AN ABSTRACT OF THE THESIS OF

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Title: DEVELOPMENT OF INTERNALLY pH-CONTROLLED BULK STARTER MEDIA FOR THE PROPAGATION OF LACTIC ACID BACTERIA

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William E. Sandine

Various pH-controlled bulk starter media were formulated for the propagation of mesophilic and thermophilic lactic acid bacteria. PH control was achieved externally with a single-shot addition of sodium carbonate, internally using controlled release alkali in the form of encapsulated sodium carbonate and internally with pH-dependent, insoluble buffers. The concept of internal pH control was also applied to laboratory plating media used for enumeration and differentiation of lactic streptococci.

Single-shot external neutralization effectively doubled the lactic cell population. Internal neutralization with controlled release alkali produced improved holdover cultures but had several commercial drawbacks. Internal neutralization with the insoluble buffers trimagnesium phosphate and calcium carbonate proved to be most suitable from a commercial standpoint. The end result of this technology is commercially manufactured and sold under the

name PHASE 4. To this date the bulk starter medium PHASE 4 has been used in the manufacture of over 650 million pounds of American and cottage cheese.

Several PHASE 4 formulations were developed using the insoluble buffer trimagnesium phosphate or ingredients necessary to generate this compound *in situ* in the bulk starter tank. A chemical interaction between trimagnesium phosphate and diammonium phosphate enabled PHASE 4 to have a high buffering capacity with a physiologically desirable starting pH. The medium was made phage-inhibitory with the addition of citrate. Even the low calcium-requiring phages found to proliferate in many commercially available bulk starter media were inhibited in PHASE 4.

High quality yeast extract was shown to be necessary for successful performance of PHASE 4 with frozen culture concentrates. However, the rich amino-acid material content of yeast extract enabled 'slow' acid-producing lactic streptococci to compete with 'fast' cells when grown together in PHASE 4.

PHASE 4 not only supported growth of *Streptococcus lactis* and *S. cremoris*, but it promoted growth of leuconostoc bacteria and *S. diacetilactis*. An actively growing starter culture suppressed growth of *Staphylococcus aureus* in PHASE 4 but could not completely suppress growth of gram negative contaminants. Other pH-controlled media were also found vulnerable to competition by gram negative contaminants.

Several commercial blending problems were encountered in transferring the PHASE 4 product from the laboratory to commercial manufacture. Formulation changes, modified agitation patterns and reduced solids usage were shown to aid in correcting many of these problems. Overall, strict specifications for all ingredients and precision blending were found to be essential factors in the uniform manufacture of PHASE 4.

Bulk starter media formulations were developed to support the balanced growth of the rod-coccus starter cultures used in the manufacture of Italian cheeses. These formulations included one most suited for propagating phosphate tolerant commercial cultures and another for phosphate sensitive cultures. An additional formulation was proposed for growing the S. thermophilus organism alone, commonly used in Swiss cheese manufacture. Greatest difficulty was encountered in formulating a medium that was phage-inhibitory without markedly suppressing growth of the Lactobacillus bulgaricus rods. All attempts to produce an acceptable phage-inhibitory medium for rod-coccus cultures were unsuccessful.

The insoluble buffer trimagnesium phosphate was used in two laboratory plating media for enumeration of lactic streptococci and for differentiation of 'fast' and 'slow' acid-producing lactic streptococci. Improved visual differentiation was possible in the trimagnesium phosphate-buffered FSDA-11 with less chance of inhibition to injured or phosphate sensitive cells.

DEVELOPMENT OF INTERNALLY pH-CONTROLLED BULK STARTER MEDIA FOR THE PROPAGATION OF LACTIC ACID BACTERIA

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DEDICATION

To my wife Cindy whose patience,
understanding and long hours of
typing made this all possible.

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CHAPTER I

INTRODUCTION

Starter culture for the industrial fermentation of milk is traditionally prepared as sequential transfers of lactic acid bacteria in increasing volumes of milk or specialized growth media (Figure 1). The mother culture (10-100 ml) is propagated in sterile milk and represents the initial seed of the starter culture. An intermediate culture (1 quart to 5 gallons) is also commonly grown in milk and provides sufficient inoculum for the bulk culture (100-1000 gallons). Bulk cultures can likewise be propagated in milk, but in the United States it has become more common to grow bulk culture in specialized starter media designed to stimulate growth of lactic acid bacteria and produce a highly active starter culture (71).

Recent technology has made optional some or many of the steps in starter culture preparation by providing culture concentrates to directly inoculate the bulk starter medium or final fermentation vat (59). More recently, the principle of pH control has been applied to the preparation of bulk starter cultures in the cultured dairy plant (66).

This research was undertaken to develop pH-controlled bulk starter media for the propagation of both mesophilic and thermophilic lactic acid bacteria. The following three approaches were taken:

1) simplified external pH control involving neutralization near the end of the fermentation by the addition of a single dose of alkali,

2) internal pH control with controlled release alkali; encapsulated pellets that gradually disintegrated during the incubation period and

3) internal pH control with temporarily insoluble buffers; solubility increasing with decreasing pH.

In addition, experiments were done to apply the technology of insoluble buffers to laboratory plating media for enumeration and differentiation of lactic acid bacteria.

Figure 1. Application of PHASE 4 in starter culture technology. Frozen bulk sets generally prepared in 70-130 ml amounts (5-50 x 10^9 cfu/ml); enough to set 150-500 gallons bulk starter medium. Frozen direct vat set concentrates usually contain 130-500 ml (50-400 x 10^9 cfu/ml) which is enough to set 5000 lb cheese milk (59).

