STIMULATION OF LACTIC STARTER CULTURES BY SUBSTANCES IN MILK CULTURES OF *PSEUDOMONAS FLUORESCENS* AND *BACILLUS CEREUS*

by

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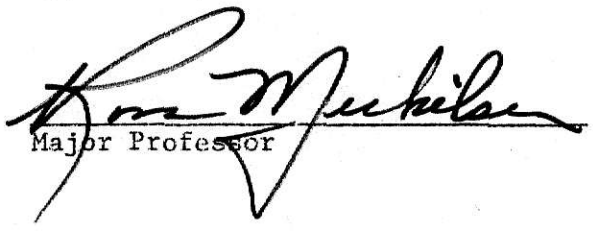

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INTRODUCTION

One of the main problems in cheese and buttermilk production is the selection and maintenance of lactic starter cultures.

Cheese manufacturers rely on starter culture activity to influence flavor, body and texture of the cheese, aid in rennet action, help in moisture expulsion, reduce the growth of undesirable bacteria in the curd and cheese and influence certain changes that take place during cheddaring.

In the manufacture of buttermilk, lactic cultures are used to produce desirable acid, and aid in developing other flavor and aroma compounds. The coagulation of milk by lactic acid production imparts certain body and texture characteristics required for good quality buttermilk.

Cheese and buttermilk industries continually face the problem of slow starter cultures. This entails lengthening the manufacturing process which often results in undesirable end-products. Some technical advances have been made to prevent the incidence of slow starter activity, thus alleviating the problem to some extent. These methods include asceptic transfer of lactic starters, and the use of milk free of sanitizers, bacteriophage and antibiotics. Rotation of lactic cultures by the development of a "starter library" and the use of milk containing the least amount of undesirable microorganisms also have proven to be useful.

In recent years, the dairy industry has used pancreas and liver extracts as growth stimulants for starter cultures. Lyophilized lactic cultures containing either liver or pancreas extracts are now available from commercial sources.

In 1961, Koburger and Claydon (28), showed that various fractions from a milk proteolysate produced by Pseudomonas fluorescens were stimulatory to lactic acid bacteria when grown in autoclaved 10 percent reconstituted non-fat-dry milk (NDM). A fraction precipitated by acetone-ethanol and an amino acid

fraction, increased titratable acidity over the control 28 and 13 percent, respectively, at 14-hour incubation at 21 C. Vincent (48), in 1963, conducted experiments evaluating the milk proteolysate of Ps. fluorescens in cottage cheese manufacture. He observed less stimulation of acid production in the cottage cheese vats than had been anticipated based on the findings of Koburger and Claydon. Overall manufacturing time was reduced from 12 to 18 percent; however, this reduction in time primarily was attributable to a decrease in cooking time for vats containing the added "stimulatory agent" rather than an increase in acid production per se.

The objective ot this study was to investigate further the stimulatory fraction precipitated by acetone-ethanol that was reported by Koburger and Claydon, and to make an additional evaluation of the observations of Vincent, relative to the stimulation of lactic cultures by the addition of milk proteolysate to pasteurized skim milk. Further experiments were undertaken to determine whether a milk proteolysate of Bacillus cereus was stimulatory to lactic starter organisms when grown in autoclaved 10 percent reconstituted nonfat dry milk and pasteurized skim milk.

REVIEW OF LITERATURE

Some Factors Affecting Acid Production by Lactic Cultures

Fermentation of lactose to lactic acid by starter cultures is essential in the manufacture of cheese, cultured buttermilk, cultured cream and butter culture. If the lactic bacteria are slow, cultured dairy products may exhibit unfavorable characteristics such as insufficient acid, lack of curd formation and undesirable flavor compounds.

Milk containing high levels of free fatty acids has been shown to inhibit the growth of Streptococcus lactis and the inhibition was in direct proportion to the degree of lipolysis (Costilow and Speck 13). Free fatty acids in lipolytic milk exert a toxic effect on lactic cultures.

Davis (18) reported that mastitic milk inhibited lactic organisms, but that the inhibitory factor was partially destroyed by pasteurization. Whitehead and Cox (49) found that lactic cultures were not able to develop appreciable quantities of lactic acid in milk containing more than 5,000,000 leucocytes per ml.

Antibiotics such as penicillin are employed in the treatment of udder infections; these same materials, when found in milk used for cheese production inhibit lactic acid organisms. Katz, Nelson and Hood (30) found penicillin to be most effective against lactic acid-producing streptococci. It was 3.3 times more effective than aureomycin, 6.6 times more effective than streptomycin and 33.3 times more effective than chloromycetin.

Hunter (27) showed that heavy bacteriophage contamination resulted in decreased acid production during the final stages of cheese manufacture. Heat treatment of milk used for cheese manufacturing purposes with the addition of phosphate salts to the starter cultures has given maximum protection against bacteriophage contamination (25).

Certain sanitizing compounds are inhibitory to lactic cultures. Calbert (8)

showed acid production by a lactic culture in sterile skim milk was retarded by 25 to 200 ppm of chlorine. Babel (5) reported that acid production was inhibited greatly by 25 ppm of chlorine. He observed also that an iodophor added to milk in amounts ranging from 5 to 50 ppm did not decrease acid production but instead stimulated lactic cultures. Miller and Elliker (39) noted that acid production by a commercial lactic culture was inhibited almost completely in milk containing quaternary ammonium compounds or a detergent sanitizer containing quaternary ammonium compounds.

Anderson and Elliker (3) demonstrated that 21 strains of commercial starter cultures required the addition of specific amino acids to initiate growth. Amino acids not required to initiate growth in any of the 21 strains were cysteine, tryptophane, aspartic acid and serine. Pantothenic acid was required for growth by all of the strains investigated. Pyridoxine, biotin and thiamin (B complex vitamins) were necessary in all instances for maximum response of lactic cultures. Most of the strains were stimulated by riboflavin and more than one-third required it for growth. If the lactic cultures were not provided the minimum nutritional requirements, the production of lactic acid decreased.

It is important to consider the milk medium used to propagate lactic acid bacteria. Howall et al. (26) determined that a medium prepared by reconstituting NDM produced cultures and starters more constant in activity from day to day than when selected whole milk was used as the growth medium.

Effect of heat treatment of milk on culture response. The heat treatment given milk intended for culture propagation influences the rate of acid production. Davis (18) obtained less acid development in unheated cow's milk inoculated with culture than in milk heated to 73.9 C for 30 minutes. Milk boiled for 24 hours formed more acid when inoculated with lactic culture than milk heated to 82.2 C by a flash method and similarly inoculated.

Foster (20), using milk from individual cows, was able to obtain greater

acid production by S. lactis in autoclaved milk than milk heated at 80 C for 10 minutes. Singh and Laxminarayana (38) found that heating cow's milk to 62.8 C and holding for 30 minutes, followed by cooling improved the growth activity of lactic cultures subsequently inoculated into the milk. Milk heated to boiling and then cooled was even more satisfactory as a lactic starter culture medium.

Greene and Jezeski (21) showed by acid production and dye reduction that reconstituted high heat NDM supported starter activity more favorably than did reconstituted low heat NDM. It was noted that excessively high heat treatment of skim milk during manufacture into NDM caused a decrease in the activity of starter cultures when the reconstituted NDM was used as growth medium. In another investigation, Greene and Jezeski (22) studied starter responses in a series of fluid milks which had been subjected to various heat treatments ranging from 62 C for 30 minutes to 120 C for 60 minutes. The primary zone of stimulation occurred at exposures from 62 C for 30 minutes to 72 C for 40 minutes. The first heat induced inhibition was initiated at exposures of 72 C for 45 minutes and extended through 82 C for 10 minutes to 120 minutes, as well as 90 C from 1 to 45 minutes. There was a marked variation in starter responses in raw and pasteurized milk, but the differences between starters were diminished considerably in milks heated to 72-75 C for 30 minutes. Greene and Jezeski (23) concluded that primary stimulatory activity observed after heating milk to 62 and 72 C for 30 minutes was due to destruction of heat labile inhibitors, the lowering of the oxidation-reduction potential, hydrolysis of protein, which usually occurs above 80 C, and heat-denaturation of serum proteins rendering the milk more susceptible to protein degradation by the lactic cultures.

Effect of peptides and amino acids on lactic starter stimulation. Numerous studies have shown that the growth of many lactic streptococci in milk are stimulated by various peptides and amino acids. Anderson et al. (4) analyzed individual cow's milk and found variations in peptide content.

The rate of acid production by mixed strains of commercial cultures and individual strains of lactic streptococci, in most instances increased with increases in peptide content of milks from individual cows. Peptide content appeared to have a greater influence on the growth rate of lactic streptococci than did total protein content of milk. The correlation between rate of growth and peptide content was poor in milk from mastitic cows or from those in early or late lactation. Krehl and Fruton (36) showed that the rate of acid production by a slow strain of Streptococcus cremoris was stimulated more compared to a faster strain of the same organism when the culture medium was supplemented with peptone or acid hydrolyzed peptone.

Zurow et al. (50) observed the presence of stimulatory factors in condensed maize fermentation solubles which were active for Lactobacillus casei in milk. The purified stimulants were identified as phenylalanine and a tyrosine peptide.

Bautista et al. (6) found that certain amino acids were produced in milk cultures from the synergistic effect of Lactobacillus bulgaricus and Streptococcus thermophilus. Glycine and histidine were shown to be responsible for stimulating S. thermophilus. In an investigation by Speck and Williamson (44), strains of starter culture showed less degradation of milk protein when fortified with stimulatory peptides, such as those contained in pancreas extract. Their data indicated that the presence of stimulatory peptides in milk precluded the necessity for cultures to hydrolyze milk proteins before initiating growth. Kennedy (31) determined the effect of several peptide rich extracts on acid production by lactic streptococci. The extracts were: pancreas extract, yeast extract, liver fraction L, tryptone, tryptase neopeptine, casein hydrolysate, milk protein hydrolysate and peptonized milk. The addition of 0.5 percent of each stimulant to the medium enabled the lactic culture to produce more acid than the control after eight hours incubation. The addition of these peptide rich extracts to lactic acid bacteria in the presence of antibiotics did not

stimulate acid production. Sandine et al. (40) demonstrated that pancreas tissues contained two stimulants for L. casei and S. lactis. One stimulant was purified and showed characteristics of a peptide. Acid and alkaline hydrolysates of the stimulant contained a large number of amino acids. Anderson and Elliker (2) showed liver fraction L, trypsinized skim milk or peptonized milk to be stimulatory to mixed strains of lactic cultures when grown in a medium consisting of low concentrations of reconstituted NDM. Liver fraction L produced marked stimulation in the initial growth rate of many slow strains. The faster strains were not stimulated by the foregoing preparations. Speck et al. (43) investigated the causes of growth response by starter streptococci in milk supplemented with certain stimulatory extracts. It was shown by paper chromatography that aqueous extracts of pancreas and yeast contained peptides responsible for the stimulation.

Speck and Ledford (42) reported that pancreas extract had commercial application in the manufacture of cottage cheese. The addition of 0.015 to 1.0 percent of pancreas extract decreased the time for acid development and the cutting of cheese 17 to 41 percent. Concurrent with the decrease in acid development time was a decrease in the cooking time of 12 to 92 percent.

The effect of other microorganisms on the stimulation of lactic acid bacteria. Cox and Whitehead (14) observed the synergistic effect of lactic streptococci and four organisms found in contaminated milk, Escherichia coli, Bacillus subtilus, a staphylococcus and Bacillus faecalis akaligenes. All the organisms except E. coli stimulated acid production by lactic cultures. Contrary to the findings of Cox and Whitehead, Hall (24) found acid production was accelerated by a mixture of lactic and coliform organisms. A sterile extract of the coliform organism grown in reconstituted NDM also stimulated acid production by S. lactis.

Claydon and Koburger (11) reported that cell-free filtrates of milk cultures of Ps. fluorescens were found to stimulate acid production. In another study

by Koburger and Claydon (28), milk cultures from a strain of Ps. fluorescens were fractionated to identify factors which stimulated production by lactic starter cultures. A stimulatory fraction produced a 28 percent increase in titratable acidity at 14 hours at 21 C. It appeared that enzyme activity was involved in stimulation, but this could not be clearly established. An amino acid fraction, which was stimulatory, contained 17 amino acids and gave an increase in titratable acidity of 13 percent. Koburger and Ormdorff (34), demonstrated that a filtrate from the milk culture of Ps. fluorescens contained at least two enzymes capable of coagulating milk. Coagulation of milk by the most active fraction was favored by lowering the pH or increasing the calcium ion concentration at constant pH. Knaut et al. (32) observed the milk coagulation phenomenon of a cell-free filtrate of Ps. fluorescens when added to cultures of S. lactis, S. helveticus and L. casei. No stimulation of lactic acid production was reported. However, coagulation of milk occurred at pH 5.65 to 5.90 instead of pH 4.5 to 4.9 as happened without addition of filtrates.

Effect of nucleic acid derivatives in stimulating lactic acid bacteria. In a study by Koburger et al. (35) an analysis was made of components contained in pancreas extract which stimulated lactic acid bacteria. The active compounds were found to be inosine, hydroxanthine and adenine. Dahyia and Speck (17) showed that acid production by single strain and mixed strain starter cultures was stimulated when milk was fortified with various nucleic acid derivatives. Inosine gave the highest acid production compared with purines and purine riboside. The development of lactic cultures was slower in fresh milk than in reconstituted 10 percent NDM; but, equal when the fresh milk was fortified with inosine. These results were similar to the findings of Taniguchi et al. (45); they reported that lactobacilli were influenced to a greater extent than lactic streptococci. Generally, nucleic acid derivatives containing pyrimidine bases had little stimulatory effect compared to those with

the purine bases adenosine and hypoxanthine which were identified as the stimulatory derivatives. The order of effectiveness of the nucleic acid derivatives was: base >, nucleoside >, nucleotide.

In contrast to the findings of Taniguchi, Kristoffersen (37) found that hypoxanthine and xanthine were inhibitory to lactic starter cultures under certain conditions; this was evident, especially when a milk source contained low levels of xanthine oxidase. Both hypoxanthine and xanthine serve as substrates for the naturally occurring milk enzyme xanthine oxidase.

Dahyia and Speck (16) observed that single strains of S. lactic and S. cremoris isolated from a commercial starter culture showed symbiotic growth in milk. Isolation of the main stimulatory factor for the slower strain was identified as adenine. Pure adenine produced the stimulatory effect on the faster strain. Crater and Mikolajcik (15) were able to show a correlation between slow acid production and a deficiency of nucleic acid derivatives. The intracellular nucleotide content of five strains of S. lactis was determined. The principal compounds isolated were: uridine, quanosine, nicotinamide adenine dinucleotide, adenosine monophosphate, uridine monophosphate, quanosine monophosphate and uridine diphosphate.

