Use of an Enzyme-Treated, Whey-Based Medium to Reduce Culture Agglutination[1]

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ABSTRACT

Rennet whey was hydrolyzed with papain during diafiltration at 40°C over a 2-h period. Permeate collected from a membrane with a molecular weight cut-off of 10,000 was freeze-dried and added to internal pH-controlled buffer salts as a replacement of the whey fraction. A control medium was prepared using dried whey. Both media were reconstituted (7.5% solids) and heat treated (85°C for 45 min). Commercial lactic cultures OS, M30, and M37 were grown in both media to prepare bulk starter. Pasteurized skim milk (1000 ml) in graduated cylinders was inoculated (5%) with bulk starter. Top and bottom pH of the skim milk was determined at 1-h intervals for 5 h. Culture agglutination was inhibited by 55 and 72% for cultures M30 and M37, respectively, when cultures were grown in the hydrolyzed whey media. Microscopic examination showed that cultures grown in hydrolyzed whey media formed considerably shorter chains and had little to no clumping.

(**Key words**: agglutination, cultures, media, enzyme)

INTRODUCTION

Agglutination is increasingly being recognized for its impact on starter culture performance. Agglutination was once considered to be a problem only in cottage cheese manufacture. However, more recently, agglutination has been suggested to be a problem also in other cultured dairy products that utilize lactococci as starter cultures (18). Therefore, agglutination may be a significant source of revenue loss to the dairy industry.

One mechanism for lactococcal agglutination results from the interaction of immunoglobulins (agglutinins) in milk with starter bacteria (5, 6). Agglutinating bacteria form long chains, or clumps, which eventually settle to the bottom of the cheese vat; acid-coagulated casein becomes entrapped between the clumps or chains (4, 9, 15). Agglutination results in uneven distribution of starter culture throughout the milk and uneven acid production in the cheese vat (3, 4, 6). Grainy, shattered curd, sediment formation, and slow acid production are problems in cottage cheese manufacture that directly relate to agglutination of lactic acid starter strains (3, 4, 6). Direct consequences of agglutination are inconsistent product quality and yield losses (7, 9), both of which decreased profits to the cottage cheese manufacturer. Minor sludge associated with agglutination in cottage cheese may routinely be responsible for yield losses of 4 to 8% (7).

The severity of agglutination depends on the cheese milk and the strain of the lactic starter culture used (6, 12). The severity of agglutination of a particular strain depends on two factors: the frequency with which a specific antigenic determinant is expressed on the cell surface and the agglutinin titer, which may indicate an antibody's specificity to certain cell surface antigenic determinants (18). Starter cultures that are proteinase-negative experience higher frequency of agglutination

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problems in the cheese vat than their protease-positive counterparts (15). Cheese milk that is high in colostral or mastitic secretions favors agglutination, but homogenized skim milk or milk pasteurized at higher than normal temperatures reduces agglutination (5, 6). Agglutination profiles also vary within the lactation cycle, and titers differ among cows at the same stage of lactation (6, 18).

Many of the methods suggested to prevent agglutination (such as pasteurizing milk at higher than normal temperatures, screening cultures, or homogenizing milk prior to cheese manufacture) in cottage cheese manufacture have not been entirely successful or commercially practical. Screening cultures to identify agglutination-sensitive strains may not be commercially feasible because of preparation time, subjectivity in interpreting test results, and lack of reliability in application to manufacturing conditions. Most commercial cultures consist of mixed or single strains. Replacement of agglutination-sensitive strains with agglutination-resistant strains would be hindered if the cultures were phage sensitive. Some agglutination-resistant strains possess agglutination-sensitive cells, which may become dominant after extended subculturing in autoclaved milk (1). Pasteurization of milk at higher than normal temperatures makes it unsuitable for cheese manufacture. Commercially feasible methods to prevent agglutination are still needed.

Research in our laboratory has shown that all commonly used commercial cultures may agglutinate when grown in commercial whey-based media (2, 14). Therefore, the overall objective of this research was to develop a bulk starter medium that would prevent or reduce agglutination of starter culture in the bulk medium to enhance cheese quality, cheese yield, and acid development during cheese manufacture.

