21 days storage.

With cultures stored at -30 C, also there were striking differences among cultures, however in trial 1, the average increases in acid production by DTEC over the controls was 71 and 62% after 4 and 6 weeks storage respectively.

Results of the study showed that Ps. fluorescens proteolysate of milk is useful in reactivating stored lactic cultures. With commercial lyophilized cultures, use of the proteolysate would permit either the preparation of larger quantities of lactic starter on the initial propagation or the use of less inoculum. It would also reduce the need for intermediate transfer to

regain normal culture activity.

Enrichment of lactic starter cultures with proteolysate prior to freezing and further addition on subsequent propagation offers possibilities in improving the activity of frozen cultures. Because of a relatively greater effect with slow starters, its use would produce greater uniformity in lactic starter cultures activity. Use of larger amounts of proteolysate than the 1% used in this study might give greater stimulation.

The use of diatomaceous earth for preserving lactic starter cultures offers promising possibilities but requires further investigation.

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REACTIVATION OF STORED LACTIC STARTER CULTURES WITH A BACTERIAL PROTEOLYSATE OF MILK AND THEIR PRESERVATION WITH DIATOMACEOUS EARTH

by

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The purpose of this study was to determine the effect of a Ps. fluorescens proteolysate of milk on reactivating stored lactic starter cultures. It was further directed towards evaluating diatomaceous earth (Micro-Cel) in preserving cultures.

The proteolysate was obtained by inoculation of reconstituted skim milk with a strain of Ps. fluorescens and incubation at 25C for 10 days. Sterile proteolysate was prepared by treatment with 25% ethyl alcohol, followed by evaporation of alcohol under vacuum. In trials with lactic starter cultures the treated proteolysate was added at the rate of 1%.

On the first propagation of ten commercial lyophilized lactic starter cultures, those with added proteolysate developed 27% more acid than the control cultures (no proteolysate) in 16 hr incubation at 21C as measured by titratable acidity. In addition coagulation occurred in seven of the ten enriched cultures. Control cultures failed to coagulate during the incubation period.

A total of 17 liquid starter cultures with and without added proteolysate were stored at 5 and -5C for 21 and 150 days respectively.

When cultures stored at 5C for 4 days continued to receive proteolysate upon subsequent propagation they developed slightly more acid than cultures which had never been enriched. Cultures from which the proteolysate was withheld developed acidity levels very much below cultures which had never been enriched (controls). On the other hand cultures enriched for the first time developed 11% greater acid than control cultures. After 14 and 21 days of storage the effect of the proteolysate was generally the same, except that acidity values were very low.

With cultures stored at -5C for 1, 2 and 5 months, the effect of the

proteolysate was generally the same as with cultures stored at 5 C but low acidity levels were obtained. However, the maximum increase in acid production due to continued enrichment with the proteolysate occurred after 1 month storage. The average increase in acid production in this case after 14 hr incubation at 21 C was 66 % above cultures which had never been enriched. The average increase in acid production by cultures enriched for the first time was 17% above cultures which had never been enriched. Cultures from which the proteolysate was withheld developed higher acidities than cultures which were never enriched.

A second propagation of cultures stored at -5 C for 1, 2 and 5 months brought acidity levels up to the normal range, with the enriched cultures producing the highest acidities. Also, slow cultures were stimulated the most due to added proteolysate, but frequently they gave lower acidities than the active cultures.

From this study it is evident that enrichment with 1% Ps. fluorescens proteolysate is of value in reactivating stored lactic cultures. With commercial lyophilized cultures, use of the proteolysate would permit either preparation of larger quantities of lactic starter on the initial propagation or the use of less inoculum.

Enrichment of liquid lactic starter cultures with proteolysate prior to freezing and further addition on subsequent propagation offers possibilities in improving the activity of frozen cultures. The ability of the proteolysate to stimulate slow cultures the most, would help to overcome the low activity problem and its economic loss in the cheese and cultured dairy products industries.

In the diatomaceous earth study, five ripened lactic starter cultures were blended with diatomaceous earth at the rate of 3:1. The final product

DTEC was stored at 5 C and -30 C for 21 and 60 days respectively. Liquid portions of the same cultures were stored under the same conditions to serve as controls.

When cultures stored at 5 C were propagated after storage, there were striking differences among the different cultures. In addition, activity of DTEC was much below the respective liquid controls, and continued to drop in activity up to 21 days of storage.

When cultures stored at -30 C were propagated after storage, there were also striking differences among the different cultures. In the first trial the average increase in acid production by DTEC above the controls was 71% and 62% after storage of 4 and 6 weeks respectively. However, in the second trial the cultures dropped in activity at initial propagation, and continued to drop on successive storage.

Although results were inconsistent, some showed promising possibilities of using diatomaceous earth in preserving lactic starter cultures. Further investigation is warranted, with particular attention to blending techniques.