MATERIAL AND METHODS

Propagation of Ps. fluorescens and B. cereus

A transfer was made into litmus milk from a stock culture of Ps. fluorescens initially used by Koburger and Claydon for investigation of its stimulatory properties (33). A B. cereus culture previously identified according to Bergey (7) was obtained from the Dairy and Poultry Science Department collection. It had been isolated and initially used in this laboratory for producing proteolyzed milk (12). Cultures of Ps. fluorescens and B. cereus were transferred into litmus milk at weekly intervals and were incubated at 25 C for 24 hours. Following incubation, they were held at 3 C until used or retransferred.

Media preparation. Ten percent autoclaved reconstituted NDM was used as the growth medium for both organisms. Also this was used as the growth medium for lactic cultures when testing for stimulatory activity. Portions from a single lot of NDM were tested to determine ability to support growth of lactic cultures. The NDM was dispensed into tin cans, sealed, and stored at room temperature until used.

The NDM was reconstituted to 10 percent solids with distilled water; 250 ml quantities were dispensed into 1 liter Erlenmeyer flasks, and autoclaved for 15 minutes at 121 C. For studies involving the stimulation of lactic cultures, 99 ml of reconstituted NDM were dispensed into prescription bottles and autoclaved.

Pasteurized skim milk obtained from the University Dairy Processing Plant also was used to test stimulatory activity. The skim milk was stored at 3 C and usually not held for more than four days before being used.

Standards Methods Medium (1) was used to determine total viable cells of Ps. fluorescens and B. cereus. The medium was resuspended by heating in distilled water and dispensed into prescription bottles followed by autoclaving for 15 minutes at 121 C.

Preparation of Proteolytic Material

Crude proteolysate. A preliminary study was conducted to determine optimum incubation time required for production of milk proteolysate from Ps. fluorescens and B. cereus that was stimulatory to lactic cultures. Transfers from the stock cultures of Ps. fluorescens and B. cereus were inoculated into 150 ml of 10 percent autoclaved reconstituted NDM in Erlenmeyer flasks and incubated for 1-14 days at 25 C.

After each day of incubation, samples were removed and evaluated to determine the period of time in the proteolytic degradation of milk at which the lactic stimulatory factor was at the highest level. The following evaluations also were made in an attempt to correlate the production of lactic stimulatory substance with specific characteristics of the proteolysates: total plate count, pH, formol titer expressed in ml of 0.1 N sodium hydroxide, and the ratio of absorbance at 280 mμ/260 mμ.

Plate counts were made according to methods prescribed by the American Public Health Association (1). Agar plates containing samples in appropriate dilutions were incubated three days at 25 C.

Formol titrations were carried out daily. Ten ml of milk proteolysate were titrated to the phenolphthalein end point with 0.1 N sodium hydroxide. One-half ml of potassium oxalate and 1.5 ml of formalin were added to the samples which caused the pink color to dissipate. Samples then were titrated with 0.1 N sodium hydroxide to the same end point as for determining titratable acidity in milk using phenolphthalein as indicator. The ml of 0.1 N sodium hydroxide used in the second titrations to adjust the reaction to the phenolthalein end point were expressed as the formol titer of the samples. Blank determinations were made to account for acidity of reagents.

Results of this preliminary study indicated that eight days incubation at 25 C was optimum for production of crude proteolysate (see subsequent discussion in RESULTS AND DISCUSSION section).

Based on the foregoing evaluations, comparisons were made on stimulatory activity, expressed in terms of titratable acidities and pH, of both heat-treated (70 C for one hour) and non-heat-treated eight-day old milk proteolysates of Ps. fluorescens and B. cereus. Crude proteolysate required for further stimulatory evaluation and fractionation was prepared as outlined in the foregoing preliminary study except that 250 ml of sterile 10 percent reconstituted NDM were used rather than 150 ml quantities.

Cell-free filtrates. Cell-free filtrates of reconstituted NDM, proteolyzed by Ps. fluorescens and B. cereus were obtained by passing the eight-day old proteolysate through Seitz filters. Usually 3-5 ml of cell-free filtrate could be obtained from 25 ml of proteolysate without replacing the Seitz filter. The filtrate was collected in large sterile tubes and transferred aseptically to 20 ml screw cap tubes. The filtrates were tested for sterility by a streak plate method and stored at 5 C until used.

Precipitation of cell-free proteolysate. At the end of an eight-day at 25 C incubation period, the milk proteolysates of Ps. fluorescens and B. cereus were stored in a refrigerator for three hours at 3 C, after which they were centrifuged at 800 relative centrifugal force. An eight-day incubation period was chosen because by that time most of the milk protein had been digested. The centrifugal sludge which consisted of cell-debris and small amounts of undigested protein was discarded. Three hundred ml of the supernatant were decanted into a two liter Erlenmeyer flask. Six hundred ml of cold 95 percent ethanol were added and the supernatant-solvent mixture was refrigerated at 0 C for four hours. A precipitate formed which was collected on Whatman No. 41 filter paper. The supernatant was discarded. The ethanol-precipitate was resuspended in cold 0.001 M phosphate buffer at pH 6.5 to a total volume of 900 ml.

Three hundred ml of the resuspended precipitate were dialyzed for 72 hours at 2-3C against 0.001 M phosphate buffer, pH 6.5. Dialyzer tubing was

soaked in 0.001 M Ethylenediamine tetracetic acid (E.D.T.A.) for a period of four hours to remove residual metal ions that may have been present from the manufacturing process. The tubing was washed exhaustively with changes of distilled water for a period of several days and then stored in a refrigerator in 0.001 M phosphate buffer at pH 6.5. Following dialysis the preparation was lyophilized and stored at -5 C. The preparation was resuspended as needed in 0.001 M phosphate buffer, pH 6.5 to 250 ml volume.

Fractionation of lyophilized proteolysates by gel-filtration. A jacketed column (Pharmacia Fine Chemicals, Inc.) was used for the fractionation experiments. Cold (2-3 C) ethylene glycol was pumped through the column jacket. Sephadex G-100 was allowed to swell for three days, cooled to 2 C and packed in the cold jacketed column to a bed volume of 2.5 by 34 cm. After the column had eluted 2-3 times its volume of 0.001 M phosphate buffer at pH 6.5, the void volume and flow rate were determined. Approximately 3-5 grams of the lyophilized material prepared and resuspended in pH 6.5 buffer as described previously was passed through the Sephadex column using 0.001 M phosphate buffer, pH 6.5, as eluant. After the initial 15 ml sample had been flushed through the column, the process was repeated several times until the total of 100 ml of resuspended material had been used. Fractionated material from the column was collected in an automatic fraction collector. Absorbancy patterns of the fractions were determined at 280 mμ and were pooled according to their elution rate and absorbancy. The fractions were lyophilized and stored at -5 C.

Evaluation of Stimulatory Activity on Lactic Cultures

Propagation of lactic starter cultures. Four lactic cultures strains were propagated initially in sterile skim milk and one ml quantities were transferred at weekly intervals into 99 ml of sterile reconstituted NDM and incubated at 21 C for 14-16 hours. Ten hours prior to beginning an experiment, fresh transfers were made into autoclaved reconstituted NDM and/or pasteurized skim milk. The

fresh transfers were incubated for 10 hours at 21 C in preparation for use as inoculum in subsequent determinations where stimulatory effects of proteolysates in lactic cultures were determined.

Assay methods. To measure acid development of lactic cultures, a series of screw cap prescription bottles was prepared containing 99 ml of cold autoclaved reconstituted 10 percent NDM or commercially pasteurized skim milk. One ml of lactic starter culture was added just prior to the addition of the cell-free proteolysate (includes both the ethanol resuspended precipitate and Sephadex fractionated material) to be tested for stimulatory effect. Volume adjustments of controls was made using sterile distilled water so that controls and samples containing stimulatory material had the same volume. The added materials were dispensed throughout the milk to insure a uniform mixture. The bottles were held in an ice water bath prior to initial incubation to retard culture activity until incubation was begun.

From each bottle, nine ml samples were pipetted in duplicate into sterile screw cap tubes and incubated at 21 C for either 10, 12, or 14 hours. Acid development was measured by titrating the samples directly with 0.1 N sodium hydroxide using phenolphthalein indicator. The results were reported as the average titer of the duplicate samples.

Specific stimulatory activity. Stimulatory activity in terms of increased lactic acid production was determined as follows:

$$\frac{\text{Acid titer of sample containing stimulatory material} - \text{Acid titer of control sample} \times (9) \times (\text{Dilution Factor}^{1})}{5.935^{2,3}} = \text{Specific Stimulatory Activity}$$

[1] Ninety-nine ml of sterile milk, plus one ml of starter culture, plus the volume (ml) of stimulatory material required to introduce 5.935 mg nitrogen, divided by 9, equals the dilution factor.

[2] A sufficient volume of stimulatory material was added so as to introduce 5.935 mg of nitrogen for Ps. fluorescens preparations.

[3] A sufficient volume of stimulatory material was added so as to introduce 5.729 mg of nitrogen for B. cereus preparations.

Evaluation of Proteolytic Activity

Assay methods. Each of the cell-free preparations described in the foregoing sections was reconstituted in 0.001 M phosphate buffer at pH 6.5 so as to contain the same concentration of nitrogenous material as the cell-free filtrate from which the fraction was obtained (5.935 or 5.729 mg/ml depending upon which milk proteolysate was utilized). By using this constant nitrogen "bench mark", the addition to lactic cultures of one ml of any stimulatory fraction introduced the same quantity of nitrogen as was present in one ml of the original cell-free filtrate.

Proteolytic enzyme activity was determined according to the methods of Hurley et al. (28). One ml of the standardized fractions described previously was added to four ml of substrate consisting of one percent NDM in sterile distilled water. Following incubation for 60 minutes in a water bath at 37 C, 10 ml of 18 percent trichloracetic acid (T.C.A.) were added to terminate the reaction and to precipitate the remaining undigested protein and peptides. The contents of the tubes were filtered through washed Whatman No. 3 filter paper. Absorbancies of T.C.A. filtrates at 280 mμ were compared to a standard curve prepared by determining the absorbancy of tyrosine at various concentrations (Figure 1). Blanks were prepared for each trial by the addition of 10 ml of 18 percent T.C.A. plus four ml of one percent NDM, followed by the addition of one ml of the standardized fraction. Blanks were incubated and filtered in the same manner as the test samples.

The semi-micro Kjeldahl procedure of Shahani and Sommers (41) was used to determine total nitrogen.

Specific proteolytic activity. One unit of proteolytic activity was arbitrarily defined as the concentration of stimulatory material needed to increase absorbancy 0.1 unit (expressed in terms of mg/ml of tyrosine, see Figure 1) at 280 mμ in 60 minutes at 37 C.

RESULTS AND DISCUSSION

Results of Preliminary Investigations

Standard plate count. Standard plate counts of Ps. fluorescens and B. cereus for growth periods of 1-14 days are shown in Figure 2. Both cultures produced maximum total viable cell counts on sterile reconstituted NDM medium between the fourth and seventh day at 25 C.

Determination of amount of proteolysis. Although greater bacterial numbers were produced by Ps. fluorescens, its ability to proteolyze autoclaved reconstituted 10 percent NDM was less than B. cereus. Evidence for this is presented in Table 1 of the appendix. Formol titration values of milk cultures containing B. cereus generally were higher than Ps. fluorescens cultures at any given time during incubation. The formol titration value indicates the presence of amino groups derived from the degradation of proteins (29). The B. cereus culture, therefore, was able to proteolyze sterile reconstituted 10 percent NDM faster than the Ps. fluorescens culture under the conditions of this experiment.

A spectrophotometric analysis was conducted on the cell-free filtrates of B. cereus and Ps. fluorescens during each of the 14 days fermentation. The purpose of this was to evaluate the relative amounts of substrate proteolyzed and cellular material released from the bacterial cells. Certain amino acids (tryptophane and tyrosine, for example) inherent in proteins have a combined maximum absorbancy near 280 mu. Cellular components rich in pyrimidine and purine bearing nucleic acids tends to have a combined maximum absorbancy at 260 mu (10). Thus, if the ratio of absorbancy at 280 mu/260 mu was plotted against incubation time, an indication of the relative bacterial enzymatic content of the cell-free extract at any given time of incubation would be shown. A high ratio of 1:75 would indicate that the light absorbancy materials were high in nucleic acid content which was derived from cellular material as the nucleic acid content of

milk protein is low (19).

Cell-free milk proteolysates of B. cereus contained more suspected enzymatic material between the fifth and ninth day of fermentation and, again, between the tenth and thirteenth day than did the cell-free milk proteolysate of Ps. fluorescens (Fig. 1). Both milk proteolysates appeared to contain increasing amounts of nucleic acid derivatives between the third and fifth day of incubation. It appears that cell autolysis had occurred resulting in a higher concentration of nucleic acid derivatives. Qualitative analysis was not carried out to determine the specific nucleic acid derivatives present.

Acid base characteristics of milk proteolysates. The pH of milk proteolysates of Ps. fluorescens and B. cereus were determined and are shown in Table 2 of the appendix. The proteolysates became progressively more alkaline as incubation was extended.

Stimulation of Lactic Starter Cultures

Cell-free filtrates. Cell-free milk proteolysates of Ps. fluorescens and B. cereus were prepared daily during 14 days of fermentation and were used to stimulate lactic starter cultures (Tables 3-30 of the Appendix). Stimulation of lactic cultures was apparent when a cell-free milk proteolysate from at least a three-day old fermentation was used as the stimulatory material (Tables 7 and 8 of the Appendix). The cell-free filtrate of B. cereus increased titratable acidity of lactic streptococci an average of 9.3 percent over the control after 14 hours incubation at 21 C. The cell-free filtrate of Ps. fluorescens produced an average increase in titratable acidity of 6.9 percent. Although there was an increase in titratable acidity over the control when using three-day old cell-free proteolysate as a stimulant, the difficulty in Seitz filtering the three-day old proteolysate made it necessary to use eight-day old proteolysate for preparing the bulk of the cell-free material.

At the eighth day, the cell-free milk proteolysate of B. cereus gave an average increase in titratable acidity of 32.7 percent after 14 hours incubation

at 21 C; Ps. fluorescens cell-free filtrate gave an increase in titratable acidity of 17.2 percent (Tables 17 and 18 of the Appendix). Milk samples containing cell-free filtrates from 13 days fermentation coagulated at lower acidities than the control after 10 hours of incubation at 21 C (Tables 27 and 28 of the Appendix). This suggested the presence of milk clotting enzymes liberated by bacterial cell autolysis.