MATERIALS AND METHODS

Cultures

Frozen commercial lactic bulk starter cultures OS, M30, and M37 (Rhône-Poulenc, Marschall Division, Madison, WI) were inoculated (.1 g) into 10-ml aliquots of reconstituted and sterilized NDM (10%, wt/vol). The tubes were incubated at 26°C for approximately 3 h or until a curd was formed. In the second propagation, the formed curd (or contents of the tube) was transferred into 100 ml of sterilized NDM (10%, wt/vol) and incubated for 18 h. Ten milliliters of the second propagation were transferred into enzyme-treated and control media, prepared as described, and incubated.

Commercial cultures OS, M30, and M37 were selected because of their varying sensitivities to agglutination. Hicks and Ibrahim (10) screened these cultures previously according to their agglutination characteristics. The OS culture is a cottage cheese culture that does not normally agglutinate. This culture was used as a control in the experimental design. Cultures M30 and M37 are Cheddar cheese cultures that are extremely sensitive to agglutination and represent a worst case scenerio if used in the manufacture of cottage cheese.

Media Preparation

Seven liters of rennet whey were treated with 1 g of crude type papain (Sigma Chemical Co., St. Louis, MO) while being ultrafiltered and diafiltered using a hollow fiber membrane (Supelco, Bellefonte, PA) with a molecular weight cutoff of 10,000. The process ran for 2 h at 40°C. The permeate was collected and freeze-dried. The freeze-dried permeate was added (41.7%, dry weight basis) to an internal pH control buffer salt mixture (19) (Galloway West, Fond du Lac, WI) to replace the whey. A control medium was prepared using untreated whey. Both media were reconstituted (75.7 g/L), split into three fractions, and pasteurized at 85°C for 45 min. Media were cooled to 26°C, inoculated with the test cultures, and incubated at 26°C for 16 h or until pH 5.3 was achieved.

Monitoring of Agglutination

Culture agglutination was monitored by determination of the pH differential in skim milk and by direct microscopic examination. Agglutination in skim milk was monitored by inoculating the fermented media at 5% (vol/vol) into 1000 ml of pasteurized (63°C for 30 min) skim milk contained in 1000-ml graduated cylinders and incubated at 32°C. The pH

differential was determined by measuring top and bottom pH of skim milk in the graduated cylinders at 1-h intervals over 5 h. Recordings were made 5 cm below the skim milk surface and at the bottom of the cylinder. A pH meter (American Scientific Products, McGaw Park, IL) was equipped with combination pH electrode (Orion Research, Inc., Boston, MA). The electrode was attached to a stainless steel rod, which was used to lower the electrode to the bottom of the cylinder. The pH differential was computed by subtracting the bottom pH from the top pH.

At the end of 5 h of incubation, the bottom of the graduated cylinders was visually inspected for sediment formation. Difference in total solids between the top and bottom of the cottage cheese vats was reported to be a sensitive indicator of agglutination (11). Milk (or curd) samples were taken from the bottom of the cylinders and stored at 4°C overnight for direct microscopic examination the following day. The next day, samples were Gram stained (21) to determine culture growth characteristics and cells per chain distribution by microscopic examination. Photomicrographs of representative fields were prepared to illustrate the differences in culture growth characteristics, the extent of chain formation, and clumping among the cultures grown in the two media.

Statistical Analysis

The experiment was replicated four times in a randomized complete block design. Data were analyzed using the GLM procedure of SAS (17) to determine differences between media with respect to agglutination characteristics of various lactic cultures. Least squares means and significance of each treatment were computed using Type IV sums of squares and the predicted difference procedure. Least significant differences were computed for the pH differential between top and bottom pH. A pH differential of .12 units or greater was significant ($P < .05$) and was an indicator of starter culture agglutination.

RESULTS AND DISCUSSION

Culture Performance

Slow or uneven acid production has been reported as an indicator of culture agglutination in previous studies (6, 11, 13, 15). In this study, acid production was also used as an indicator of agglutination and, thus, as an indicator of culture performance. Culture performance over the 5-h incubation in skim milk improved ($P < .01$) for two commercial cultures studied (M30 and M37) when grown in enzymically treated whey medium compared with performance of the control (untreated whey) medium (Table 1).