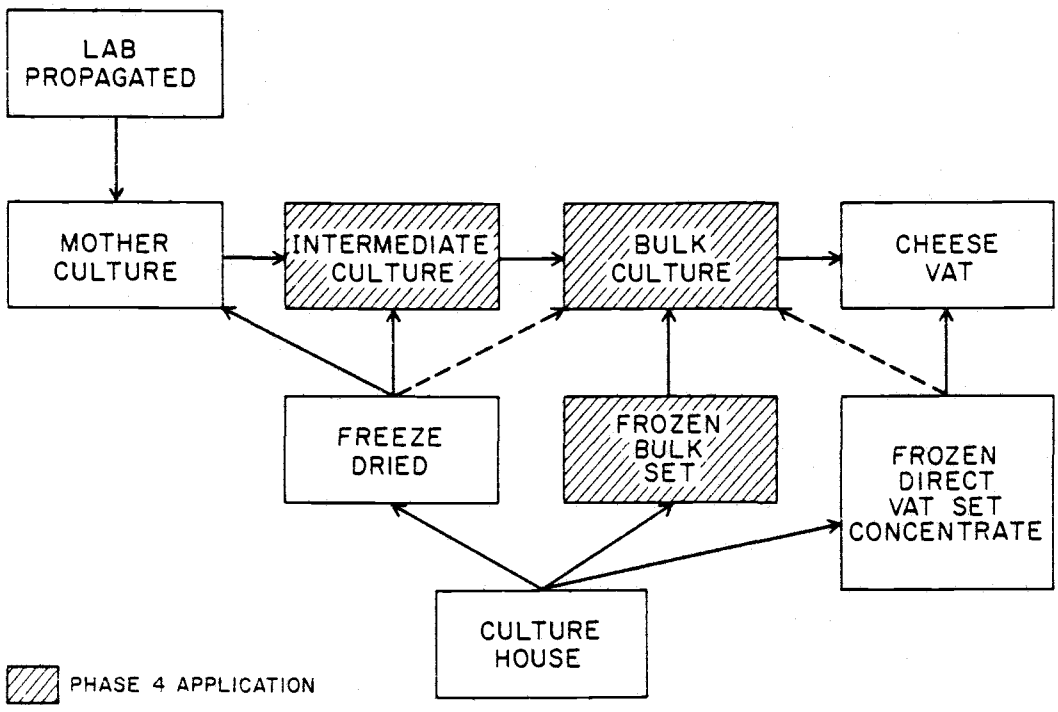

LAB PROPAGATED
MOTHER CULTURE
INTERMEDIATE CULTURE
BULK CULTURE
CHEESE VAT
FREEZE DRIED
FROZEN BULK SET
FROZEN DIRECT VAT SET CONCENTRATE
CULTURE HOUSE
PHASE 4 APPLICATION

Figure 1.

CHAPTER II

HISTORICAL REVIEW

Origin of Bulk Starter Cultures

Early attempts to control acid development and flavor in butter and cheese involved holding back clean flavored sour milk or whey and using it the following day to inoculate fresh cream or milk. The first starter system evolved around the beginning of the 20th century when Storch in Denmark, Conn in the United States and Weigmann in Germany observed bacteria to be responsible for the desired fermentation in buttermaking (72). Starter prepared daily was used to inoculate fresh cream to achieve a more reproducible product. These practices were soon adopted for cheesemaking as well.

Until the early 1950's, cheesemakers prepared starters in their own untested fluid skim milk or reconstituted skim solids. Milk with high bacteria counts or containing antibiotics from mastitis treatment often resulted in starters with poor activity in the cheese vat. Reproducible starter cultures were dependent on identifying a reliable producer of high quality raw milk that could be used for growing starter. Low heat nonfat milk powder, guaranteed free of antibiotics and pretested to support the growth of lactic streptococcal strains, soon became commercially available and was widely used as a bulk starter medium.

Lactic Streptococci and Bacteriophage

In 1935, Whitehead and Cox (90) first reported the presence of bacteriophages (phage) in lactic streptococcal bulk cultures. Phage inhibition of lactic starter cultures still remains the single most important problem in the dairy fermentation industry (71). Elaborate measures to exclude phage from bulk starter have been employed by the industry. Isolated rooms equipped with chlorine foot baths and specialized bulk starter tanks (69) which are maintained under positive pressure with filter-sterilized air are commonly used in the production of bulk starter. Strict sanitation programs and restricted traffic in these starter rooms help prevent phage contamination (72).

Bulk starter media are routinely pasteurized at 80-90 C since most lactic phages survive normal pasteurization procedures (94). Even though lactic phages are destroyed in less than 90 seconds at 88 C (72), extended holding times (40-60 min) at these temperatures are required to inactivate phage in the head space of the starter tank.

In New Zealand (27), Australia (32) and to a lesser extent in the United States (16,67) and other parts of the world, phage inhibition is dealt with at the level of the cultures themselves. Carefully selected starter strains that are resistant or insensitive to high frequency phage attack are used to prepare bulk cultures. In Holland, yet another approach is taken in which no precautions

are taken against phage infection (78). Instead, constant exposure to phage results in a relatively stable balance of susceptible and resistant bacteria.

In the United States the predominant method for phage control is rotation of several mixed strain cultures in phage inhibitory media (71). Development of these media resulted from the observation by Reiter (65) in 1956 that lactic phage replication was inhibited in milk in which calcium ions had been removed. Babel (3) reported lactic streptococcal cultures were slightly inhibited in milk with calcium removed. Hargrove et al. (25) demonstrated that a suitable growth medium which could be used to propagate phage-free starters was possible by adding orthophosphates to milk. Terminal heat treatment of phosphated milk was necessary to bind the free calcium. Most lactic phages that he tested were suppressed by a 2% phosphate concentration. Zottola and Marth (93) later formulated various combinations of orthophosphates that could be dry-blended with nonfat milk powder-electrodialyzed whey combinations and upon reconstitution, pasteurization and inoculation successfully inhibited most phage replication. Subsequently, several commercial phage inhibitory media (11) emerged that were phosphated with a nonfat dry milk or more recently, dried sweet whey base. These products are still widely used by the dairy industry for production of bulk starter cultures.