Effect of heat treatment on cell-free milk proteolysates when using pasteurized skim milk as growth medium. An eight-day old cell-free milk proteolysate of B. cereus coagulated pasteurized skim milk after 10 hours incubation at 21 C (Table 31 of the Appendix). The low titratable acidity and relatively high pH at the time a clot was observed suggests the presence of milk clotting enzymes. Also, there was stimulation of lactic starter cultures after 12 and 14 hours incubation in addition to that observed at 10 hours. Coagulation at the longer incubation times probably was due to the production of lactic acid, as evidenced by pH readings which were near the isoelectric point of casein.

The cell-free proteolysates of Ps. fluorescens coagulated pasteurized skim milk at 10 hours with a relatively high pH (Table 32 of the Appendix). Again, this suggests the presence of milk clotting enzymes. Both stimulation and coagulation occurred after 12 and 14 hours incubation. The relatively low pH suggests stimulation of lactics by the cell-free material and coagulation of the milk by the production of lactic acid.

The cell-free eight-day old milk proteolysate of Ps. fluorescens and B. cereus were heated in a water bath for one hour at 70 C. There was no noticeable precipitation of protein in either proteolysate at the end of the heating period. However, stimulation and milk clotting ability of the two milk proteolysates were inhibited by the heat treatments (Tables 33 and 34 of the Appendix). These findings are in agreement with those of Koburger and Claydon (33). They found that autoclaving milk proteolysates of Ps. fluorescens for 15 minutes at 250 F reduced its ability to stimulate lactic starter cultures and to clot milk.

Effect of Fractionating Cell-Free Milk Proteolysates of Ps. fluorescens and B. cereus

Koburger and Claydon (33) reported two stimulatory fractions from the milk proteolysate of Ps. fluorescens; namely, amino acids present in the proteolysate, and a second stimulatory factor which was present in a precipitate material prepared from the proteolysate. An objective of the present investigation was to evaluate further the precipitated material studied by Koburger and Claydon. Also studies were initiated to determine whether a milk proteolysate of B. cereus would exhibit similar stimulatory properties.

Stimulatory effect of the original eight day cell-free milk proteolysate of Ps. fluorescens. Addition of one percent cell-free filtrate of Ps. fluorescens to lactic starter cultures when grown in 10 percent sterile reconstituted NDM resulted in an average increase in titratable acidity of 31.1 percent at the end of 14 hours (Table 1). The stimulatory effect on lactic cultures decreased when pasteurized skim milk was used as the growth medium (Table 2). In this latter instance the cell-free filtrate of Ps. fluorescens increased the average titratable acidity over the control 17.2 percent at the end of 14 hours incubation compared to 31 percent when autoclaved reconstituted NDM was the growth medium. This agrees with the findings of Vincent with regard to less stimulation of lactics when grown in pasteurized skim milk compared to autoclaved reconstituted NDM as growth medium (48).

Stimulatory effect of an ethanol precipitate obtained from a proteolyzed milk culture of Ps. fluorescens. Addition of the cell-free ethanol precipitated milk proteolysate of Ps. fluorescens stimulated lactic starter cultures when grown in autoclaved 10 percent reconstituted NDM at 21 C (Table 3). The average increase in titratable acidity was 26 percent after 14 hours compared to 22 percent when the original filtrate was used with autoclaved 10 percent reconstituted NDM as the growth medium (Table 1). When pasteurized skim milk was used as growth medium for lactic starter culture stimulation, the cell-free

ethanol precipitated fraction of Ps. fluorescens resulted in an average increase in titratable acidity of 15.1 percent (Table 4). Coagulation was evident at the end of 10 hours incubation at 21 C. Since the average titratable acidity was low, early coagulation suggested the action of milk clotting enzymes.

Support of this latter postulation is found in Table 14 which shows steps used in purification of the cell-free ethanol precipitated fraction of Ps. fluorescens. Specific activity expressed in terms of proteolysis showed there was approximately a 20-fold increase over the original filtrate.

Stimulatory effect on lactic cultures of the cell-free lyophilized ethanol-precipitated milk proteolysate fraction of Ps. fluorescens obtained from Sephadex G-100 Gel Filtration. The lyophilized ethanol-precipitated cell-free material upon being resuspended, and subsequently fractionated through Sephadex G-100, was stimulatory when added to lactic starter cultures. Figure 4, shows two major peaks which were obtained from column fractionation of resuspended lyophilized ethanol precipitate. The first peak from Ps. fluorescens produced the greatest stimulation for lactic starter cultures resulting in an average increase of titratable acidity of over 33 percent above the control at 14 hours incubation at 21 C (Table 5). The growth medium was autoclaved reconstituted 10 percent NDM. When pasteurized skim milk was used as the growth medium there was an average increase of 10 percent titratable acidity (Table 6). At the end of 10 hours of incubation there also was coagulation of the pasteurized milk sample. The coagulation was associated with a comparatively low titratable acidity, suggesting again the action of milk clotting enzymes. Table 15 shows, there was a 50 fold increase in specific activity in terms of proteolysis. These latter data support evidence for the presence of milk-clotting enzymes with their ability to clot milk at relatively short incubation periods, i.e. $\leq$ 10 hours.

The cell-free filtrate of Ps. fluorescens obtained from the second gel-filtration peak was less stimulatory to lactic cultures than the first peak. Table 7 shows the effect from the second peak of the cell-free milk proteolysate

of Ps. fluorescens on the stimulation of lactic starter cultures. Pasteurized skim milk was used as the growth medium. There was an average increase in titratable acidity of approximately 13 percent after 14 hours incubation at 21 C. Also, coagulation occurred at this incubation time, due to development of lactic acid.

Stimulatory effect by the original eight-day old cell-free filtrate obtained from a proteolyzed milk culture of B. cereus. Addition of one percent cell-free filtrate of B. cereus to lactic starter cultures increased the titratable acidity an average of 17.2 percent using autoclaved reconstituted NDM as growth medium (Table 8). The stimulatory affect on lactic starter cultures was decreased, however, when pasteurized skim milk was used as the growth medium (Table 9). The cell-free filtrate of B. cereus increased the average titratable acidity over the control 14 percent after 14 hours incubation at 21 C.

Stimulatory effect of an ethanol precipitate obtained from a proteolyzed milk culture of B. cereus. Addition of the cell-free ethanol precipitated fraction of B. cereus resulted in stimulation of lactic starter cultures when grown in autoclaved 10 percent reconstituted NDM at 21 C (Table 10). The average increase in titratable acidity after 14 hours was 32.2 percent. However, compared to the original cell-free filtrate (17.2 percent increase after 14 hours incubation at 21 C, Table 8) there was a substantial increase in lactic acid production when autoclaved 10 percent reconstituted NDM was used as the growth medium. The addition of cell-free filtrates that had been precipitated with ethanol produced a 24.7 percent average increase in titratable acidity, after 14 hours incubation at 21 C, when pasteurized skim milk was used as the growth medium (Table 11).

Coagulation of pasteurized skim milk was noted after 12 hours incubation at 21 C with the cell-free ethanol precipitated filtrate of B. cereus. Coagulation was considered to be caused by the presence of lactic acid.

Stimulatory effect of the lyophilized ethanol-precipitated milk proteolysate fraction of B. cereus obtained from Sephadex G-100 Gel-Filtration. Figure 4 shows two major peaks which were obtained from column fractionation of resuspended ethanol precipitate of B cereus. The first peak produced greater stimulation for lactic starter cultures than the second peak. There was an average stimulation in titratable acidity exceeding 9.5 percent when using material from the first peak with autoclaved 10 percent reconstituted NDM as growth medium (Table 12). In contrast, there was an average increase in titratable acidity of approximately 24.7 percent when pasteurized skim milk was used as the growth medium (Table 13). Coagulation of pasteurized skim milk occurred at the end of 10 hours of incubation. The pH of the coagulated milk samples was not determined; therefore, no definite correlation could be made between coagulation of the milk and the presence of milk-clotting enzymes.

Table 15 shows, however, that there was approximately a 20 fold increase in specific activity in terms of proteolysis over the original filtrate of B. cereus. By increasing the specific activity of the preparation, it appears that a greater amount of enzymatic material was present, consequently responsible for the coagulation of pasteurized skim milk after only 10 hours of incubation.

Cell-free filtrate of B. cereus obtained from the second gel-filtration peak was less stimulatory than the fraction from the first peak (Table 16). The average increase in titratable acidity was 18 percent over the control when pasteurized skim milk was used as the growth medium. Coagulation was observed only in one case at 14 hours of incubation at 21 C.

The combining of two Sephadex fractions, i.e. peaks 1 and 2 for stimulating lactic cultures was not carried out. The possibility exists that greater stimulation could have been achieved had this been done. Dilution from subsequent fractionation, might alter in some way the stimulatory property of the test fraction. However, the effect of diluting the cell-free filtrate was not demonstrated.

Specific Stimulatory Acitivity of Various Test Fractions Upon Lactic Starter Cultures

The development of lactic starter cultures as affected by various cell-free fractions is shown in Tables 17-20. The objective of this experiment was to compare the specific stimulatory activity of various test fractions. Specific stimulatory activity was expressed as mg of lactic acid produced per mg of nitrogen per sample.

Table 16 shows development of lactic cultures by addition of various fractions of B. cereus cell-free milk proteolysate; the growth medium used was autoclaved 10 percent reconstituted NDM. Results indicated the greatest increase in specific stimulatory activity occurred when cell-free Sephadex-gel filtration fractions were used. As is evident in Table 16, lactic starter culture "D" showed the greatest response to overall specific stimulatory activity to the cell-free preparation of B. cereus.

SUMMARY

Principle findings from this investigation include the following:

1. The B. cereus culture was able to proteolyze autoclaved 10 percent reconstituted NDM faster than Ps. fluorescens culture over a 1-14 day fermentation period.
2. Cell-free milk proteolysates of Ps. fluorescens or B. cereus obtained after three days or longer fermentation were stimulatory to lactic starter cultures.
3. Heat treatment of cell-free milk proteolysates of Ps. fluorescens and B. cereus inhibited the stimulation of lactic starter cultures, as well as reducing the milk-clotting capability of respective cell-free filtrates.
4. The addition of cell-free ethanol precipitated proteolysates of Ps. fluorescens and B. cereus stimulated lactic starter cultures; growth media for the lactic cultures included 10 percent sterile reconstituted NDM and pasteurized skim milk.
5. The cell-free fractions of Ps. fluorescens and B. cereus obtained from Sephadex G-100 gel-filtration were stimulatory to lactic starter cultures. There was greater stimulation of the lactic organisms using 10 percent autoclaved reconstituted NDM as the growth medium, as compared to pasteurized skim milk.
6. Cell-free fractions of Ps. fluorescens produced the greatest specific stimulatory activity when compared to that of B. cereus. Specific stimulatory activity was expressed in terms of both proteolysis and stimulation of lactic acid production.

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Table 1. Effect of a cell-free eight-day old milk proteolysate from Pseudomonas fluorescens on acid development of lactic starter cultures grown in autoclaved 10 percent reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.27	.31	.33	.45	.42	.68[b]
	.27	.31	.34	.48	.43	.68[b]
Avg.	.27	.31	.33	.46	.42	.68
		14.8[c]		32.3[c]		61.9[c]
B	.42	.46	.57	.72[b]	.72[b]	.81[b]
	.42	.46	.56	.70[b]	.74[b]	.81[b]
Avg.	.42	.46	.56	.71	.73	.81
		9.5[c]		26[c]		10.9[c]
C	.31	.37	.41	.59[b]	.54	.74[b]
	.30	.37	.41	.60[b]	.52	.70[b]
Avg.	.30	.37	.41	.59	.53	.72
		23.3[c]		43[c]		35.8[c]
D	.37	.44	.50	.64[b]	.69[b]	.80[b]
	.40	.44	.52	.66[b]	.69[b]	.80[b]
Avg.	.37	.44	.51	.65	.69	.80
		18.9[c]		27.4[c]		15.9[c]
Increase over controls (%)		16.6		32.5		31.1

[a] Results are the average of duplicate samples.

[b] Indicates coagulation.

[c] Percentage average increase in titratable acidity of sample containing filtrate over that of control.

Table 2. Effect of a cell-free eight-day old milk proteolysate of *Pseudomonas fluorescens* on acid development of lactic starter cultures grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.34	.42	.56[b]	.53	.63[b]
	.29	.36	.44	.59[b]	.50	.64[b]
Avg.	.30	.35	.43	.56	.51	.63
		16.6[c]		30.2[c]		23.5[c]
B	.31	.36	.53	.65[b]	.62[b]	.71[b]
	.31	.36	.54	.66[b]	.60[b]	.72[b]
Avg.	.31	.36	.53	.65	.61	.71
		16.1[c]		22.6[c]		16.3[c]
C	.29	.29	.41	.47	.50	.53
	.28	.31	.43	.48	.49	.50
Avg.	.28	.30	.42	.47	.49	.51
		2.1[c]		11.9[c]		4.0[c]
D	.31	.32	.45	.54	.43	.65[b]
	.31	.33	.43	.57	.52	.66[b]
Avg.	.31	.32	.44	.55	.52	.65
		3.2[c]		25.0[c]		25.0[c]
Increase over control (%)		9.5		22.4		17.2

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage average increase in titratable acidity of sample containing filtrate over that of control.