The OS culture, which is a mixed-strain culture that infrequently agglutinates in agglutinin-rich milk or under normal conditions, showed the least ($P < .01$) amount of culture agglutination (compared with that for M30 and M37 cultures) when grown in either media. These results were expected and were consistent with our previous experiments using this culture in our laboratory. The OS culture (grown in either media) during the 5-h incubation had faster acid development (decrease in pH) ($P < .01$) (Figure 1a and Table 1) and a smaller pH differential ($P < .01$) (Table 1) than did M30 and M37 cultures (Figure 1, b and c, and Table 1). Skim milk inoculated with OS culture grown in control or enzymically treated media and incubated had a similar rate of acid production (rate of pH decrease) at the top and bottom of the graduated cylinders. The pH decreased steadily up to 4 h of incubation and leveled off at pH 4.5 when skim milk coagulated (Figure 1a). However, over the 5-h incubation, the pH differentials were smaller in skim milk inoculated with culture grown in the enzymically treated whey media. The pH

TABLE 1. Effect of enzymically treated whey internal pH control media on rate of acid development in skim milk.

		Cultures		
pH	Media[1]	OS	M30	M37
Top	C	5.25[2]	6.23	6.22
	ET	5.26	5.40	5.81
Bottom	C	5.10	5.55	5.57
	ET	5.17	5.08	5.63
Differential[3]	C	.15	.68	.64
	ET	.09	.31	.18

[1]C = Control; EC = enzymically treated.

[2]Least squares means of observations at 1, 2, 3, 4, and 5 h. Four replicates for all treatments; n = 20. Least significant difference ($P = .05$) of the least squares means = .12.

[3]Top pH minus bottom pH.

differentials were .09 and .15 for enzymically treated and control media, respectively (Table 1), suggesting that the performance of the OS culture was improved by 40%. When the skim milk cylinders were visually inspected at the end of 5 h, no visual sediment was observed in either cylinder.

When commercial M30 culture was grown in enzymically treated whey medium and inoculated into skim milk contained in graduated cylinders, rate of acid production at the top of the cylinder improved ($P < .01$) over the 5-h incubation period compared with that of the same culture grown in the control medium (Table 1; Figure 1b). In the cylinders inoculated with the M30 culture grown in control media, top pH decreased by only .8 pH units over 5 h. This culture agglutinated within the 1st h of incubation, which produced a rapid decline in the bottom pH of the graduated cylinders (Figure 1b). Russell-Campbell and Hicks (16) suggested that the time to the first sign of visible agglutination was dependent on the formation of a critical mass of bacteria clumps, chains, and entrapped casein. Milton et al. (15) reported that agglutinated lactic cultures could be observed as early as 15 min in the cheese vats. Our results are consistent with these reports (15, 16). When M30 culture was grown in the enzymically treated media and inoculated into skim milk, the pH differential over the 5-h incubation period was reduced from .68 (cultures grown in the control medium) to .31 (that grown in enzymically treated medium) (Table 1 and Figure 1b). The pH differential was decreased ($P < .01$) by 55%, suggesting that culture agglutination was being inhibited and that culture performance was improved.