While the exact mechanism(s) by which Ca^{+2} affects phage growth in lactic streptococci remains unknown, it is well

established that Ca^{+2} is necessary for maximum phage proliferation (10,14,60,64,74). Potter and Nelson (61) showed Ca^{+2} to function during the early stages of the lactic phage growth cycle, and they concluded that Ca^{+2} appears to be required for invasion of the host cells. More recently, Watanabe and Takesue (87) have demonstrated with a 32-P-labelled phage that calcium ions were required for penetration of phage genomes into host cells of Lactobacillus casei. There have also been reports of lactic phage-host combinations not requiring Ca^{+2} for lysis (76) and other phage-host systems that apparently have very low Ca^{+2} requirements as evidenced by their proliferation in several commercial phage inhibitory media (24,31).

Early evaluation of the first commercial phage inhibitory medium (PIM) supported manufacturers' claims of phage inhibition but showed that not all strains of lactic streptococci grew equally well in the medium, and Leuconostoc grew very poorly (29). Gulstrom et al. (24) recently evaluated the growth-promoting and phage-inhibitory properties of several commercial bulk starter media and found only 2 out of 7 media to effectively suppress phage replication. The four most effective PIM of that study contained citrate buffers in addition to phosphates to bind the free calcium.

pH Control in Bulk Starters

Lactic streptococci are homofermentative and produce predominantly lactic acid during growth on lactose or glucose. Unless the growth medium is well buffered, the hydrogen ion

concentration becomes growth limiting near pH 5.0 (70) although lactic acid production continues (85). Growth in conventional phosphated PIM, even though more highly buffered than milk, is limited by low pH.

Cell numbers of lactic streptococci can be greatly increased by controlling the pH of the growth medium between 5.4 and 6.3 with pH 6.0 most often preferred (57,58,59). The fermentation pH is generally maintained by external addition of gaseous NH_3 or concentrated alkali. These practices are utilized by culture manufacturers in the production of frozen culture concentrates (59).

With hydrogen ion concentration no longer growth limiting with pH control, other factors are responsible for stopping growth of lactic streptococci. Accumulations of hydrogen peroxide (22,34), D-leucine (21) and lactates (7,58,59) have all been shown to act as auto-inhibitors. PH-control also protects the cells from acid injury that occurs below pH 5.0 (26,44).

Only recently has the concept of pH control been applied to production of bulk starter cultures in individual cheese plants. An economic medium utilizing liquid whey with added phosphates and growth stimulants in which pH is controlled by injection of anhydrous NH_3 was used to propagate lactic bulk starter cultures (9,66). These cultures were more active than when grown in commercial PIM and they made possible shorter make times or less

starter usage and more reliable acid production in American and cottage cheese manufacture.

Reduced phosphate is possible in the whey-based medium using external pH control developed by Ausavanodom et al., without compromising phage inhibition (2). This seems worthwhile since Ledford and Speck (39) reported metabolic injury and diminished proteinase activity for lactic streptococci cultured in commercial bulk starter media containing high concentrations of phosphate.

A modified approach to external pH control has been recently reported in New Zealand (41). Bulk starter is allowed to grow in the usual manner in milk to pH 5.0 or less. The pH is then raised to 7.0 by adding NaOH to the ripened culture and starter growth is allowed to continue for an additional 2 h, resulting in a doubling of the cell number. Greater activity per unit volume is achieved with these single-shot neutralized cultures.

Direct to Vat Inoculation

Concentrated cultures (DVS) of lactic streptococci are sometimes used to directly inoculate the cheese vat, obviating the need for bulk cultures. Porubcan and Sellars (59) outlined several advantages of DVS over bulk culture systems, including energy and labor savings, better strain balance and simple vat-to-vat rotations. These culture concentrates are used with success in the industry; however, bulk starter produced in individual plants

is still more economic and remains the predominant form of inoculum for cheese manufacture.

Thermophilic Starter Cultures

Commercial bulk starter media for propagation of thermophilic starter bacteria, including Streptoccus thermophilus, Lactobacillus bulgaricus, L. helviticus and L. lactis, are few in number compared to bulk starter media for lactic cultures. Unlike with growing lactic cultures in this country, the use of whey-grown starters or propagation in milk is still relatively common with thermophilic bulk cultures for manufacture of Swiss and Italian cheese varieties. PIM developed for lactic streptococci are not satisfactory with thermophilic starters (86) and special formulations are required to support balanced growth of rod-coccus cultures for Italian cheese manufacture, and additional changes are necessary to propagate S. thermophilus alone (12).

Reddy and Richardson (62) adapted the principle of external pH control to propagation of S. thermophilus and L. bulgaricus, separately, in a whey-based medium similar to that used for lactic cultures. However, no attempt was made to grow numerically balanced cultures of rod-coccus combinations.

Using thermochemical measurements to investigate growth of S. thermophilus and L. bulgaricus, Monk (47) found that when the two organisms were grown in combination during pH control (pH 6.5) the maximum heat of fermentation effect was nearly double the sum of

that for the individual cultures. However, upon repeated subculture, the maximum heat effect showed a large decrease and the thermograms became irregular when compared to repeated subculture without pH control.

Buffered Plating Media for Enumeration and Differentiation of Lactic Acid Bacteria

Hunter (33) demonstrated that with addition of sodium phosphate (33.7 mM) to a simple plating medium, more growth and larger colonies resulted. Buffering provided by the phosphate allowed more cell divisions before the hydrogen ion concentration became inhibitory to further growth.

Improved growth of lactic streptococcal results when disodium-B-glycerophosphate (62 mM) is incorporated into a complex medium (81). This medium is also lactose-limiting which enables the final pH to remain well above pH 5.0 and avoid any acid injury to the cells. Douglas (17) claimed one of the advantages of glycerophosphates over inorganic phosphates was that microbiological media could be formulated with high phosphate concentrations without precipitating out many essential metals. This feature is especially useful in plaquing lactic bacteriophages where Ca^{+2} is required for optimum formation of plaques (64,74).

Sodium-B-glycerophosphate is also utilized in two different plating media designed to differentiate 'fast' and 'slow' colonies of lactic streptococci (30,42). These buffered media enable fast

acid-producing colonies to grow to a larger size so that they can be distinguished from smaller slow colonies.

Shankar and Davies (73) found B-glycerophosphate inhibited some strains of L. bulgaricus and more recently there have been reports of inhibition of a few strains of lactic streptococci (36,49).