Table 3. Effect of cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Pseudomonas fluorescens on acid development of lactic starter cultures grown in autoclaved 10 percent reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.34	.44[b]	.50	.60[b]	.62[b]	.78[b]
	.36	.42[b]	.48	.59[b]	.64[b]	.77[b]
Avg.	.35	.43	.49	.59	.63	.77
		22.8[c]		20.4[c]		22.2[c]
B	.40	.48	.54	.65[b]	.75[b]	.80[b]
	.41	.46	.55	.67[b]	.74[b]	.79[b]
Avg.	.40	.47	.54	.66	.74	.79
		17.5[c]		22.2[c]		6.7[c]
C	.27	.30	.39	.51[b]	.52	.69[b]
	.26	.28	.40	.52[b]	.50	.70[b]
Avg.	.26	.27	.39	.51	.51	.69
		3.8[c]		30.7[c]		35.2[c]
D	.31	.50[b]	.39	.51[b]	.50	.70[b]
	.32	.48[b]	.40	.52[b]	.51	.71[b]
Avg.	.31	.49	.39	.51	.50	.70
		58[c]		30.7[c]		40.0[c]
Increase over control (%)		25.5		26.0		26.0

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 4. Effect of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Pseudomonas fluorescens on acid development of lactic starter cultures, grown in pasteurized skim milk.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.28	.31	.39	.45[b]	.46	.50[b]
	.27	.27	.38	.47[b]	.46	.51[b]
Avg.	.27	.29	.38	.46	.46	.50
		2.4[c]		21.0[c]		8.6[c]
B	.28	.29	.46	.55[b]	.54	.58[b]
	.28	.30	.45	.53[b]	.52	.59[b]
Avg.	.28	.29	.45	.57	.53	.58
		3.5[c]		26.6[c]		9.4[c]
C	.25	.27	.37	.41[b]	.43	.53[b]
	.26	.26	.37	.43[b]	.42	.55[b]
Avg.	.25	.26	.37	.42	.42	.54
		4.0[c]		13.5[c]		28.5[c]
D	.26	.30	.39	.43	.50[b]	.56[b]
	.27	.27	.37	.43	.50[b]	.59[b]
Avg.	.26	.28	.38	.43	.50	.57
		7.6[c]		13.1[c]		14.0[c]
Increase over control (%)		5.6		18.5		15.1

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 5. Effect by gel-filtration (peak I) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Pseudomonas fluorescens on acid development of lactic starter cultures in autoclaved 10 percent NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.35	.40	.54[b]	.60[b]	.75[b]
	.30	.36	.38	.54[b]	.60[b]	.74[b]
Avg.	.30	.35	.39	.54	.60	.74
		16.6[c]		38.4[c]		23.3[c]
B	.34	.40	.42	.58[b]	.66[b]	.79[b]
	.35	.41	.43	.60[b]	.68[b]	.80[b]
Avg.	.34	.40	.42	.59	.67	.79
		17.6[c]		40.4[c]		17.7[c]
C	.34	.44	.43	.59[b]	.64[b]	.74[b]
	.35	.43	.42	.60[b]	.63[b]	.76[b]
Avg.	.34	.43	.42	.59	.63	.75
		26.4[c]		40.4[c]		17.0[c]
D	.30	.39	.36	.59[b]	.43	.76[b]
	.28	.37	.35	.60[b]	.45	.77[b]
Avg.	.29	.38	.35	.59	.44	.76
		31.0[c]		68.5[c]		72.7[c]
Increase over control (%)		22.9		46.9		33.2

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 6. Effect of gel-filtration (peak I) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Pseudomonas fluorescens on acid development of lactic starter cultures grown in pasteurized skim milk.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.33	.41[b]	.50	.72[b]	.77[b]	.90[b]
	.32	.42[b]	.50	.73[b]	.79[b]	.84[b]
Avg.	.32	.41	.50	.72	.78	.87
		28.1[c]		44.0[c]		11.5[c]
B	.39	.43[b]	.60[b]	.77[b]	.83[b]	.85[b]
	.37	.43[b]	.58[b]	.79[b]	.84[b]	.85[b]
Avg.	.38	.43	.59	.78	.83	.85
		13.1[c]		32.2[c]		2.4[c]
C	.38	.43[b]	.60[b]	.79[b]	.78[b]	.88[b]
	.38	.44[b]	.59[b]	.79[b]	.77[b]	.89[b]
Avg.	.38	.43	.59	.79	.77	.88
		13.1[c]		33.8[c]		14.2[c]
D	.34	.38[b]	.53	.73[b]	.77[b]	.88[b]
	.34	.39[b]	.52	.74[b]	.76[b]	.87[b]
Avg.	.34	.38	.52	.73	.77	.87
		11.7[c]		40.3[c]		12.9[c]
Increase over control (%)		11.5		37.5		10.2

[a]Results are the average of duplicate samples.

[b]Indicates coagulation

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 7. Effect by gel-filtration (peak II) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Pseudomonas fluorescens on acid development of lactic starter cultures grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.27	.27	.30	.32	.38	.41
	.26	.27	.30	.33	.39	.40
Avg.	.26	.27	.30	.32	.38	.40
		3.9[c]		6.6[c]		5.2[c]
B	.30	.34	.47	.53	.61	.65[b]
	.32	.34	.46	.52	.60	.66[b]
Avg.	.31	.34	.46	.52	.60	.65
		9.6[c]		13.0[c]		8.3[c]
C	.30	.35	.45	.51	.59	.66
	.33	.37	.44	.51	.57	.68
Avg.	.32	.33	.44	.51	.58	.67
		3.1[c]		15.9[c]		15.5[c]
D	.33	.36	.42	.47	.50	.61
	.32	.36	.42	.48	.51	.62
Avg.	.32	.36	.42	.47	.50	.51
		12.5[c]		11.9[c]		22.0[c]
Increase over control (%)		17.2		11.8		12.7

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 8. Effect of a cell-free eight-day old milk proteolysate from Bacillus cereus on acid development of lactic starter cultures grown in autoclaved 10 percent reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.26	.35	.36	.56[b]	.57	.76[b]
	.28	.36	.35	.56[b]	.55	.72[b]
Avg.	.27	.35	.35	.56	.56	.74
	29.6[c]		60.0[c]		32.1[c]	
B	.41	.52[b]	.62[b]	.74[b]	.80[b]	.84[b]
	.40	.54[b]	.63[b]	.74[b]	.82[b]	.85[b]
Avg.	.40	.53	.62	.74	.81	.84
	32.5[c]		19.3[c]		3.7[c]	
C	.32	.38	.45	.60[b]	.61[b]	.75[b]
	.33	.37	.44	.62[b]	.63[b]	.76[b]
Avg.	.32	.37	.44	.61	.62	.75
	15.6[c]		38.6[c]		20.7[c]	
D	.41	.51[b]	.55[b]	.71[b]	.69[b]	.84[b]
	.41	.54[b]	.56[b]	.73[b]	.70[b]	.85[b]
Avg.	.41	.52	.55	.72	.69	.84
	26.8[c]		30.9[c]		12.1[c]	
Increase over control (%)	26.1		37.2		17.2	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage average increase in titratable acidity of sample containing filtrate over that of control.

Table 9. Effect of a cell-free eight-day old milk proteolysate of Bacillus cereus on acid development of lactic starter cultures, grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.32	.48	.56	.51	.58[b]
	.30	.32	.47	.53	.50	.60[b]
Avg.	.29	.32	.47	.54	.51	.59
		10.3[c]		14.8[c]		15.6[c]
B	.33	.34	.60[b]	.63[b]	.64[b]	.66[b]
	.32	.35	.58[b]	.60[b]	.61[b]	.69[b]
Avg.	.32	.34	.59	.61	.62	.67
		6.2[c]		3.3[c]		8.0[c]
C	.30	.32	.48	.49	.48	.48
	.30	.30	.49	.50	.50	.52
Avg.	.30	.31	.48	.49	.49	.50
		3.3[c]		2.0[c]		2.0[c]
D	.30	.34	.46	.54	.51	.58[b]
	.31	.35	.46	.56	.52	.67[b]
Avg.	.30	.34	.46	.55	.51	.62
		13.3[c]		19.5[c]		31.3[c]
Increase over control (%)		8.2		9.9		14.2

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage average increase in titratable acidity of sample containing filtrate over that of control.

Table 10. Effect of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Bacillus cereus on acid development of lactic starter cultures grown in autoclaved 10 percent reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.34	.50[b]	.51	.64[b]	.64[b]	.76[b]
	.36	.48[b]	.52	.64[b]	.65[b]	.77[b]
Avg.	.35	.49	.51	.64	.64	.76
	40.0[c]		25.4[c]		40.7[c]	
B	.42	.52	.59[b]	.70[b]	.76[b]	.87[b]
	.42	.54	.59[b]	.70[b]	.75[b]	.86[b]
Avg.	.42	.53	.59	.70	.75	.86
	26.1[c]		18.6[c]		14.6[c]	
C	.29	.33	.38	.49	.52	.68[b]
	.27	.32	.36	.50	.54	.69[b]
Avg.	.28	.32	.37	.49	.53	.68
	14.2[c]		32.4[c]		28.3[c]	
D	.39	.45[b]	.40	.59[b]	.54	.77[b]
	.38	.47[b]	.41	.60[b]	.53	.78[b]
Avg.	.38	.46	.40	.59	.53	.77
	21.0[c]		47.5[c]		45.2[c]	
Increase over control (%)	25.3		30.9		32.2	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 11. Effect of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Bacillus cereus on acid development of lactic starter cultures, grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.27	.28	.37	.44[b]	.44	.53[b]
	.28	.30	.37	.42[b]	.45	.55[b]
Avg.	.27	.29	.37	.41	.44	.54
		7.4[c]		10.8[c]		22.7[c]
B	.28	.30[b]	.46	.51[b]	.51	.61[b]
	.27	.30[b]	.47	.52[b]	.50	.62[b]
Avg.	.27	.30	.46	.51	.50	.61
		11.1[c]		10.8[c]		22.0[c]
C	.27	.27	.37	.41[b]	.42	.51
	.25	.27	.37	.42[b]	.43	.53
Avg.	.26	.27	.37	.41	.42	.52
		3.8[c]		10.8[c]		23.8[c]
D	.27	.29	.40	.48[b]	.49	.63[b]
	.28	.29	.39	.47[b]	.50	.65[b]
Avg.	.27	.29	.39	.47	.49	.64
		7.4[c]		20.5[c]		30.6[c]
Increase over control (%)		7.4		13.2		24.7

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 12. Effect of gel-filtration (peak I) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Bacillus cereus on acid development of lactic starter cultures grown in autoclaved 10 percent NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.35	.42	.57[b]	.67[b]	.73[b]
	.31	.36	.43	.58[b]	.66[b]	.74[b]
Avg.	.30	.35	.42	.57	.66	.73
		16.6[c]		35.7[c]		10.6[c]
B	.34	.44	.49	.64[b]	.71[b]	.77[b]
	.35	.43	.48	.65[b]	.72[b]	.79[b]
Avg.	.34	.43	.48	.64	.71	.78
		26.4[c]		33.3[c]		9.8[c]
C	.34	.43	.47	.56[b]	.67[b]	.72[b]
	.35	.44	.47	.58[b]	.68[b]	.74[b]
Avg.	.34	.43	.57	.67	.73	
		26.4[c]		21.2[c]		8.9[c]
D	.31	.38	.36	.57[b]	.67[b]	.72[b]
	.29	.40	.34	.59[b]	.68[b]	.74[b]
Avg.	.30	.39	.35	.58	.67	.73
		30.0[c]		65.7[c]		8.9[c]
Increase over control (%)		24.8		38.9		9.5

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 13. Effect of gel-filtration)peak I) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Bacillus cereus on acid development of lactic starter cultures, grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.32	.38[b]	.57	.72[b]	.83[b]	.85[b]
	.33	.39[b]	.58	.71[b]	.85[b]	.85[b]
Avg.	.32	.38	.57	.71	.84	.85
		18.7[c]		24.5[c]		1.1[c]
B	.38	.45[b]	.62[b]	.81[b]	.83[b]	.91[b]
	.33	.46[b]	.60[b]	.82[b]	.84[b]	.89[b]
Avg.	.35	.45	.61	.81	.83	.90
		28.5[c]		32.7[c]		8.4[c]
C	.40	.42[b]	.66[b]	.76[b]	.81[b]	.96[b]
	.40	.43[b]	.64[b]	.75[b]	.80[b]	.95[b]
Avg.	.40	.42	.65	.75	.80	.95
		5.0[c]		15.3[c]		18.7[c]
D	.35	.40[b]	.59[b]	.77[b]	.81[b]	.86[b]
	.34	.39[b]	.58[b]	.76[b]	.80[b]	.87[b]
	.34	.39	.58	.76	.80	.86
		14.7[c]		31.0[c]		7.5[c]
Increase over control (%)		16.7		25.8		8.7

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 14. Purification of milk-clotting enzymes obtained from cell-free filtrates of Ps. florescens when grown in autocalved 10 percent NDM for 8 days at 25 C.

Fraction Number	Fractionation Step	Volume (ml)	Tyrosine Equivalent (mg/ml)	Total[1] Units	Nitrogen (mg/ml)	Specific Activity[2]	Yield (%)	Purification
I.	Original Cell-free Filtrate	900	2.085	1,876.50	5.9350	0.3513	100	1
II.	Resuspended Ethanol Precipitate	250	6.000	1,500.00	0.8690	6.9410	80	19.65
III.	Peak I Gel-Filtration Sephadex G-100	100	3.400	340	0.1764	19.2400	18	54.76

[1]Total units expressed by vol/ml x Mg tyrosine equivalent/ml.

[2]Specific activity expressed in terms of proteolysis, i.e., tyrosine equivalent (mg/ml) ÷ nitrogen content (mg/ml).

Table 15. Purification of milk-clotting enzymes obtained from cell-free filtrates of B. cereus when grown in autoclaved 10 percent NDM for 8 days at 25 C.

Fraction Number	Fractionation Step	Volume (ml)	Tyrosine Equivalent (mg/ml)	Total[1] Units	Nitrogen (mg/ml)	Specific[2] Activity	Yield (%)	Purification
I.	Original Cell-free Filtrate	900	4.040	3636	5.729	0.7051	100.00	1.00
II.	Resuspended Ethanol Precipitate	250	6.000	1500	0.9221	6.5070	41.00	9.22
III.	Peak I	100	2.500	250	0.1802	13.8700	6.9	19.67

[1]Total units expressed by vol/ml x Mg tyrosine equivalent/ml.

[2]Specific activity expressed in terms of proteolysis, i.e., tyrosine equivalent (mg/ml ÷ nitrogen content (mg/ml).

Table 16. Effect by gel-filtration (peak II) of a cell-free eight-day old resuspended ethanol precipitate from a milk proteolysate of Bacillus cereus on acid development of lactic starter cultures grown in pasteurized skim milk.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.26	.33	.33	.41	.36	.49
	.27	.34	.31	.41	.35	.50
Avg.	.26	.33	.32	.41	.35	.49
		26.9[c]		28.1[c]		40.0[c]
B	.31	.33	.45	.48	.60	.62[b]
	.32	.32	.46	.49	.60	.60[b]
Avg.	.31	.32	.45	.48	.60	.61
		3.2[c]		6.6[c]		1.6[c]
C	.31	.32	.47	.52	.52	.62
	.33	.34	.46	.50	.52	.63
Avg.	.32	.33	.46	.51	.52	.62
		3.1[c]		10.8[c]		19.2[c]
D	.32	.37	.44	.51	.51	.57
	.32	.38	.44	.50	.52	.58
Avg.	.32	.37	.44	.50	.51	.57
		15.6[c]		13.6[c]		11.7[c]
Increase over control (%)		12.2		14.7		18.1

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

[c]Percentage of average increase in titratable acidity of sample containing filtrate over that of control.