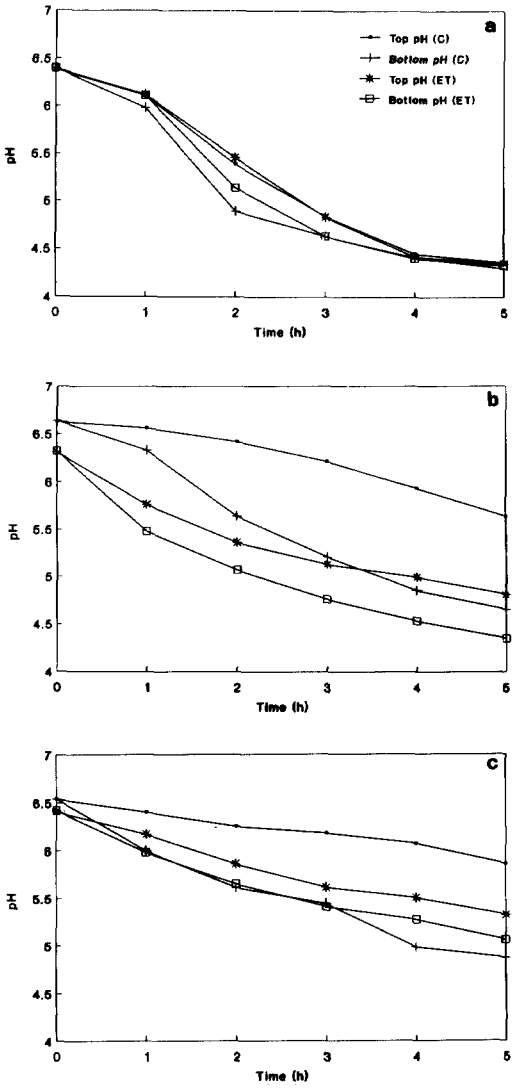

Figure 1. Rate of top and bottom pH decrease of commercial cultures OS (a), M30 (b), and M37 (c) inoculated into skim milk contained in 1000-ml cylinders. C = Control media; ET = enzymically treated media.

Among the three commercial cultures studied, M37 showed the greatest improvement in culture performance when grown in the enzymically treated whey media compared with that of the control (Table 1 and Figure 1c). When M37 was grown in the control medium and inoculated into skim milk, rate of acid production at the top of the cylinder was slow. The pH decreased by only .6 units over 5 h, and the strain agglutinated within the 1st h of incubation. The pH decrease at the bottom of the cylinders was fairly rapid during the 5-h incubation (Figure 1c). When the M37 culture was grown in the enzymically treated media and inoculated into skim milk, a steady decrease in pH occurred at the top and at the bottom of the cylinders (Figure 1c). The pH differentials (over 5-h incubation) decreased from .64 for cultures grown in the control medium to .18 for that grown in enzymically treated medium (Table 1). The pH differential was reduced ($P < .01$) by 72%, suggesting inhibition of culture agglutination and improvement of culture performance.

The cylinders were visually inspected at the end of 5 h for sediment formation. Cylinders inoculated with M30 and M37 cultures grown in the control medium both had heavy sedimentation (5 and 6 cm deep, respectively) at the bottom of the cylinders. This sediment was greatly reduced when M30 and M37 cultures were grown in the enzymically treated medium. Milton et al. (15) reported that, when a lactic bulk starter culture that is extremely agglutinated is added to a vat of milk, the cell complex settles to the bottom of the vat so rapidly that little additional casein from skim milk precipitates around the cells. In that situation, the sludge layer is light brown and similar to the color of the bulk starter. The sediment also had a slight brownish tint in this experiment for the cultures grown in the control medium. Statistical analyses showed the media by culture interaction to be significant ($P < .01$) for rate of decrease of top and bottom pH and for pH differentials. The extent to which the culture performance was improved when grown in the enzymically treated whey medium appeared to depend on the specific culture and perhaps was related to the severity of agglutination associated with a particular strain. The severity of agglutination of a particular strain depends on several factors, such as the frequency that a specific antigenic determinant is expressed on the cell surface, or the agglutinin titer, which may indicate an antibody's specificity to certain cell surface antigenic determinant (18). Perhaps these factors contributed in this study to the differences in the extent of improvement in performance of the various commercial cultures investigated.

Culture Morphology

When lactic cultures agglutinate, long chains or clumps of chains are formed in the sediment, but nonagglutinating cultures formed smaller clumps, or no clumps and shorter chains, and appeared to be more evenly dispersed throughout the skim milk (6, 9, 10, 15). Photomicrographs of the three cultures grown in the control medium and enzymically treated whey medium showed that all three cultures (OS, M30, and M37) formed considerably shorter chains and almost no clumping of chains when grown in enzymically treated whey medium (Figure 2) compared with those of controls. For all photomicrographs, samples were taken after 5-h incubation from the bottom of the cylinders.

Figure 2a shows the OS culture grown in the control medium and inoculated into skim milk. Although this culture is a nonagglutinating culture and technically did not agglutinate, some grouping of cells was observed with the microscope when this culture was grown in the control medium. However, no visible coagulation occurred around the cells, nor was casein entrapped in the cell complex. When the OS culture was grown in the enzymically treated whey media, no visible grouping of cells occurred. The cells were mostly short chains (four to six cells per chain) and seemed more evenly dispersed (Figure 2b).