Barach (6) improved the enumeration of lactic streptococci by as much as 7.75 times by incorporating 30 mM diammonium phosphate into unbuffered Elliker agar.

Insoluble $CaCO_3$ has been added to agar media to help preserve neutral conditions for isolation and cultivation of acid-producing bacteria. Production of acid is localized around colonies and can be detected by clear halos resulting from acid-solubilization of $CaCO_3$ immediately surrounding the colonies (79). Such a buffering system is used in a plating medium for differential enumeration of lactic streptococci (63) and in another for differentiating colonies of S. thermophilus from L. bulgaricus (40).

CHAPTER III

MATERIALS AND METHODS

Cultures and bacteriophages

Several single strain cultures and bacteriophages from the Oregon State University Department of Microbiology collection were used throughout this research. Plasmid-characterized slow variants of S. lactis C2 were provided by Dr. L. L. McKay (Dept. of Food Science and Nutrition, Univ. of Minnesota, St. Paul, MN). Pathogenic strains of S. aureus 265-1 and Salmonella jacksonville were furnished by Robert Williams (OSDA, Salem, OR). Frozen commercial cultures from three culture houses, Marschall Products (Madison, WI), Chr. Hansen's Laboratories (Milwaukee, WI) and Microlife Technics (Sarasota, FL) were also used. Frozen culture LAM was provided by Gaylord Fowler (Swiss Valley Farms, Luana, IA). Bacteriophage r2 was provided by Dr. E. R. Vedamuthu (Microlife Technics, Sarasota, FL). A complete list of cultures and phages used is presented in Table 1.

All lactic acid bacterial cultures were maintained in sterile 11% (w/w) reconstituted nonfat dry milk (NDM, autoclaved 10 min - 121 C). Lactic streptococci were grown at 21 C for 16-18 and S. thermophilus, L. bulgaricus and L. helveticus strains were propagated at 37-40 C for 8-16 h. Each culture was then transferred to fresh NDM (1-2% inoc rate) and stored at 4-7 C in the unincubated state. This sequence was repeated weekly. Cultures not being

Table 1. Origin of cultures and bacteriophages.

OSU Collection	Marschall Products
Streptococcus cremoris 134, 205, HP, H2, C13, 108, 224, 290, $SK_{11}G$	MFS, MRD, MI3, M21, MI1, VT6, VT7, SG1, WP, OS, EV4, C5, CS24, CC1
Streptococcus lactis B1, ML_8, BA-1, C2	CR3, CR4, CR5, CR7, CR9, CR12, CR14, CR15
Streptococcus thermophilus 404, 410 440, 19987, L12, S133, C3	ST, ST4, ST135, LAM[a]
Lactobacillus bulgaricus 404, 448	
Lactobacillus helveticus L112, 15807, 450	H4, H52, H70, H86, H92, H99
Leuconostoc cremoris L2, L6	R1, R2, R5, R6, R39 CH1, CH2, CH3
Enterobacter cloacae	
Pediococcus cerevisiae 1220	
Phage hp, h2, ml_8, c2, T189	Microlife Technics 408, 410 Phage r2
OSDA	Univ of Minnesota
Staphylococcus aureus 265-1	LM0210, LM0220, LM0231
Salmonella jacksonville	

[a]Private label for Swiss Valley Farms, Luana, IA

actively used were stored as 10% transfers in sterile litmus milk at -80 C. Staphylococcus aureus 265-1 was maintained on Trypticase soy agar (BBL) slants, incubated 24 h at 35 C and then stored at 4 C (monthly transfers). Salmonella jacksonville and E. cloacae were maintained on Nutrient agar (Difco) slants; incubated 24 h at 37 C and stored at 4 C (monthly transfers). Multiple uses of frozen commercial lactic cultures were achieved by pre-softening the cultures overnight at -20 C; then cutting the frozen culture mass into 5-10 chunks with a sterile spatula, distributing the chunks into Whirlpaks (NASCO) and storing at -80 C before the culture chunks thawed.

Lactic phages were maintained and propagated in M17 as previously described (81). Streptococcus thermophilus phage r2 was maintained and propagated in HJ broth as previously described (77).

Ingredients and commercial bulk starter media

Various ingredients were used in formulating different internally pH-controlled media. These ingredients along with several commercial bulk starter media that were used as comparative controls are summarized in Table 2. Other chemicals used as ingredients in laboratory trials were of reagent grade quality.

Table 2. Bulk starter media, ingredients and suppliers.

Ingredient	Supplier
Dried sweet whey	
Cheddar	Tillamook County Creamery Assn., Tillamook, OR
Italian	Polly O, Campbell, NY
Yeast extract	
YEP	Yeast Products, Inc., Clifton, NY
Amberex 1003	Amber Laboratories, Juneau, WI
Nonfat dry milk (NDM)	
Instant Peake	Galloway West Co., Fond du Lac, WI
Magnesium hydroxide	
MHT 100	Dow Chemical Co., Midland, MI
Trimagnesium phosphate	Stauffer Chemical Co., Westport, CT
Calcium carbonate	Pfizer, Inc., New York, NY
GW4-4 base	Galloway West Co., Fond du Lac, WI
Stimulac	Biolac, Logan, UT
American Actilac	Galloway West Co., Fond du Lac, WI
Italian Actilac	Galloway West Co.
CFS	Marschall Products, Madison, WI
1-2-1	Marschall Products
Thermostar	Marschall Products
HBM	Dederich Corp., Germantown, WI
RCT 164	Dederich Corp.
Phage-Stat	Pfizer, Milwaukee, WI

Fermentation equipment

Laboratory fermentations were run on four different apparatus. Single fermentations were run on two bench-scale fermentors, Fermentation Design, Inc. (Allentown, PA) and a New Brunswick Multigen F-2000 (Edison, NJ). Working volumes were 700 and 1500 ml, respectively. Both fermenters were equipped with automatic external pH control, temperature control and variable-speed agitation. The FDI fermenter was modified to provide top stirring with a stainless steel paddle powered by a variable-speed electric motor and controller (GT-21, GK Heller Corp., Floral Park, NY).