Table 17. Acid development by lactic starter cultures as affected by various fractions of Ps. fluorescens proteolysate (Lactic cultures grown in autoclaved 10 percent reconstituted NDM).

		ACID DEVELOPMENT BY LACTIC CULTURES — Incubation at 21 C						INCREASE IN LACTIC ACID (SAMPLE CONTAINING FILTRATE MINUS CONTROL			SPECIFIC STIMULATORY ACTIVITY OF THE FILTRATE		
		10 hrs.		12 hrs.		14 hrs.		10 hrs.	12 hrs.	14 hrs.	10 hrs.	12 hrs.	14 hrs.
Culture		Titratable acidity in percent[a]											
		Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	mg/100 ml sample			mg lactic acid/100 ml sample/mg N in 1 ml filtrate		
I	A	.27	.31	.33	.46	.42	.68	40.3	131.2	201.6	7.0	22.0	34.0
Original Cell-free Filtrate	B	.42	.46	.56	.71[d]	.73	.81[d]	40.3	141.1	106.8	7.0	22.1	18.0
	C	.30	.37	.41	.59[d]	.53	.72[d]	60.5	181.7	191.5	10.2	30.6	32.3
5.935 mg N/ml	D	.39	.44	.51	.65[d]	.69[d]	.80[d]	40.3	141.1	110.9	6.8	23.8	18.7
II	A	.35	.43[d]	.49	.59[d]	.63[d]	.77[d]	85.0	133.5	159.3	14.3	22.5	26.8
Resuspended Ethanol Cell-free Filtrate	B	.40	.47	.54	.66[d]	.74[d]	.79[d]	63.7	125.2	53.1	10.7	21.1	8.9
	C	.26	.29	.39	.57	.51[d]	.69[d]	21.2	192.1	191.1	32.3	40.5	32.2
5.935 mg N/ml	D	.31	.49[d]	.39	.57	.50	.70	191.3	192.1	212.5	32.3	21.5	35.8
III	A	.30	.35	.39	.54[d]	.60[d]	.74[d]	66.7	200.3	186.9	11.2	33.7	31.5
Cell-free Filtrate Peak I Sephadex Gel G-100	B	.34	.40	.42	.59[d]	.67[d]	.79[d]	80.1	227.0	198.8	13.5	38.2	33.5
	C	.34	.53	.42	.59[d]	.63[d]	.75[d]	253.7	226.5	160.2	42.7	38.1	27.0
5.935 mg N/ml	D	.29	.38	.35	.59[d]	.44	.76[d]	120.2	320.0	427.0	20.2	54.0	72.0

[a] Results are the average of duplicate samples.

[b] 99 ml milk, 1 ml of lactic acid culture and sterile water if required for dilution purposes.

[c] 99 ml milk, 1 ml of lactic culture and known quantity of test fraction.

[d] Indicates coagulation.

Table 18. Acid development by lactic starter cultures as affected by various fractions of B. cereus cell-free milk proteolysate (Lactic cultures grown in autoclaved 10 percent reconstituted NDM).

Culture		ACID DEVELOPMENT BY LACTIC CULTURES — Incubation at 21 C — 10 hrs. Cont.[b]	10 hrs. Filt.[c]	12 hrs. Cont.[b]	12 hrs. Filt.[c]	14 hrs. Cont.[b]	14 hrs. Filt.[c]	INCREASE IN LACTIC ACID (SAMPLE CONTAINING FILTRATE MINUS CONTROL) 10 hrs.	12 hrs.	14 hrs.	SPECIFIC STIMULATORY ACTIVITY OF THE FILTRATE AT 10 hrs.	12 hrs.	14 hrs.
		Titratable acidity in percent[a]						mg/100 ml sample			mg lactic acid/100 ml sample/mg N in 1 ml filtrate		
I	A	.27	.35	.35	.56[d]	.56	.74[d]	80.7	212.0	181.7	14.0	37.0	31.7
Original Cell-free Filtrate	B	.40	.53	.62[d]	.74[d]	.81[d]	.84[d]	131.2	121.1	30.2	22.9	21.1	5.2
	C	.32	.37	.45	.61	.62[d]	.75[d]	50.4	161.5	257.1	8.8	28.2	44.8
5.729 mg N/ml	D	.41	.52[d]	.55[d]	.72[d]	.69[d]	.84[d]	90.8	171.6	151.4	15.8	29.9	26.4
II	A	.35	.49[d]	.51	.64[d]	.64	.76[d]	148.6	138.0	127.4	25.9	24.0	22.2
Resuspended Ethanol Cell-free Filtrate	B	.42	.53	.59[d]	.70[d]	.75[d]	.86[d]	116.8	138.0	116.8	20.3	24.0	20.3
	C	.28	.32	.37	.49	.53	.68[d]	42.4	127.4	154.3	7.4	22.2	27.8
5.729 mg N/ml	D	.38	.46	.40	.59[d]	.53	.77[d]	84.9	201.7	254.9	14.8	35.2	44.4
III	A	.30	.35	.42	.57[d]	.66[d]	.73[d]	68.9	197.6	96.5	12.0	34.6	16.1
Cell-free Filtrate Peak I Sephadex Gel G-100	B	.34	.43	.48	.64[d]	.71[d]	.78[d]	124.0	210.8	96.5	21.6	36.8	16.8
	C	.34	.43	.47	.57[d]	.67[d]	.73[d]	118.5	131.7	63.7	20.7	23.0	11.1
5.729 mg N/ml	D	.30	.37	.35	.58[d]	.67[d]	.73[d]	118.5	317.1	77.0	20.7	55.3	11.1

[a]Results are the average of duplicate samples.

[b]99 ml milk, 1 ml of lactic culture and sterile water if required for dilution purposes.

[c]99 ml milk, 1 ml of lactic culture and known quantity of test fraction.

[d]Indicates coagulation.

Table 19. Acid development by lactic starter cultures as affected by various fractions of Ps. fluorescens proteolysate (Lactic cultures grown in pasteurized skim milk).

		ACID DEVELOPMENT BY LACTIC CULTURES						INCREASE IN LACTIC ACID (SAMPLE CONTAINING FILTRATE MINUS CONTROL			SPECIFIC STIMULATORY ACTIVITY OF THE FILTRATE AT		
		Incubation at 21 C											
Culture		10 hrs.		12 hrs.		14 hrs.		10 hrs.	12 hrs.	14 hrs.	10 hrs.	12 hrs.	14 hrs.
		------Titratable acidity in percent[a]------											
		Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	----mg/100 ml sample----			mg lactic acid/100 ml sample/mg N in 1 ml filtrate		
I	A	.30	.35	.43	.56[d]	.51	.63[d]	50.4	131.2	111.0	8.5	22.1	18.7
Original Cell-free Filtrate	B	.31	.36	.53	.65[d]	.61[d]	.71[d]	50.4	132.3	100.9	8.5	22.3	17.0
	C	.28	.30	.42	.47	.49	.51	20.1	50.4	20.1	3.4	8.5	3.4
5.935 mg N/ml	D	.31	.32	.44	.55	.52	.65[d]	10.0	100.0	43.4	1.7	17.0	73.1
II	A	.27	.29	.38	.46	.46	.50	127.5	85.0	42.5	21.4	14.3	7.2
Resuspended Ethanol Cell-free Filtrate	B	.28	.29	.45	.57[d]	.53	.58[d]	10.6	170.2	53.1	1.7	28.6	8.9
	C	.25	.26	.37	.42[d]	.42	.54[d]	10.6	53.1	127.5	1.7	8.9	21.4
5.935 mg N/ml	D	.26	.28	.38	.43	.50[d]	.57[d]	21.2	53.1	74.4	3.5	8.9	12.5
III	A	.32	.41[d]	.50	.72[d]	.78[d]	.87[d]	120.2	293.8	120.2	20.2	49.5	20.2
Cell-free Filtrate Peak I Sephadex Gel G-100	B	.38	.43	.59[d]	.78[d]	.83[d]	.85[d]	66.7	253.7	26.7	11.2	42.7	4.5
	C	.38	.43[d]	.59[d]	.79[d]	.77[d]	.88[d]	66.7	267.0	146.9	11.2	45.0	25.7
5.935 mg N/ml	D	.34	.38[d]	.52	.73[d]	.77[d]	.87[d]	53.4	186.9	133.5	9.0	31.5	22.5

[a] Results are the average of duplicate samples.

[b] 99 ml milk, 1 ml of lactic culture and sterile water if required for dilution purposes.

[c] 99 ml milk, 1 ml of lactic culture and known quantity of test fraction.

[d] Indicates coagulation.

Table 20. Acid development by lactic starter cultures as affected by various fractions of B. cereus proteolysate (Lactic cultures grown in pasteurized skim milk).

		ACID DEVELOPMENT BY LACTIC CULTURES						INCREASE IN LACTIC ACID (SAMPLE CONTAINING FILTRATE MINUS CONTROL)			SPECIFIC STIMULATORY ACTIVITY OF THE FILTRATE AT		
		Incubation at 21 C											
Culture		10 hrs.		12 hrs.		14 hrs.		10 hrs.	12 hrs.	14 hrs.	10 hrs.	12 hrs.	14 hrs.
		------Titratable acidity in percent[a]-------											
		Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	Cont.[b]	Filt.[c]	----mg/100 ml sample----			mg lactic acid/100 ml sample/mg N in 1 ml filtrate		
I	A	.29	.32	.47	.54	.50	.59[d]	30.2	70.6	90.8	5.2	12.3	15.8
Original Cell-free Filtrate	B	.32	.34	.59[d]	.61[d]	.62[d]	.67[d]	20.1	20.1	50.4	3.5	3.5	8.8
	C	.30	.31	.48	.49	.49	.50	10.0	10.0	10.0	1.7	1.7	1.7
5.729 mg N/ml	D	.30	.34	.46	.55	.51	.62[d]	40.3	90.7	111.0	7.0	15.8	19.4
II	A	.27	.29	.37	.41	.44	.54	21.2	42.4	106.2	3.7	9.6	18.5
Resuspended Ethanol Cell-Free Filtrate	B	.27	.30	.46	.51	.51	.61[d]	31.8	68.9	106.2	5.5	12.0	18.5
	C	.26	.27	.37	.41	.42	.52	10.6	53.1	106.2	1.8	9.6	18.5
5.729 mg N/ml	D	.27	.29	.39	.47	.49	.64[d]	21.2	84.9	53.1	3.7	14.8	9.2
III	A	.32	.38[d]	.57[d]	.71[d]	.84[d]	.85[d]	79.0	220.6	13.1	13.7	38.5	2.2
Cell-free Filtrate Peak I Sephadex Gel G-100 5.729 mg N/ml	B	.35	.45[d]	.61[d]	.81[d]	.83[d]	.90[d]	131.7	275.7	96.5	22.9	48.1	16.0
	C	.40	.42[d]	.65[d]	.75[d]	.81[d]	.95[d]	27.5	137.8	193.0	4.8	24.0	33.6
	D	.34	.39[d]	.58[d]	.76[d]	.81[d]	.86[d]	65.8	248.1	68.9	11.4	43.3	12.4

[a]Results are the average of duplicate samples.

[b]99 ml milk, 1 ml of lactic culture and sterile water if required for dilution purposes.

[c]99 ml milk, 1 ml of lactic culture and known quantity of test fraction.

[d]Indicates coagulation.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

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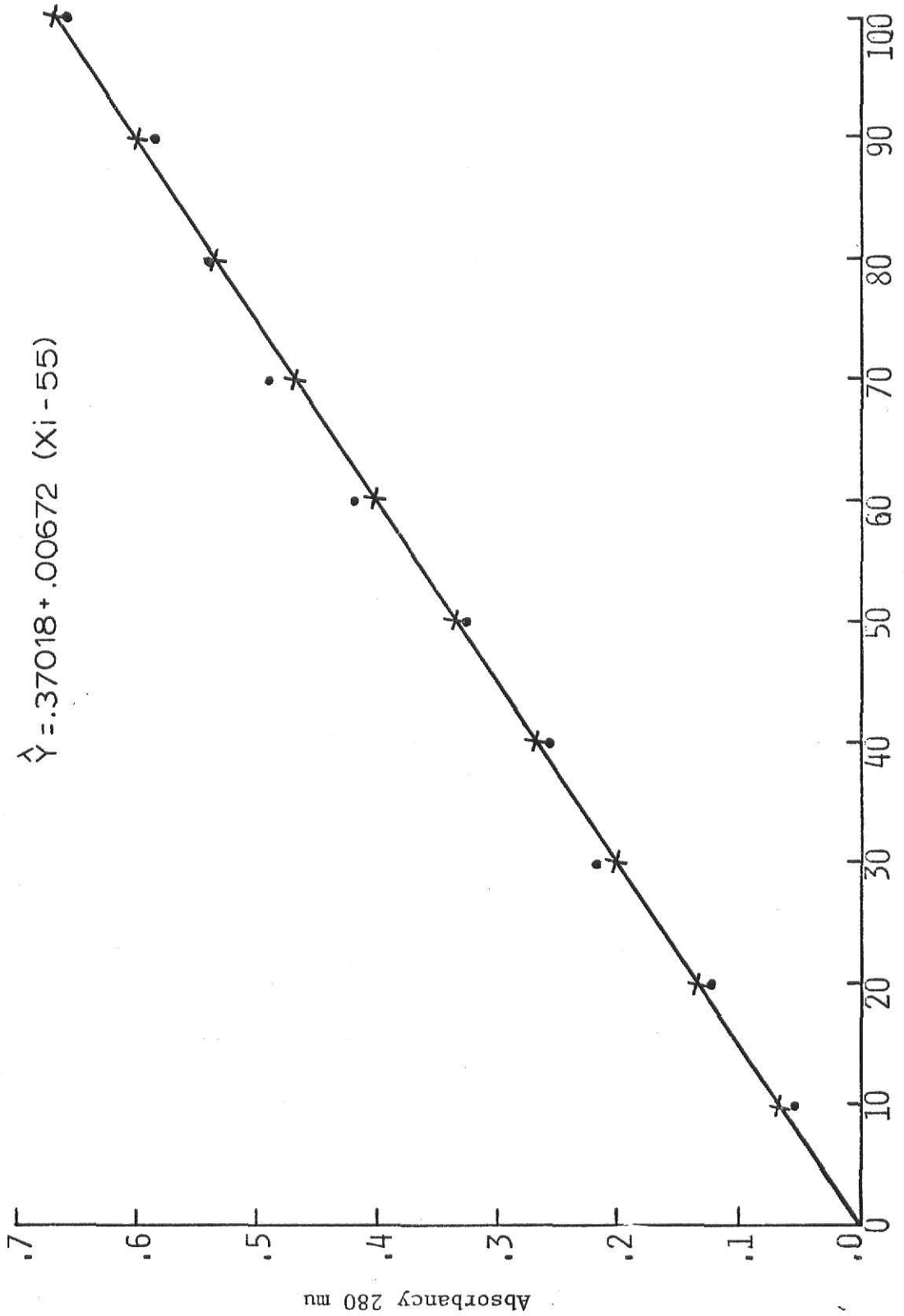