Figure 2 (c and d) shows the M30 culture grown in the control and enzymically treated whey media. The M30 culture grown in the control media at the end of 5 h was extremely agglutinated, as seen in Figure 2c. Large clumps of cells were observed. These cell complexes were still capable of producing acid, which coagulated the casein around them, as was seen by the heavy dark masses around the cells. Some of the casein was entrapped within the cell complexes. When M30 was grown in the enzymically treated whey media, no long-chain formation or cell clumping occurred. The cell density at the bottom of the cylinders was greatly reduced. The cells were mostly in very short chains, diplococci or triplets. No large clumps, casein precipitation, or casein entrapment was observed (Figure 2d).

Observations of the photomicrographs of M37 culture grown in either media were similar to those with M30 (Figure 2, e and f). When M37 was grown in the control medium, the culture severely agglutinated (Figure 2e). Long chains were present in the sediment, and several of the chains were clumped together. Protein sediments were apparent around the clumps of cells (Figure 2e). When M37 culture was grown in the enzymically treated whey medium, the cell density at the bottom of the cylinders was greatly reduced. Fewer and shorter chains occurred. Only a few chains folded upon themselves, and no protein deposits were found among these cells (Figure 2f). The shorter chains appeared to be more evenly dispersed in the sample.

Short chains, lacking the critical mass needed to sink to the bottom of the cylinders,

were more evenly dispersed throughout the skim milk, providing for improved culture performance throughout the cylinder during incubation and, thus, improved acid production, particularly at the top of the cylinders. These photomicrographs support the data on the pH differential and visible sedimentation that were discussed previously.

Agglutination resulted from the interaction of the bulk starter culture cells with agglutinins (IgM and IgG) in media or milk to form long chains or clumps of chains. Previous research in our laboratory showed that other proteolytic enzymes, such as trypsin, chymotrypsin, and pepsin, liberated peptides that do not inhibit agglutination, probably because of their specific site of hydrolysis. In some cases, agglutination actually increased. Papain has been shown to hydrolyze agglutinins just below the branching point (8); the initial peptides [Fab