Up to six one-liter vessels (800-ml working volume) were run at one time with a pharmaceutical dissolution apparatus (Hanson Research Corp., Northridge, CA) equipped with overhead, variable-speed agitation with teflon-coated paddles. Fermentation vessels were suspended in a temperature-controlled, circulating water bath. Bath temperatures as high as 90 C were attainable with two circulating bath heaters (Precision Scientific).

A Lab-Line Multi-Magnestir (Lab-Line Instruments, Inc., Melrose Park, IL) was also used to run up to six fermentations at once. Agitation was provided by variable-speed magnetic stirring and the incubation temperature was dependent on room air temperature (about 25 C).

Note that all fermentations involving either externally or internally pH-controlled media were run with continuous, low-speed agitation unless otherwise indicated in the text.

Laboratory batch pasteurization

Pasteurization of reconstituted bulk starter media was at 85-90 C for 40-60 minutes with a high rate of mixing. Such time-temperature relationships conformed to manufacturers' recommendations for batch pasteurization of bulk starter media. The fermentor vessel or flask containing reconstituted media was rapidly heated to 85 C on a Corning hot plate-stirrer and held between 85 and 90 C for the desired time followed by rapid cooling in an ice water bath. To prevent localized burning near the bottom of the vessel during heating, an outer bath filled with water was used to evenly distribute heat around the vessel.

Pasteurization of the six vessels in the dissolution apparatus was done simultaneously by preheating the bath to 80 C with the suspended vessels in place containing only water. Appropriate amounts of test media were then added to the vessels and the contents rapidly agitated throughout the pasteurization period. Cooling was accomplished by draining the bath approximately 10 minutes before the end of the pasteurization period and refilling the bath with cold water. The desired fermentation temperature was set by using circulator heaters or adding additional cold water. Preliminary trials showed that adding dry media to preheated (80 C) water did not adversely affect their performance with cultures.

Activity tests

Ten-ml quantities of pasteurized (62.8 C-30 minutes) 11% (w/w) reconstituted NDM (Galloway West Co., Fond du Lac, WI), in duplicate, were inoculated at various levels, and along with uninoculated controls were incubated in a temperature-controlled water bath. Final pH measurements were made of all tubes at the end of the activity test. Reported values were calculated as the difference in pH (ΔpH) between uninoculated control and the average of duplicate tubes for each sample.

Four different activity tests were used to evaluate culture activity resulting from various starter fermentations.

Activity for mesophilic lactic streptococci was measured as the change in pH (ΔpH) after incubation for 6 h at 30 C. A controlled temperature profile (CTP) activity test, a modification of an activity test developed by Pearce (55) and improved by Heap and Lawrence (27), was also used to determine activity for mesophilic streptococci. Cultures were subjected to a time-temperature profile typical of Cheddar cheese manufacture (Figure 2).

Titratable acidity

Percent lactic acid was determined by titrating a 9 g (9.0 ml) sample with 0.1 N NaOH to a phenolphthalein end point (pH 8.3). Percent titratable acidity (TA) was calculated as:

$$\frac{(\text{ml NaOH})\ (0.1\ \text{N})\ (0.090)\ (100)}{9}$$

Figure 2. Time-temperature profiles for mesophilic (top) and thermophilic (bottom) activity tests. Approximately 30 minutes of natural cooling was required to drop from 39 to 38 C while circulating cooling coils were necessary to lower the temperature from 38 to 32 C during the 40-minute period at the end of the mesophilic CTP activity test. Mesophilic CTP without the temperature programmer or circulating cold water required time-temperature modifications (—•—•—).

Only natural cooling was used in the thermophilic activity test, and use of the temperature programmer was optional.

The CTP activity test was done in a 14-liter stainless steel water bath (B. Braun Melsengen, West Germany) housing a Thermomix 1480 (B. Braun) circulator-heater and integrated with a Thermograd 1491 (B. Braun) temperature programmer. This equipment enabled precise control of temperature, especially during the gradual temperature increase, simulating the cooking stage of cheese manufacture.

Activity measurements for thermophilic starters were made using (a) pH after 5 h-35 C and (b) thermophilic CTP-modified to typify Italian cheese manufacture (Figure 2).