Fig. 1 Standard tyrosine curve

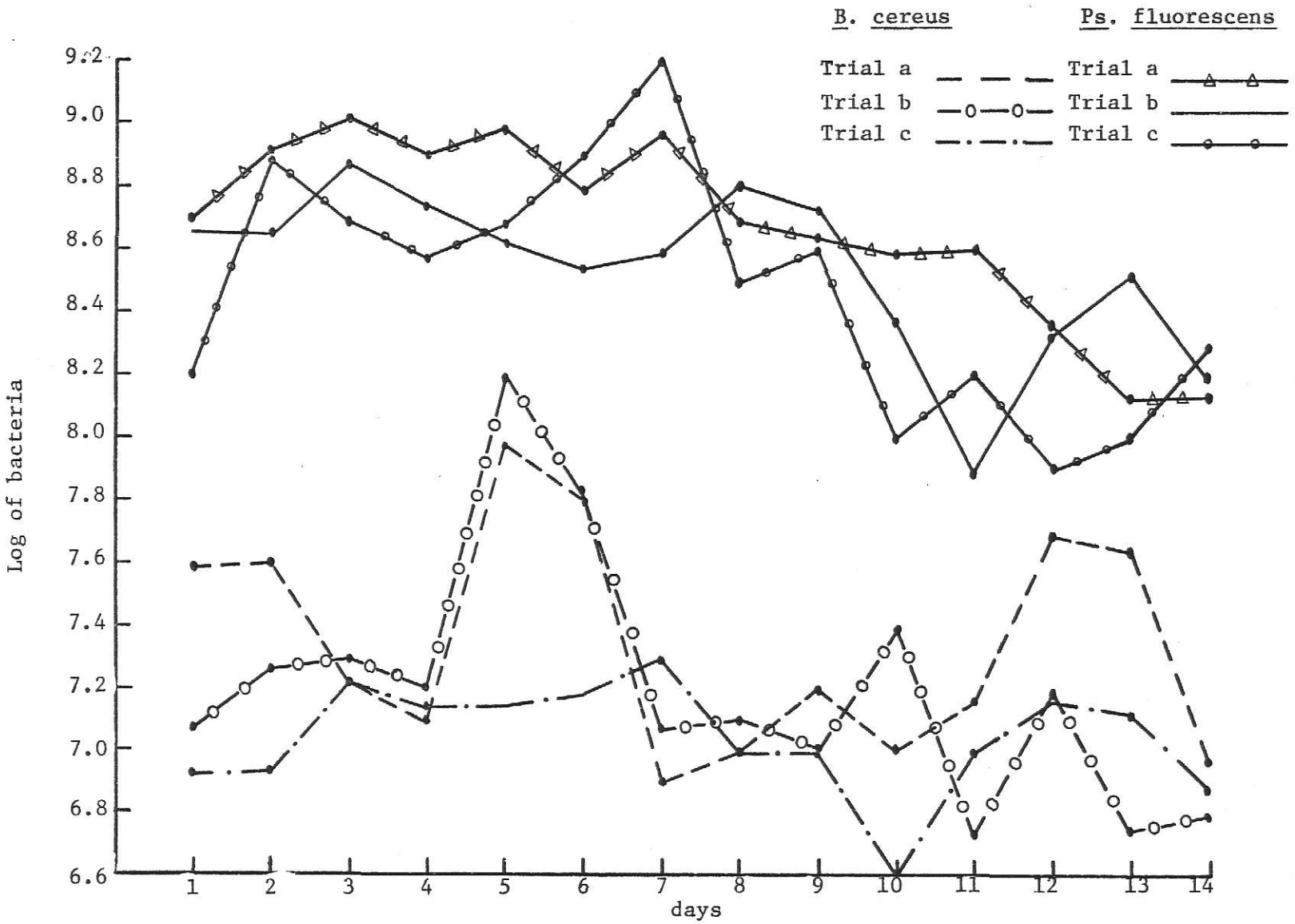

Fig. 2 Standard plate count representing three different trials of B. cereus and Ps. fluorescens for periods of 1-14 days incubated at 25 C.

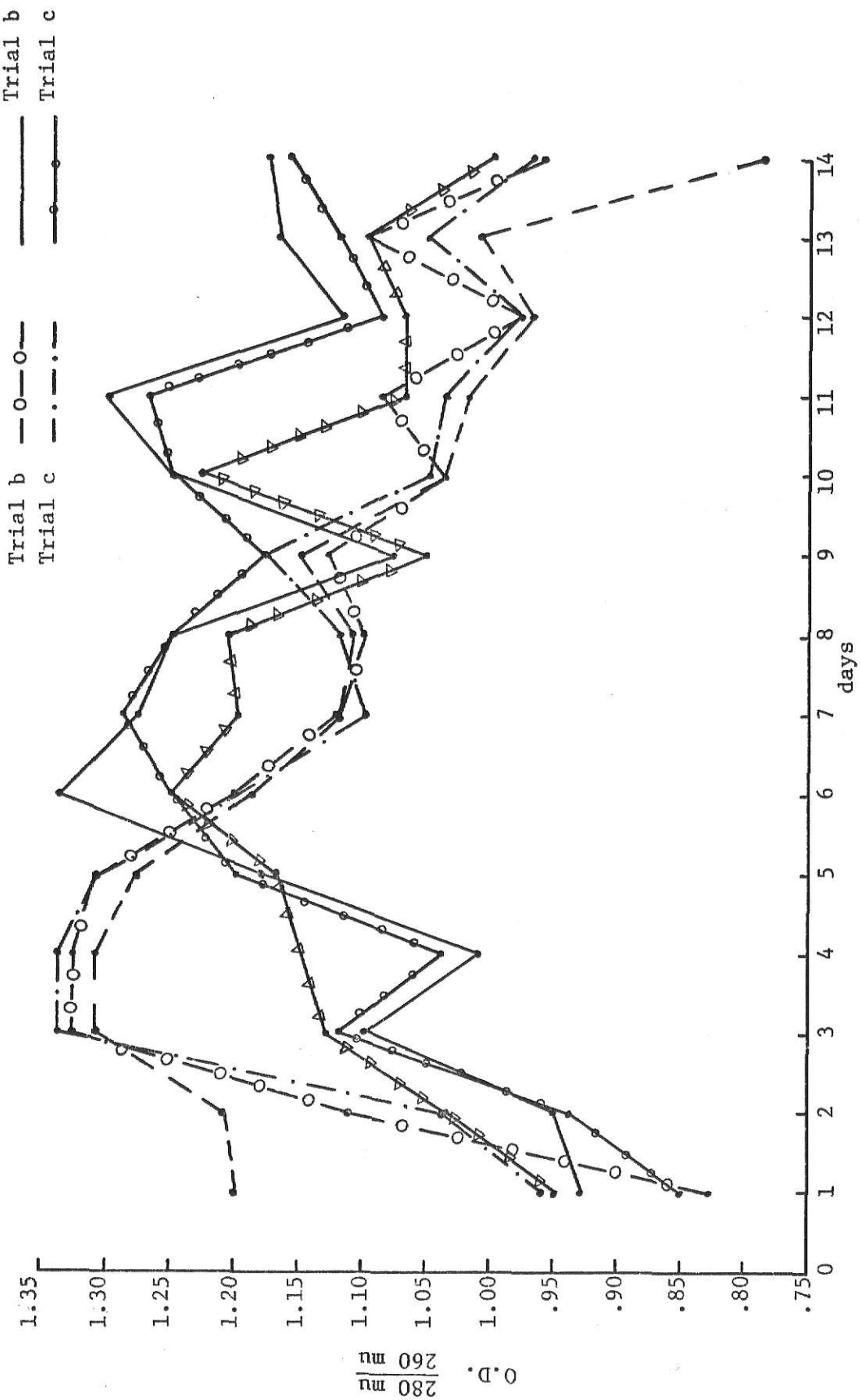

Fig. 3. Spectrophotometric assay of cell-free milk proteolysates of Ps. fluorescens and B. cereus grown in autoclaved 10 percent reconstituted NDM for 14 days.

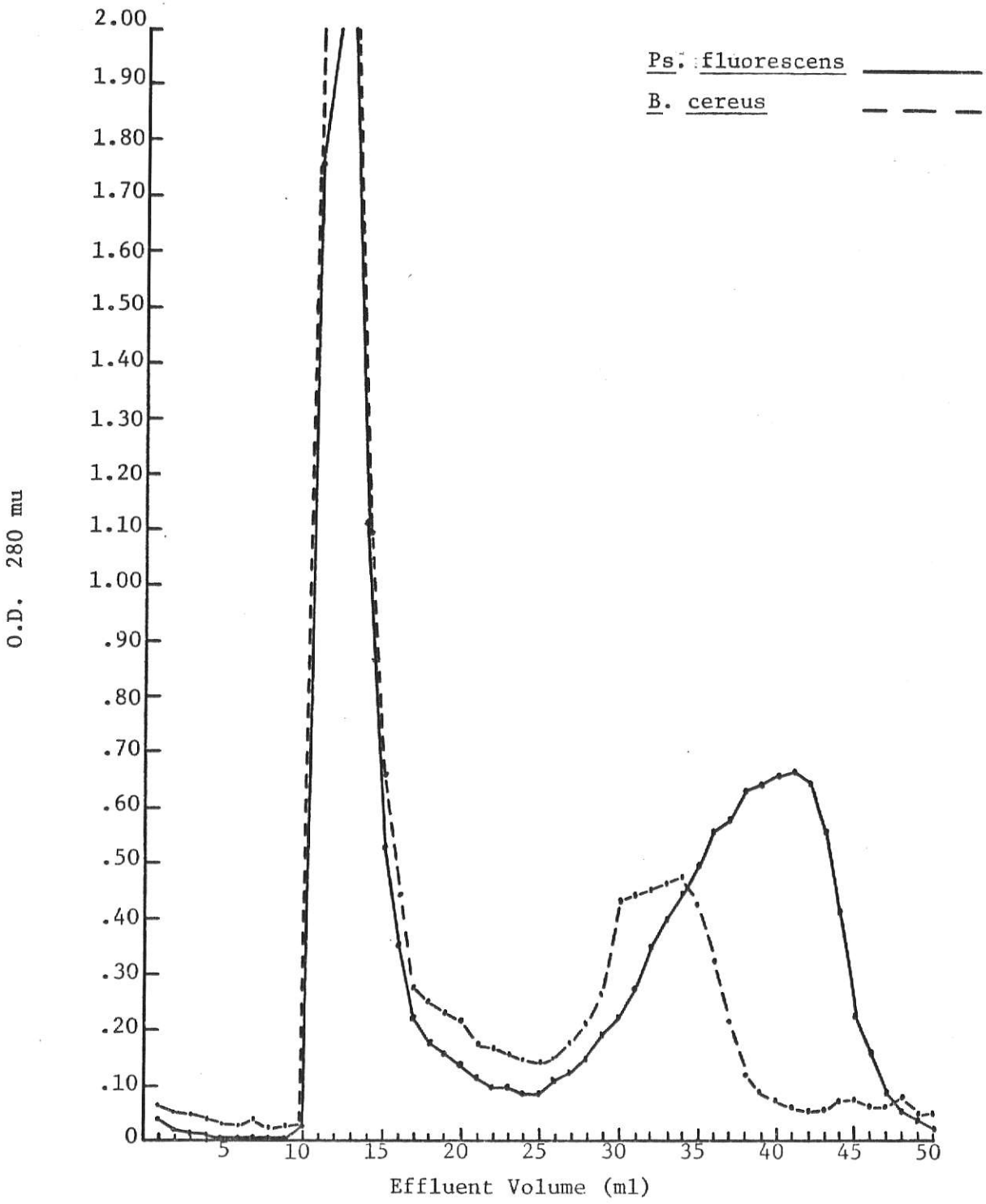

Fig. 4. Absorbancy patterns at 280 mu of resuspended ethanol precipitate fractionated on G-100 Sephadex of B. cereus and Ps. fluorescens. Cultures grown in autoclaved 10 percent reconstituted NDM at 25 C for eight days.

A P P E N D I X

Table 1. The formol titer in ml of 0.1 N NaOH in milk proteolysates of Ps. fluorescens and B. cereus. (1-14 days)

Ps. fluorescens				B. cereus			
Days	Trial a	Trial b	Trial c	Days	Trial a	Trial b	Trial c
1	.29	.27	.37	1	.40	.34	.37
2	.29	.29	.38	2	.56	.47	.48
3	.30	.30	.43	3	.64	.58	.62
4	.31	.31	.44	4	.67	.66	.65
5	.40	.34	.52	5	.80	.70	.75
6	.51	.40	.55	6	.83	.77	.80
7	.41	.50	.60	7	1.10	.81	.82
8	.65	.51	.62	8	.83	.85	.85
9	.66	.55	.80	9	.82	.88	.90
10	.55	.73	.85	10	.96	.90	.93
11	.70	.69	.84	11	1.33	.97	.95
12	.85	.82	.95	12	1.25	.99	.99
13	.75	.80	.90	13	1.32	1.02	1.15
14	.88	.96	.96	14	1.33	1.03	1.20

Table 2. The pH of milk proteolysates of Ps. fluorescens and B. cereus. (1-14 days).