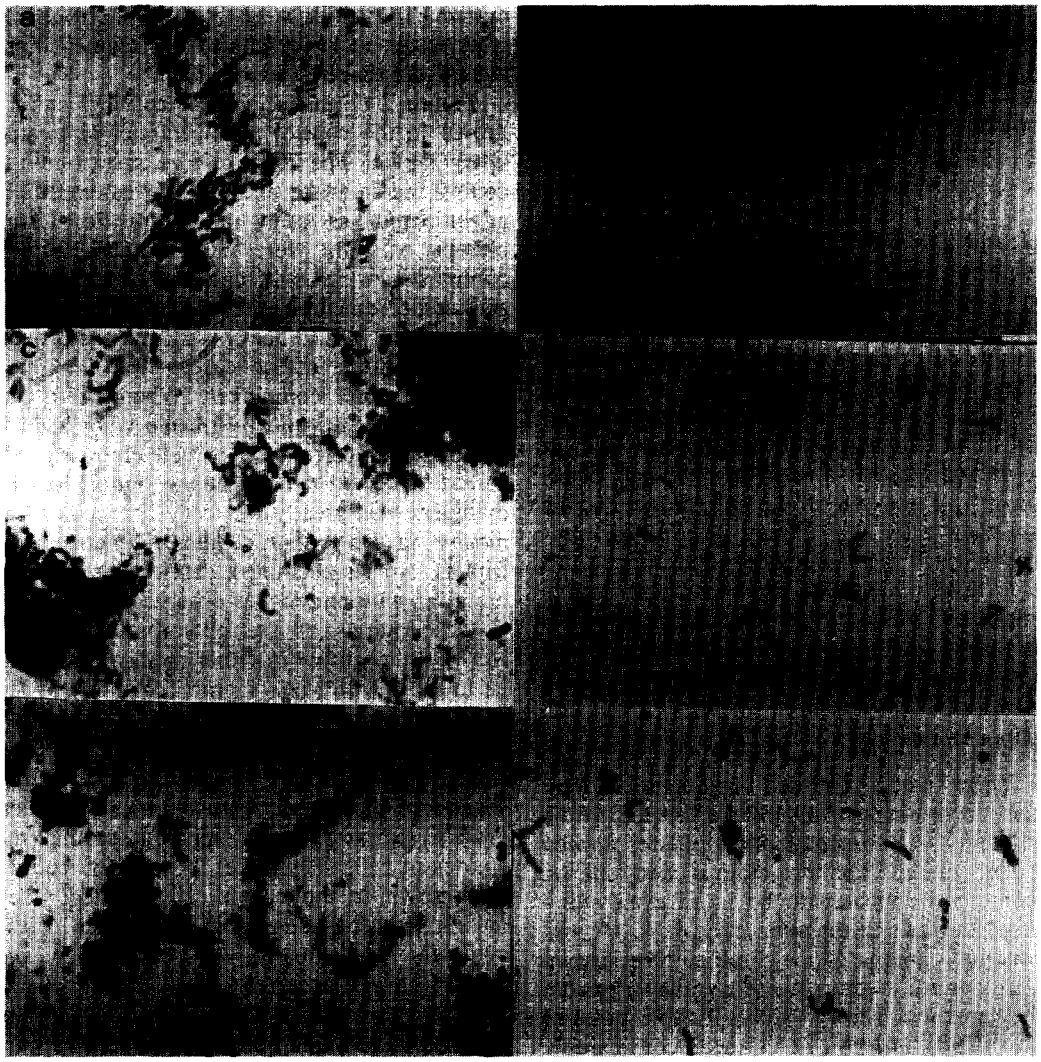

Figure 2. Growth characteristics and cell distribution of commercial cultures OS (a and b), M30 (c and d), and M37 (e and f) cultures grown in enzymically treated whey and control media. ×1000. a, c, and e = Control; b, d, and f = enzymically treated.

has a molecular weight of approximately 45,000 and Fc a molecular weight of 50,000 (8)] did not reduce culture agglutination. In this study, the fraction collected through an UF membrane that had a molecular weight cutoff of 10,000 did inhibit culture agglutination, thus suggesting that papain might further hydrolyze the branched chains. These hydrolyzed fragments might inhibit agglutination by binding to the antigenic sites on the cell surface in the same manner as they did when they were part of the intact immune protein. However, because the bridging part of the immune protein has been removed, this cell can no longer interact with other cells to form chains or clumps. The fragment instead acts as blocker to prevent intact immune proteins from binding to the antigenic site. Thus, starter culture agglutination was inhibited in the enzymically treated whey medium as well as in the skim milk. If agglutinins were merely removed by enzyme hydrolysis and ultrafiltration, agglutination in the growth medium would be prevented, but cultures prone to agglutination would agglutinate once transferred into skim milk. However, this agglutination did not occur in our study. Our results showed that agglutination was significantly reduced in skim milk inoculated with cultures grown in enzymically treated whey media, suggesting that the antigenic sites on the cell surface were blocked; thus, further interactions with milk agglutinins were prevented. Also, enzyme hydrolyzed whey proteins probably are more readily utilized as a nutrient by the lactic cultures. Experiments to test these hypotheses are currently in progress.

Hicks and Ibrahim (10) recommended homogenization of the bulk culture as a method to prevent agglutination. However, homogenization requires additional labor and contributes to the cost of culture preparation. Thunell et al. (20) suggested that selection of starter bacteria from nonagglutinating strains would be the most effective method to reduce agglutination. Unfortunately, culture supply companies have difficulty finding compatible agglutination-resistant lactic strains to produce sufficient mixed- and single-strain cultures for rotations to inhibit phage problems (10). Many mixed-strain cultures recommended for cottage cheese manufacture consistently agglutinate. Even the most resistant cultures may agglutinate in some milk during certain seasons.

A medium that inhibits starter culture agglutination is an economical and labor-saving alternative to the homogenization of the bulk starter culture to reduce agglutination. Such media would eliminate some of the problems associated with culture selection and increase the number of cultures (such as higher cheese yielding protease-negative cultures) that could be used in the manufacture of cottage cheese. These results also suggest that this technology could reduce the variation in culture performance between vats, days, and weeks and allow cheesemakers to adhere to their cheesemaking schedule. Improvements in culture performance of this type also may be beneficial for cultures used for hard cheese manufacture.

CONCLUSIONS

Our studies showed that culture agglutination (as evidenced by culture performance and culture morphology) was inhibited by 55 and 72% for commercial cultures M30 and M37, respectively, and that culture performance (as demonstrated by a more uniform pH decrease throughout the skim milk) was improved when cultures were grown in enzymically hydrolyzed whey media. The direct microscopic examination of these cultures showed that cultures grown in hydrolyzed whey media formed considerably shorter chains with little to no clumping. Media that prevent agglutination of starter culture and reduce the daily variation in culture performance would greatly benefit the dairy industry.