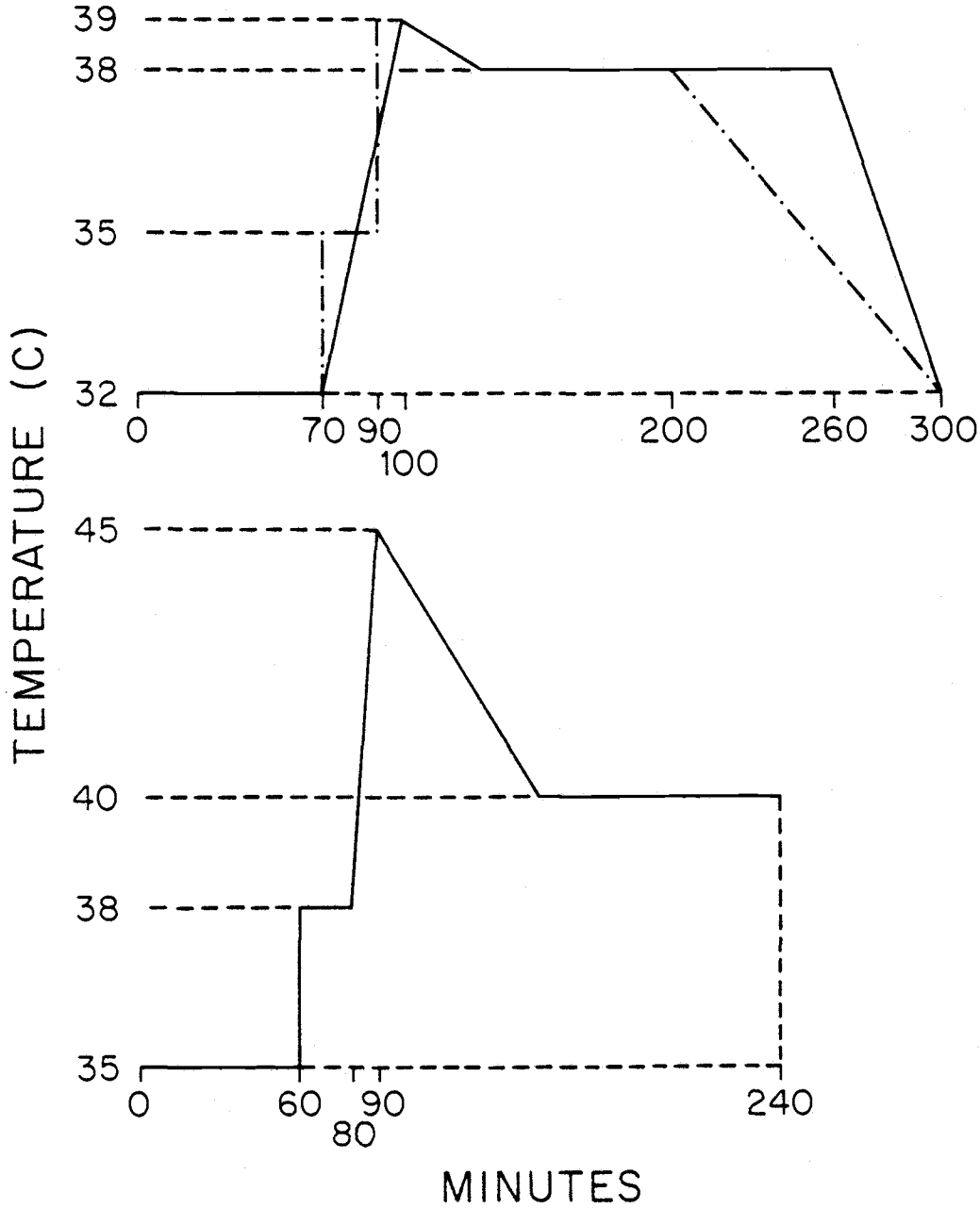

39
38
35
32
0
70 90
100
200
260
300
45
40
38
35
0
60 90
80
240
TEMPERATURE (C)
MINUTES

Figure 2.

Bacteria counts

Samples (0.1 ml) from starter fermentations were blended with a Waring blender in chilled 0.1% (w/v) peptone (Difco) diluent (100 ml) for 60 sec (mesophilic) or 120 sec (thermophilic) to break up the cocci and/or rods. Blended samples were then serially diluted and plated using either the spread plate technique or a micro-drop technique. Mesophilic streptococci were plated on M17 agar (81) or PMP agar for total cell counts (24-48 h at 30 C) while the thermophilic starters were enumerated on Lee's Agar (40) or LB agar (18) with incubation at 37-40 C for 24 h under anaerobic conditions (BBL Gaspak).

Phage assay

For mesophilic phage assays, one ml of phage-infected starter was transferred to nine ml of chilled sterile saline (0.85% NaCl), centrifuged at 5000 x g for 10 minutes, and the supernatant was serially diluted and titered by the standard double layer plaque assay of Adams (1) on M17 agar (with 0.0025% bromcresol purple). A micro-drop phage assay was also used in some instances, primarily with the T189-*S. cremoris* 205 combination. Incubation for both procedures was at 30 C for 12-24 h.

Thermophilic phage assays were done by the procedure described by Sozzi et al. (77) using HJ Agar under CO_2.

EXTERNAL NEUTRALIZATION WITH SODIUM CARBONATE

Three separate fermentations were performed using Cottage Actilac (Galloway West Co., Fond du Lac, WI), reconstituted to 11% solids (w/w) and pasteurized and cooled as previously described. A one percent inoculum of S. cremoris 134 was added to each fermentation vessel and incubated at 27 C for 16 h with continuous low-speed agitation. A control fermentation was run without any pH control while the other two fermentations were externally neutralized with sterile 5N Na_2CO_3, one continuously neutralized to pH 6.0 ± .05 and the other with a single addition of 5N Na_2CO_3 after 12 h to raise the pH to 7.0. Total cell counts were determined after 16 h for the unneutralized control and at two-hour intervals for the externally neutralized fermentations.

INTERNAL NEUTRALIZATION WITH CONTROLLED RELEASE ALKALI

A fermentation medium was prepared from the following ingredients (w/v): whey powder, 3.5%; yeast extract, 0.5%; disodium-B-glycerophosphate, 0.5%; trisodium citrate dihydrate, 0.83% and diammonium citrate, 0.17%. The medium was pasteurized and cooled to 27 C. The initial pH of the pasteurized medium was 6.5.

The controlled release ingredient consisted of Na_2CO_3 coated with ethyl cellulose (Ethocel 45, Dow Chemical Co.). The coating was applied by spraying a solution of 20% Ethocel dissolved in methylene chloride onto Na_2CO_3 tumbling in a pharmaceutical coating pan to form agglomerated granules. The granules were washed with water, oven dried and 7.5 g added to the fermentation medium.

The complete medium (CRA-7.5) was then inoculated with S. lactis B1 (1%) and incubated at 27 C with gentle agitation for 16 h. The fermentation pH was monitored with a strip chart recorder.

A second fermentation (CRA-13) was run using the same conditions except that 13.0 g (10.4 g Na_2CO_3) of the controlled release alkali (larger granules) were added to the medium.

A control fermentation (CRA-0) without the coated Na_2CO_3 and a fourth fermentation (A-10.4) to which 10.4 g of uncoated Na_2CO_3 was added in place of the controlled release granules were also performed under the same conditions.

Following the 16 h incubation period, activity (6 h-30 C) and total cell counts were measured for each of the trials. Samples from each of the four fermentations were held at 21 C for 10 days, during which continued activity and cell number measurements were made.

INTERNAL NEUTRALIZATION WITH INSOLUBLE BUFFERS

Bulk Stater Media for Mesophilic Starters

Trimagnesium phosphate and calcium carbonate

Ten percent lactic acid (w/w) was added in one ml increments to the following three media: (a) reconstituted NDM (11% w/w); (b) whey, 3.5% (w/v); yeast extract, 0.5% (w/v); $(NH_4)_2HPO_4$, 1.0% (w/v) and (c) whey, 3.5% (w/v); yeast extract, 0.5% (w/v); $Mg_3(PO_4)_2$, 2.0% (w/v). The pH was measured after each lactic acid addition with five-minute intervals between lactic acid additions in order for the pH to stabilize.