	Ps. fluorescens				B. cereus		
Days	Trial a	Trial b	Trial c	Days	Trial a	Trial b	Trial c
1	6.3	6.6	6.5	1	6.3	6.4	6.2
2	6.55	7.0	6.6	2	6.4	6.4	6.3
3	6.6	7.1	6.7	3	6.5	6.5	6.4
4	6.8	7.1	6.9	4	6.7	6.5	6.5
5	6.8	7.3	6.8	5	7.0	6.6	6.7
6	7.0	7.4	7.0	6	7.1	6.6	6.6
7	7.0	7.6	6.9	7	7.3	6.7	6.7
8	7.4	7.6	6.9	8	7.8	6.7	6.8
9	7.4	7.7	7.1	9	7.2	6.8	6.8
10	7.5	7.7	7.3	10	7.4	6.8	6.9
11	7.7	7.9	7.4	11	8.0	7.1	7.1
12	7.9	8.3	7.7	12	8.1	7.0	7.1
13	7.8	8.0	7.5	13	8.2	7.1	7.1
14	7.8	8.0	7.7	14	8.1	7.1	7.1

Table 3. Effect of cell-free filtrate from one day fermentation of Pseudonomas fluorescens on the acid developmnnt of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.32	.35	.35	.57[b]	.59[b]
	.27	.28	.35	.35	.56[b]	.61[b]
B	.38	.38	.52	.53	.73[b]	.78[b]
	.35	.37	.50	.52	.74[b]	.77[b]
C	.37	.37	.44	.45	.74[b]	.75[b]
	.34	.34	.47	.47	.75[b]	.78[b]
D	.32	.32	.44	.44	.74[b]	.78[b]
	.32	.34	.45	.45	.73[b]	.80[b]
Mean	.33	.34	.44	.45	.69	.73
Increase over Control (%)	3.0		2.2		5.7	

[a] Results are the average of duplicate samples.

[b] Indicates coagulation.

Table 4. Effect of cell-free filtrate from one day fermentation of Bacillus cereus on the development of lactic starter cultures grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.29	.33	.36	.55	.63[b]
	.29	.31	.34	.39	.56	.65[b]
B	.34	.43	.46	.46	.75[b]	.81[b]
	.35	.45	.44	.48	.74[b]	.79[b]
C	.33	.42	.42	.43	.72[b]	.78[b]
	.32	.44	.43	.45	.75[b]	.82[b]
D	.33	.37	.40	.50	.74[b]	.81[b]
	.34	.35	.42	.54	.75[b]	.80[b]
Mean	.33	.34	.44	.45	.69	.73
Increase over Control (%)	3.0		2.2		5.7	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 5. Effect of cell-free filtrate from two day fermentation of Pseudonomas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.36	.37	.51	.58	.71[b]	.74[b]
	.37	.37	.51	.57	.69[b]	.73[b]
B	.36	.36	.50	.54	.72[b]	.84[b]
	.35	.37	.49	.57	.69[b]	.73[b]
C	.35	.37	.51	.51	.63[b]	.66[b]
	.34	.38	.49	.50	.62[b]	.71[b]
D	.45	.45	.57	.58	.73[b]	.86[b]
	.44	.45	.59	.61	.73[b]	.82[b]
Mean	.37	.39	.52	.55	.69	.76
Increase over control (%)	5.4		5.7		10.1	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 6. Effect of cell-free filtrate from two day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	filtrate	Control	filtrate	Control	filtrate
	Titratable acidity in percent[a]					
A	.38.	.42	.56	.66[b]	.71[b]	.80[b]
	.36	.43	.57	.68[b]	.68[b]	.81[b]
B	.35	.40	.49	.63[b]	.70[b]	.77[b]
	.34	.40	.50	.64[b]	.71[b]	.78[b]
C	.34	.42	.47	.57	.66[b]	.73[b]
	.34	.45	.46	.57	.64[b]	.75[b]
D	.45	.52	.55	.62[b]	.75[b]	.78[b]
	.46	.53	.56	.64[b]	.74[b]	.76[b]
Mean	.37	.44	.52	.62	.69	.77
Increase over control (%)	11.8		18.9		11.5	

[a]Results are the average of duplicate results.

[b]Indicates coagulation.

Table 7. Effect of cell-free filtrate from three day fermentation of Pseudonomas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.36	.39	.56	.61[b]	.75[b]	.76[b]
	.36	.39	.59[b]	.67[b]	.72[b]	.78[b]
B	.34	.38	.59[b]	.62[b]	.71[b]	.79[b]
	.36	.38	.57[b]	.67[b]	.73[b]	.79[b]
C	.35	.41	.54	.61[b]	.65[b]	.76[b]
	.36	.42	.55	.64[b]	.67[b]	.75[b]
D	.44	.45	.64[b]	.67[b]	.77[b]	.80[b]
	.46	.46	.64[b]	.69[b]	.76[b]	.80[b]
Mean	.37	.41	.58	.64	.72	.77
Increase over control (%)	10.8		10.3		6.9	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 8. Effect of cell-free filtrate from three day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.44	.53	.64[b]	.71[b]	.76[b]	.83[b]
	.47	.55	.64[b]	.71[b]	.76[b]	.83[b]
B	.40	.50	.61[b]	.70[b]	.76[b]	.81[b]
	.35	.46	.63[b]	.72[b]	.77[b]	.80[b]
C	.36	.47	.54	.76[b]	.73[b]	.83[b]
	.35	.44	.55	.68[b]	.72[b]	.81[b]
D	.35	.50	.63[b]	.72[b]	.78[b]	.84[b]
	.36	.46	.65[b]	.73[b]	.77[b]	.83[b]
Mean	.38	.48	.61	.71	.75	.82
Increase over control (%)	26.3		11.3		9.3	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 9. Effect of cell-free filtrate from four day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.28	.28	.37	.38	.51	.59
	.27	.29	.36	.40	.48	.61
B	.28	.29	.31	.42	.47	.57
	.28	.28	.32	.39	.49	.58
C	.31	.32	.40	.41	.53	.54
	.29	.30	.40	.41	.52	.53
D	.28	.31	.33	.35	.47	.49
	.27	.28	.34	.38	.46	.50
Mean	.28	.29	.35	.39	.49	.55
Increase over control (%)		3.5		11.4		12.2

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 10. Effect of cell-free filtrate from four day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.27	.29	.39	.48	.54	.69[b]
	.27	.29	.38	.48	.50	.68[b]
B	.27	.29	.37	.50	.51	.67[b]
	.27	.29	.36	.50	.51	.66[b]
C	.31	.32	.41	.46	.59	.61
	.30	.32	.42	.45	.59	.64
D	.28	.30	.36	.40	.48	.55
	.27	.31	.33	.41	.47	.56
Mean	.28	.30	.37	.46	.52	.63
Increase over control (%)	7.1		24.3		21.1	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 11. Effect of cell-free filtrate from five day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.29	.42	.48[b]	.69[b]	.78[b]
	.30	.30	.40	.52[b]	.71[b]	.77[b]
B	.28	.29	.39	.49[b]	.70[b]	.76[b]
	.27	.29	.39	.52[b]	.69[b]	.74[b]
C	.28	.31	.39	.49	.62[b]	.72[b]
	.29	.30	.38	.50	.63[b]	.76[b]
D	.36	.38	.46	.54[b]	.71[b]	.78[b]
	.30	.36	.43	.57[b]	.69[b]	.75[b]
Mean	.31	.31	.40	.51	.68	.75
Increase over control (%)	0.0		27.5		10.2	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 12. Effect of cell-free filtrate from five day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.35	.50	.62[b]	.74[b]	.78[b]
	.30	.35	.49	.63[b]	.74[b]	.81[b]
B	.30	.33	.45	.59[b]	.72[b]	.81[b]
	.30	.33	.45	.53	.71[b]	.79[b]
C	.30	.34	.43	.52	.67[b]	.77[b]
	.31	.36	.40	.53	.66[b]	.75[b]
D	.35	.42	.48	.58[b]	.72[b]	.76[b]
	.36	.44	.47	.59[b]	.71[b]	.77[b]
Mean	.31	.36	.46	.57	.71	.78
Increase over control (%)	16.1		23.9		9.8	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 13. Effect of cell-free filtrate from six day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.33	.40	.49	.73[b]	.80[b]
	.31	.32	.41	.48	.72[b]	.79[b]
B	.31	.32	.43	.50	.71[b]	.78[b]
	.29	.34	.42	.49	.72[b]	.77[b]
C	.34	.36	.46	.53	.67[b]	.70[b]
	.34	.36	.47	.53	.67[b]	.72[b]
D	.38	.41	.54	.66[b]	.73[b]	.75[b]
	.36	.38	.53	.65[b]	.72[b]	.75[b]
Mean	.33	.35	.45	.54	.71	.75
Increase over control (%)	6.0		20.0		5.6	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 14. Effect of cell-free filtrate from six days fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.36	.45	.55[b]	.72[b]	.80[b]
	.29	.39	.42	.59[b]	.71[b]	.79[b]
B	.31	.42	.42	.61[b]	.74[b]	.79[b]
	.32	.41	.43	.61[b]	.73[b]	.80[b]
C	.35	.42	.47	.60[b]	.70[b]	.75[b]
	.34	.43	.47	.63[b]	.69[b]	.78[b]
D	.35	.43	.57	.70[b]	.74[b]	.80[b]
	.36	.44	.57	.69[b]	.73[b]	.80[b]
Mean	.32	.41	.47	.62	.72	.78
Increase over control (%)	26.1		31.9		8.3	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 15. Effect of cell-free filtrate from seven day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.32	.37	.56	.75[b]	.65[b]	.79[b]
	.31	.37	.59	.77[b]	.64[b]	.81[b]
B	.32	.40	.56	.76[b]	.64[b]	.82[b]
	.31	.41	.56	.74[b]	.66[b]	.80[b]
C	.42	.47	.69[b]	.76[b]	.75[b]	.80[b]
	.43	.46	.70[b]	.77[b]	.77[b]	.81[b]
D	.44	.49	.70[b]	.74[b]	.74[b]	.80[b]
	.45	.46	.70[b]	.73[b]	.73[b]	.78[b]
Mean	.37	.42	.63	.75	.69	.80
Increase over control (%)	13.5		19.0		15.9	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 16. Effect of cell-free filtrate from seven day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.32	.42	.62[b]	.79[b]	.69[b]	.85[b]
	.33	.44	.62[b]	.80[b]	.70[b]	.83[b]
B	.31	.40	.60[b]	.79[b]	.68[b]	.84[b]
	.32	.43	.58	.77[b]	.67[b]	.86[b]
C	.46	.47	.68[b]	.76[b]	.80[b]	.81[b]
	.44	.45	.66[b]	.77	.80[b]	.84[b]
D	.48	.50	.70[b]	.76[b]	.75[b]	.80[b]
	.46	.55	.69[b]	.79[b]	.76[b]	.81[b]
Mean	.39	.45	.64	.77	.73	.73
Increase over control (%)	11.5		20.3		0.0	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 17. Effect of cell-free filtrate from eight day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.37	.53	.73[b]	.59[b]	.77[b]
	.31	.36	.53	.75[b]	.56	.76[b]
B	.30	.37	.48	.68[b]	.55	.74[b]
	.31	.36	.49	.69[b]	.57	.75[b]
C	.31	.31	.52	.57	.60[b]	.79[b]
	.31	.35	.53	.56	.61[b]	.78[b]
D	.35	.41	.51	.60[b]	.60[b]	.80[b]
	.34	.43	.50	.61[b]	.61[b]	.79[b]
Mean	.31	.37	.51	.64	.58	.77
Increase over control (%)	19.3		25.4		32.7	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 18. Effect of cell-free filtrate from eight day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.33	.52	.56	.59[b]	.75[b]
	.29	.34	.51	.64	.56	.77[b]
B	.31	.32	.48	.56	.57	.70[b]
	.31	.31	.50	.59	.57	.68[b]
C	.32	.33	.47	.48	.58	.60[b]
	.31	.31	.48	.48	.57	.60[b]
D	.37	.40	.51	.53	.62[b]	.68[b]
	.36	.39	.50	.51	.61[b]	.69[b]
Mean	.32	.34	.49	.61	.58	.68
Increase over control (%)	6.2		24.4		17.2	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 19. Effect of cell-free filtrate from nine day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.41	.48	.66[b]	.57	.79[b]
	.32	.42	.47	.68[b]	.56	.79[b]
B	.33	.40	.48	.65[b]	.62[b]	.74[b]
	.31	.39	.47	.64[b]	.60[b]	.75[b]
C	.33	.40	.47	.68[b]	.65[b]	.74[b]
	.32	.38	.47	.69[b]	.66[b]	.72[b]
D	.40	.54[b]	.61[b]	.73[b]	.70[b]	.77[b]
	.39	.56[b]	.62[b]	.76[b]	.69[b]	.76[b]
Mean	.33	.53	.50	.68	.63	.75
Increase over control (%)	60.6		36.0		19.0	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 20. Effect of cell-free filtrate from nine day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.34	.52	.65[b]	.66[b]	.76[b]
	.29	.34	.50	.66[b]	.66[b]	.76[b]
B	.30	.33	.51	.62[b]	.64[b]	.77[b]
	.29	.34	.54	.67[b]	.65[b]	.77[b]
C	.31	.34	.56	.64[b]	.70[b]	.77[b]
	.32	.36	.57	.66[b]	.69[b]	.79[b]
D	.39	.40	.66[b]	.69[b]	.77[b]	.83[b]
	.38	.45	.68[b]	.70	.73[b]	.81[b]
Mean	.32	.36	.56	.66	.69	.78
Increase over control (%)	12.5		17.8		13.0	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 21. Effect of cell-free filtrate from 10 day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.35	.45	.54	.63[b]	.78[b]
	.31	.36	.48	.54	.62[b]	.79[b]
B	.32	.36	.42	.55	.63[b]	.80[b]
	.33	.36	.44	.53	.63[b]	.79[b]
C	.33	.37	.46	.49	.68[b]	.72[b]
	.33	.36	.47	.50	.67[b]	.74[b]
D	.40	.40	.52	.60[b]	.73[b]	.79[b]
	.37	.42	.52	.62[b]	.74[b]	.81[b]
Mean	.33	.37	.47	.54	.66	.77
Increase over control (%)	12.1		14.8		16.6	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 22. Effect of cell-free filtrate from 10 day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.31	.40	.49	.70[b]	.71[b]	.81[b]
	.33	.40	.54	.71[b]	.69[b]	.80[b]
B	.30	.40	.47	.72[b]	.69[b]	.83[b]
	.31	.40	.48	.73[b]	.68[b]	.84[b]
C	.38	.43	.51	.68[b]	.71[b]	.80[b]
	.37	.44	.55	.68[b]	.70[b]	.81[b]
D	.42	.52	.59	.73[b]	.72[b]	.84[b]
	.43	.54	.60	.72[b]	.71[b]	.83[b]
Mean	.35	.44	.52	.70	.70	.82
Increase over control (%)	25.7		34.6		17.1	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 23. Effect of cell-free filtrate from eleven day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.38	.44	.48	.65[b]	.67[b]	.80[b]
	.36	.46	.50	.67[b]	.66[b]	.81[b]
B	.43	.45	.54	.66[b]	.67[b]	.81[b]
	.42	.46	.56	.66[b]	.66[b]	.79[b]
C	.38	.50	.63[b]	.65[b]	.73[b]	.80[b]
	.40	.50	.61[b]	.64[b]	.72[b]	.81[b]
D	.43	.45	.56	.63[b]	.69[b]	.72[b]
	.42	.44	.58	.64[b]	.70[b]	.73[b]
Mean	.40	.46	.55	.65	.68	.78
Increase over control (%)	11.5		18.1		14.7	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 24. Effect of cell-free filtrate from eleven day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.39	.53	.57	.74[b]	.66[b]	.84[b]
	.38	.56	.58	.75[b]	.68[b]	.81[b]
B	.48	.53	.62[b]	.70[b]	.67[b]	.82[b]
	.47	.47	.60[b]	.74[b]	.67[b]	.81[b]
C	.53	.57	.66[b]	.78[b]	.71[b]	.80[b]
	.54	.54	.65[b]	.77[b]	.70[b]	.79[b]
D	.46	.56	.62[b]	.75[b]	.72[b]	.82[b]
	.45	.58	.63[b]	.74[b]	.71[b]	.83[b]
Mean	.46	.54	.61	.74	.69	.81
Increase over control (%)	17.3		21.3		17.3	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 25. Effect of cell-free filtrate from twelve day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
Culture	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.43	.56	.52	.73[b]	.73[b]	.94[b]
	.45	.54	.52	.74[b]	.73[b]	.94[b]
B	.43	.54	.57	.73[b]	.72[b]	.95[b]
	.45	.53	.56	.72[b]	.73[b]	.94[b]
C	.47	.51[b]	.60	.65[b]	.75[b]	.83[b]
	.45	.50[b]	.58	.68[b]	.74[b]	.84[b]
D	.46	.61	.66[b]	.76[b]	.76[b]	.99[b]
	.47	.62	.62[b]	.75[b]	.75[b]	.98[b]
Mean	.45	.55	.57	.72	.73	.92
Increase over control (%)		12.2		26.3		26.0