REFERENCES

1 Auclair, J. E., and Y. Vassal. 1963. Occurrence of variants sensitive to agglutinins and to lactoperoxidase in a lactenin-resistant strain of *Streptococcus lactis*. J. Dairy Res. 30:345.

2 Ekart, L., J. O'Leary, and C. L. Hicks. 1986. Use of proteinase negative starter cultures to increase cottage cheese yield. J. Dairy Sci. 69(Suppl. 1):68.(Abstr.)

3 Elliott, J. A., and D. B. Emmons. 1979. Solving the agglutination problem. Page 577 *in* Proc. 1st Bienn. Marschall Int. Cheese Conf. Marschall Products, Rhône-Poulenc Inc., Madison, WI.

4 Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1963. Agglutination of starter bacteria, sludge formation and slow acid development in cottage cheese manufacture. J. Dairy Sci. 46:600.(Abstr.)

5 Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1965. Sensitive test for lactic streptococcal agglutinins. J. Dairy Sci. 48:1245.

6 Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1966. Effect of lactic-streptococcal agglutinins in milk on curd formation and manufacture of cottage cheese. J. Dairy Sci. 49:1357.
7 Gradison, A. S., B. E. Brooker, P. Young, and A. S. Wigmore. 1986. Yield loss of cottage cheese curd due to the formation of minor sludge: the beneficial effect of homogenization. J. Soc. Dairy Technol. 29:123.
8 Herman, N. E. 1974. Antibody structure: the immunoglobulins. Page 409 *in* Immunology. Harper and Row, Hagerstown, MD.
9 Hicks, C. L., and B. Hamzah. 1992. Effect of culture agglutination on cottage cheese yield. Cult. Dairy Prod. J. 27:4.
10 Hicks, C. L., and S. Ibrahim. 1992. Lactic bulk starter homogenization affects culture agglutination. J. Food Sci. 57:1086.
11 Hicks, C. L., K. Milton, S. Riddell-Lawrrence, D. Wang, and J. O'Leary. 1989. Simplified method to detect agglutination in cottage cheese vats. Cult. Dairy Prod. J. 24:5.
12 Jago, G. R., and M. F. Swinbourne. 1954. Factors influencing the lactic acid producing properties of streptococci used in the production of cheddar cheese. II. Observation relating susceptibility with insusceptibility. J. Dairy Res. 26:123.
13 Jose, C., C. L. Hicks, and J. O'Leary. 1989. Effect of lecithin and milk fat with and without homogenization on bulk starter performance and skim milk agglutination. J. Dairy Sci. 72(Suppl. 1):146.(Abstr.)
14 Marsh, D. J. 1986. Effect of commercial culture systems on cottage cheese yield. M.S. Thesis, Univ. Kentucky, Lexington.
15 Milton, K. J., C. L. Hicks, J. O'Leary, and B. E. Langlois. 1990. Effect of lecithin addition and homogenization on bulk starter agglutination. J. Dairy Sci. 73:2259.
16 Russell-Campbell, E., and C. L. Hicks. 1992. Culture agglutination as affected by homogenization of skim milk. J. Dairy Sci. 75:3282.
17 SAS/STAT® User's Guide, Release 6.03 Edition. 1988. SAS Inst., Inc., Cary NC.
18 Scheuble, T. L., J. K. Kondo, and M. A. Salih. 1989. Agglutination behavior of lactic streptococci. J. Dairy Sci. 72:1103.
19 Sinkoff, B. A., and R. H. Bundus, inventors. 1983. Bulk starter media. Stauffer Chem. Co., assignee. US Pat. No. 4,403,986. Sept. 6, 1983.
20 Thunell, R. K., W. E. Sandine, and F. W. Bodyfelt. 1984. Defined strains and phage-insensitive mutants for commercial manufacture of cottage cheese and cultured buttermilk. J. Dairy Sci. 67:1175.
21 Wu, W. G., ed. 1986. Stains and mounting media. Page 33 *in* Medical Microbiology: A Laboratory Study. Star Publ. Co., Belmont, CA.