Reconstituted NDM (11% w/w), $Mg_3(PO_4)_2$-buffered (whey, 3.5% w/v; yeast extract, 0.5% w/v; diammonium citrate, 0.6% w/v; trisodium citrate dihydrate, 0.4% w/v; $Mg_3(PO_4)_2$, 4.3% w/v) and $CaCO_3$-buffered (whey, 5.0% w/v; yeast extract, 0.5% w/v; $CaCO_3$, 2.5% w/v) media were inoculated with S. cremoris 134 (1%) and incubated for 16 h at 27 C with continuous agitation. Activity (4 h-30 C with 1% inoculation rate) and total cell count determinations were made at the end of the fermentation period. Samples from each fermentation were collected and held at 5 C with daily activity and cell counts measured through four days post-fermentation.

Abilities of the previously described $Mg_3(PO_4)_2$ (except $Mg_3(PO_4)_2$ reduced to 3.5%) and $CaCO_3$-buffered media to prevent phage replication were determined by measuring the phage titer

following infection with approximately 10^5 pfu/ml of phage. For the $Mg_3(PO_4)_2$ medium, phage h2 with its homologous host S. cremoris H2 was used while hp/HP was used in the $CaCO_3$ medium. Nonfat milk controls were run with both phage/host systems. Each medium was inoculated with host (1%) and phage and incubated for 16 h at 27 C in a temperature-controlled bench top shaker (New Brunswick G24 Environmental Incubator Shaker) at 200 rpm. Phage titer was determined at the end of a 16 h incubation period.

Effect of $Mg_3(PO_4)_2$ concentration

Trimagnesium phosphate was added at 3.0, 3.5 and 4.3% (w/v) to flasks containing whey, 3.5% (w/v); yeast extract, 0.5% (w/v); diammonium citrate, 0.55% (w/v); and trisodium citrate dihydrate, 0.45% (w/v). The flasks were steamed for 30 minutes and cooled at 27 C. The pH was adjusted in each flask to 7.0 with sterile 1N HCl (pH after steaming ranged from 7.1-7.2). Reconstituted NDM (11% w/w) was pasteurized in the same manner and served as control. Each flask was inoculated with S. cremoris 134 (1%) and incubated at 27 C in a bench top incubator shaker for 16 h at 200 rpm. The final pH, activity (4 h-30 C with 1% inoculation rate) and total cell count were determined after the 16 h fermentation and again after a 48 h holdover period at ambient temperature (21-24 C) without agitation.

Interaction between $Mg_3(PO_4)_2$ and $(NH_4)_2HPO_4$

Dibasic sodium and ammonium phosphate salts were added in four 0.25 g increments to 100 ml each of two basal medium (w/v): whey, 3.5%; yeast extract, 0.5% and whey, 3.5%; yeast extract, 0.5%; $Mg_3(PO_4)_2$, 2.0%. In addition, $(NH_4)_2HPO_4$ was added to a 2.0% (w/v) suspension of $Mg_3(PO_4)_2$ in 100 ml distilled water. The media were continuously stirred on a magnetic stirring plate while pH measurements were made 15 minutes after each addition of phosphate.

Three separate mixtures consisting of a basal medium (whey, 3.5% w/v and yeast extract, 0.5% w/v) plus (a) $Mg_3(PO_4)_2$, 2.0% (w/v), (b) $Mg_3(PO_4)_2$, 2.0% (w/v) and $(NH_4)_2HPO_4$, 1.0% (w/v) and (c) $Mg_3(PO_4)_2$, 3.0% (w/v) were titrated with 10% lactic acid. After each milliliter addition of lactic acid, a pH measurement was made.

Addition of citrate for phage inhibition

Phage T189 (approx 10^5 pfu/ml), apparently a low calcium-requiring phage (A. R. Huggins, Ph.D. Thesis, Oregon State University, 1980), was used to challenge the growth of S. cremoris 205 (homologous host) in different formulations. A basal medium consisting of whey, 3.5% (w/v) and yeast extract, 0.5% (w/v) was combined with trimagnesium phosphate, diammonium phosphate, trisodium citrate dihydrate and disodium tartrate dihydrate at various concentrations to prevent replication of T189 phage. The host was added at a one percent inoculation rate followed by T189

phage. Each fermentation mixture was incubated with continuous agitation at 21 C for 16 h. A final phage titer was determined by the phage micro-drop technique.

From screening of formulations with T189 phage, the following medium was selected for further study with other phage-host combinations: Trimagnesium phosphate, diammonium phosphate, and trisodium citrate dihydrate each at 1.5% (w/v) in the basal medium (PHASE 4-M). Phages ml_8, c2, hp and h2 were used to challenge this medium in the presence of their respective homologous hosts S. lactis ML_8 and C2 and S. cremoris HP and H2 under the same conditions used with T189/205.

In situ generation of $Mg_3(PO_4)_2$ and $Mg\ NH_4PO_4$

Trimagnesium phosphate was generated in situ by combining magnesium hydroxide with citric acid monohydrate and trisodium phosphate .12 H_2O; magnesium hydroxide with phosphoric acid and magnesium hydroxide with ammonium phosphates. These combinations were incorporated into three different formulations that were compositionally (theoretical) equivalent to the PHASE 4-M medium (Table 3). Titrations with lactic acid were used to confirm the buffering capacity of each medium. Fermentations (16 h-24 C) were run using S. cremoris 205 and three commercial cultures (frozen bulk sets M13 and M21 and an intermediate culture of H4 prepared in reconstituted NDM) to compare the activity (CTP) to that attained

Table 3. PHASE 4 formulations with *in situ* generated $Mg_3(PO_4)_2$.