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 26. Effect of cell-free filtrate from twelve day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.47	.53	.54	.80[b]	.75[b]	1.00
	.46	.54	.56	.82[b]	.79[b]	.99[b]
B	.40	.52	.55	.80[b]	.83[b]	1.00[b]
	.38	.54	.58	.77[b]	.82[b]	.99[b]
C	.49	.60[b]	.67[b]	.75[b]	.80[b]	.85[b]
	.49	.62[b]	.69[b]	.74[b]	.79[b]	.86[b]
D	.53	.61	.57	.84[b]	.79[b]	1.00[b]
	.50	.63	.59	.87[b]	.78[b]	.99[b]
Mean	.46	.57	.66	.79	.79	.96
Increase over control (%)		23.9		19.6		21.5

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 27. Effect of cell-free filtrate from thirteen day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.43	.49	.56	.65[b]	.80[b]	.85[b]
	.44	.51	.56	.65[b]	.79[b]	.84[b]
B	.39	.52	.50	.62[b]	.73[b]	1.00[b]
	.41	.57	.49	.63[b]	.74	.98[b]
C	.54	.61	.67[b]	.68[b]	.79[b]	.95[b]
	.52	.60	.67[b]	.69[b]	.78[b]	.94[b]
D	.55	.60[b]	.62	.67[b]	.78[b]	.81[b]
	.56	.60[b]	.60	.68[b]	.77[b]	.83[b]
Mean	.48	.56	.58	.65	.77	.90
Increase over control (%)	16.6		12.0		16.8	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 28. Effect of cell-free filtrate from thirteen day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.48	.59	.62	.83[b]	.90[b]	1.09[b]
	.47	.61	.63	.85[b]	.89	1.08[b]
B	.48	.60	.57	.82[b]	.78[b]	1.03[b]
	.48	.61	.56	.85[b]	.76[b]	1.00[b]
C	.56	.60[b]	.73[b]	.75[b]	.76[b]	.98[b]
	.56	.61[b]	.72[b]	.74[b]	.75[b]	.96[b]
D	.55	.77[b]	.66[b]	.91[b]	.85[b]	1.03[b]
	.55	.75[b]	.65[b]	.93[b]	.84[b]	1.00[b]
Mean	.51	.64	.64	.83	.81	1.02
Increase over control (5)	25.4		27.6		28.3	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 29. Effect of cell-free filtrate from fourteen day fermentation of Pseudomonas fluorescens on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.30	.35	.37	.53	.52	.72[b]
	.31	.35	.38	.56	.50	.74[b]
B	.30	.33	.39	.52	.53	.72[b]
	.30	.35	.38	.55	.51	.73[b]
C	.40	.43	.55	.62[b]	.71[b]	.75[b]
	.39	.46	.55	.64[b]	.70[b]	.76[b]
D	.33	.44[b]	.48	.67[b]	.67[b]	.79[b]
	.36	.46[b]	.46	.64[b]	.67[b]	.79[b]
Mean	.33	.39	.44	.59	.60	.75
Increase over control (%)	18.1		34.0		25.0	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 30. Effect of cell-free filtrate from fourteen day fermentation of Bacillus cereus on the acid development of lactic starter cultures when grown in 10 percent autoclaved reconstituted NDM.

Culture	Incubation at 21 C					
	10 hrs.		12 hrs.		14 hrs.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acidity in percent[a]					
A	.29	.33	.42	.51	.54	.67[b]
	.30	.34	.40	.51	.53	.69[b]
B	.30	.35	.41	.52	.55	.69[b]
	.31	.35	.42	.55	.55	.69[b]
C	.40	.55	.60	.69[b]	.73[b]	.91[b]
	.41	.57	.61	.71[b]	.72[b]	.92[b]
D	.35	.46	.48	.63[b]	.71[b]	.79[b]
	.36	.49	.45	.63[b]	.70[b]	.79[b]
Mean	.34	.43	.47	.57	.62	.76
Increase over control (%)	26.4		21.2		22.5	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 31. Effect of a non-heat treated eight day cell-free milk proteolysate of Bacillus cereus on titratable acidity and pH of lactic starter cultures when grown in pasteurized skim milk at 21 C.

Culture		Incubation Time: 10 hr. Control Titratable acidity (Percent[a])	10 hr. Filtrate Titratable acidity (Percent[a])	10 hr. Filtrate pH	12 hr. Control Titratable acidity (Percent[a])	12 hr. Filtrate Titratable acidity (Percent[a])	12 hr. Filtrate pH	14 hrs. Control Titratable acidity (Percent[a])	14 hrs. Filtrate Titratable acidity (Percent[a])	14 hrs. Filtrate pH
A		.31	.33[b]	5.4	.50	.59[b]	4.8	.73[b]	.75[b]	4.5
		.33	.36[b]	5.4	.52	.57[b]	4.8	.72[b]	.76[b]	4.5
	Avg.	.32	.34		.51	.58		.72	.75	
B		.32	.39[b]	5.2	.62	.68[b]	4.7	.80[b]	.82[b]	4.5
		.33	.40[b]	5.0	.63	.70[b]	4.7	.79[b]	.83[b]	4.5
	Avg.	.32	.39		.62	.69		.79	.82	
C		.34	.38[b]	5.2	.57	.69[b]	4.8	.78[b]	.79[b]	4.6
		.34	.38[b]	5.1	.61	.70[b]	4.8	.77[b]	.80[b]	4.6
	Avg.	.34	.38		.59	.69		.77	.79	
D		.36	.46[b]	4.9	.59	.73[b]	4.6	.72[b]	.85[b]	4.6
		.37	.45[b]	4.9	.61	.72[b]	4.6	.73[b]	.84[b]	4.6
	Avg.	.36	.45		.60	.72		.72	.84	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 32. Effect of a non-heat treated eight day cell-free milk proteolysate of Pseudomonas fluorescens on titratable acidity and pH of lactic starter cultures when grown in pasteurized skim milk.

Culture		Incubation Time								
		10 hrs.			12 hrs.			14 hrs.		
		Control	Filtrate	Filtrate	Control	Filtrate	Filtrate	Control	Filtrate	Filtrate
		Titratable acidity		pH	Titratable acidity		pH	Titratable acidity		pH
		---Percent[a]---			---Percent[a]---			---Percent[a]---		
A		.28	.31	5.5	.54	.56	4.9	.70[b]	.77[b]	4.5
		.28	.30	5.5	.54	.57	4.9	.69[b]	.78[b]	4.5
	Avg.	.28	.30		.54	.56		.69	.77	
B		.33	.36	5.7	.57	.63[b]	4.8	.78[b]	.82[b]	4.6
		.32	.35	5.8	.56	.65[b]	4.9	.77[b]	.84[b]	4.6
	Avg.	.32	.35		.56	.64		.77	.83	
C		.33	.36	5.4	.63[b]	.67[b]	4.8	.75[b]	.79[b]	4.6
		.32	.35	5.3	.62[b]	.66[b]	4.8	.74[b]	.80[b]	4.6
	Avg.	.32	.35		.62	.66		.74	.79	
D		.37	.40	5.2	.62[b]	.70[b]	4.8	.70[b]	.80[b]	4.6
		.36[b]	.40	5.2	.63[b]	.71[b]	4.8	.71[b]	.82[b]	4.6
	Avg.	.36	.40		.62	.71		.70	.81	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 33. Effect of an eight day cell-free filtrate of Pseudomonas fluorescens heat-treated for one hour at 70 C on titratable acidity and pH of lactic starter cultures when grown in pasteurized skim milk.

Culture		Incubation Time								
		10 hrs.			12 hrs.			14 hrs.		
		Control	Filtrate	Filtrate	Control	Filtrate	Filtrate	Control	Filtrate	Filtrate
		Titratable acidity		pH	Titratable acidity		pH	Titratable acidity		pH
		-----Percent[a]-----			-----Percent[a]-----			-----Percent[a]-----		
A		.31	.31	5.6	.46	.54	4.7	.54	.55	4.9
		.30	.32	5.5	.49	.54	4.7	.53	.53	4.9
	Avg.	.31	.31		.47	.54		.53	.54	
B		.32	.32	5.5	.53	.56[b]	4.7	.59	.64[b]	5.0
		.34	.35	5.5	.55	.58[b]	4.7	.60	.65[b]	5.0
	Avg.	.33	.33		.54	.57		.59	.64	
C		.34	.35	5.8	.52	.53	4.9	.54	.58	4.9
		.34	.34	5.8	.51	.54	4.9	.53	.59	4.9
	Avg.	.34	.34		.51	.53		.53	.58	
D		.34	.41	5.5	.53	.65[b]	4.8	.60	.67[b]	4.8
		.35	.40	5.5	.54	.67[b]	4.8	.59	.68[b]	4.8
	Avg.	.34	.40		.53	.66		.59	.67	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

Table 34. Effect of an eight day cell-free filtrate of Bacillus cereus heat-treated for one hour at 70 C on titratable acidity and pH of lactic starter cultures when grown in pasteurized skim milk.

Culture		Incubation Time: 10 hrs. Control Titratable acidity (Percent[a])	10 hrs. Filtrate Titratable acidity (Percent[a])	10 hrs. Filtrate pH	12 hrs. Control Titratable acidity (Percent[a])	12 hrs. Filtrate Titratable acidity (Percent[a])	12 hrs. Filtrate pH	14 hrs. Control Titratable acidity (Percent[a])	14 hrs. Filtrate Titratable acidity (Percent[a])	14 hrs. Filtrate pH
A		.30	.32	5.7	.58	.63[b]	4.6	.51	.55	4.9
		.30	.32	5.7	.56	.62[b]	4.6	.53	.53	4.9
	Avg.	.30	.32		.57	.62		.52	.54	
B		.32	.32	5.9	.60	.63	4.6	.56	.64	5.0
		.33	.34	6.0	.59	.64	4.6	.58	.65	5.0
	Avg.	.32	.33		.59	.63		.57	.64	
C		.31	.32	5.6	.53	.56	4.8	.54	.58	4.9
		.32	.32	5.7	.54	.58	4.8	.56	.59	4.9
	Avg.	.31	.32		.53	.57		.55	.58	
D		.35	.41	5.2	.55[b]	.63	4.5	.59[b]	.67[b]	4.8
		.34	.40	5.2	.56[b]	.64	4.5	.59[b]	.68[b]	4.8
	Avg.	.34	.40		.55	.63		.59	.67	

[a]Results are the average of duplicate samples.

[b]Indicates coagulation.

STIMULATION OF LACTIC STARTER CULTURES BY SUBSTANCES IN MILK CULTURES OF *PSEUDOMONAS FLUORESCENS* AND *BACILLUS CEREUS*

by

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An investigation was conducted to evaluate further previous work done in this laboratory on lactic culture stimulation by cell free milk proteolysates of Pseudomonas fluorescens and Bacillus cereus.

Growth media used for lactic starter propagation were sterile, ten percent reconstituted nonfat dry milk (NDM), and pasteurized skimmilk. These media, when inoculated with lactic cultures and the added stimulatory proteolytic materials, were titrated with 0.1 N NaOH to determine whether or not there was an increase in lactic acid development after 10, 12 and 14 hours at 21 C.

The ability of the two proteolytic organisms to develop stimulatory potency was determined at various intervals during fourteen day growth periods in ten percent reconstituted NDM at 25 C. The B. cereus culture proteolyzed the milk substrate faster than the Ps. fluorescens culture. Cell-free milk proteolysates of both cultures prepared after three or more days fermentation were stimulatory to lactic culture. However, it was difficult to prepare cell-free proteolysate from cultures less than eight days old; therefore, eight-day old cultures were used as the source of material from which cell-free proteolysates were prepared for assay and subsequent fractionations.

Heat treatment of cell-free milk proteolysates from either Ps. fluorescens or B. cereus inhibited their ability to stimulate lactic cultures. This indicated that the stimulatory agents were heat labile.

Eight-day old proteolysates were fractionated by ethanol precipitation, dialysis, and separation on Sephadex G-100 columns in an attempt to increase the specific stimulatory activities (mg of lactic acid produced per mg of nitrogen in cell free stimulatory material).

The addition to lactic cultures of the ethanol precipitate of the cell-free proteolysates from Ps. fluorescens resulted in greater stimulation after 12 hours at 21 C than when unfractionated cell-free proteolysate was used as the stimulatory agent. The corresponding B. cereus ethanol precipitate fraction was stimulatory to lactic cultures only when they were grown in sterile reconstituted NDM. Further fractionation of ethanol precipitates from both Ps. fluorescens and B. cereus on Sephadex G-100 columns and the addition of these fractions to lactic cultures resulted in further increase in stimulation after 12 hours incubation. The greatest stimulatory response was noted when the lactic culture medium was pasteurized skimmilk and the stimulatory agent was obtained from a Sephadex fractionation of B. cereus produced proteolysate.