PHASE 4-RM	Dried sweet whey	3.5% (w/v)
	Yeast extract	0.5
	Diammonium phosphate	1.5
	Magnesium hydroxide	0.44
	Trimagnesium phosphate	0.5
	Trisodium phosphate • 12 H_2O	1.94
	Citric acid • H_2O	1.07
PHASE 4-P	Dried sweet whey	3.5% (w/v)
	Yeast extract	0.5
	Magnesium hydroxide	0.7
	Phosphoric acid (85% w/w)	0.92
	Diammonium phosphate	1.5
	Trisodium Citrate • 2 H_2O	1.5
PHASE 4-MH	Dried sweet whey	3.5% (w/v)
	Yeast extract	0.5
	Magnesium hydroxide	0.7
	Monoammonium phosphate	1.8
	Diammonium phosphate	0.5
	Trisodium citrate • 2 H_2O	1.5

in PHASE 4-M. Phage inhibition studies were also performed with the T189-S. cremoris 205 combination.

Uniform commercial blending

PHASE 4-MH was commercially dry blended (Galloway West Co., Fond du Lac, WI) in 3000-lb batches. Individual ingredients were added to rectangular mixing bins. Mixing times varied from 10-30 minutes, after which each batch was bagged in 75-lb bags (40/batch). Samples were taken from every other bag during bagging and assayed for citrate to determine the uniformity of the blended medium.

Citrate was determined by the method of Babad and Shtrikman (4). PHASE 4 samples (2.0 g) were suspended in 50 ml of distilled water. Five ml of trichloroacetic acid was mixed with five ml of the PHASE 4 suspensions, allowed to stand for two hours and filtered. Exactly 0.2 ml of the filtrate was added to dry, stoppered test tubes. Seven ml of acetic anhydride was added to each tube which was then held at 60 ± 1 C in a temperature-controlled water bath. After 10 minutes, one ml of pyridine was added to each tube followed by continuous shaking for an additional 40 minutes in the bath. The tubes were cooled for five minutes in ice water and absorbances read on a Bausch and Lomb Spectronic 20 at 420 mu.

Standard curves were prepared by combining anhydrous citric acid or sodium citrate (100, 150, 200, 250, 300 mg) with amounts of PHASE 4 basal (whey, 52.5% w/w; yeast extract, 6.0% w/w; diammonium

phosphate, 33.0% w/w; $Mg(OH)_2$, 8.5% w/w) to make 2.0 g. Standard preparations of citrate + PHASE 4 basal were then treated in the same manner as the PHASE 4 samples.

PHASE 4-MC was produced as follows: 120 mMoles of magnesium hydroxide solids in the form of a powder (0.7% w/v) or slurry was added to a 51 mM solution of citric acid (1.07% w/v). The combination of these ingredients formed a more soluble suspension of magnesium citrate (25.5 mMoles) and 43.5 mMoles of unreacted magnesium hydroxide. The remaining ingredients, whey, 3.5% (w/v), yeast extract, 0.5% (w/v) and diammonium phosphate, 2.6% (w/v) were then dry blended with an intermediate spray-dried mixture of magnesium citrate and magnesium hydroxide. The chemical composition of PHASE 4-MC was theoretically very similar to the previous PHASE 4 formulations, PHASE 4-M, RM, P and MH. The citrate content was assayed from samples taken from the first and last bags of 10 successive batches made with the PHASE 4-MC formulation (Batch No. 0-144-PH4, Galloway West Co.). A frozen bulk set culture (H86) was used to inoculate (0.062 ml/l) PHASE 4-MH and PHASE 4-MC. Following 20 h of incubation at 24 C, CTP activity (1%) was determined for each starter.

Milk-based PHASE 4 and adaptation to HTST pasteurization

One school of thought generally feels the use of a completely whey-based bulk starter medium results in cheese with whey-tainted

flavors (Grant Krugor - personal communication). Therefore, they recommend the use of a medium containing a minimum NDM : whey solids ratio of 3:1. PHASE 4-MH was reformulated using NDM solids (PHASE 4-MBØ) and evaluated for phage inhibition against phage T189 (S. cremoris 205 host) and activity (CTP) with four commercial frozen bulk set cultures. PHASE 4-MBØ consisted of NDM, 1.125% (w/v); whey, 0.375% (w/v); lactose, 1.6% (w/v); yeast extract, 0.5% (w/v); magnesium hydroxide, 0.7% (w/v); monoammonium phosphate, 1.8% (w/v); diammonium phosphate, 0.5% (w/v) and trisodium citrate dihydrate, 1.5% (w/v).

PHASE 4-MC was divided into two parts, Part A containing magnesium hydroxide and citric acid and Part B consisting of whey, yeast extract and diammonium phosphate. A milk-based variation of this A + B PHASE 4-MC was made by substituting 5.0% (w/v) NDM for whey solids and adjusting the phosphate content to 2.3% (w/v) diammonium phosphate and 0.3% (w/v) monoammonium phosphate.

These formulations were also compared to PHASE 4-MH for their abilities to support culture growth.

High temperature short time (HTST) field studies were done by Galloway West personnel with the PHASE 4-MC (A + B) formulation.

Improved PHASE 4 formulation using $MgSO_4$: PHASE 4-MS

Trimagnesium phosphate was generated in situ by combining 22 mM magnesium hydroxide (0.125% w/v) and 65 mM magnesium sulfate (1.59% w/v $MgSO_4 \cdot 7H_2O$ or 0.78% w/v $MgSO_4$) with 185 mM diammonium phosphate

(2.44% w/v). Sodium citrate, 52 mM (1.35% w/v) and the nutrients dried sweet whey (3.5% w/v) + yeast extract (0.5% w/v) were added to complete the medium. Total solids (w/w) for PHASE 4-MS was 8.7% using the heptahydrate form of magnesium sulfate and 8.0% with anhydrous form. Not to be confused with the actual solids in the reconstituted medium, total solids was based on the weight of ingredients before reconstitution and not correcting for moisture or chemically combined water.

PHASE 4-MS (with $MgSO_4 \cdot 7H_2O$) was evaluated for supporting growth of S. cremoris 205 (1.25% inoculation rate) and in a separate fermentation for inhibition of the T189 phage (10^5 pfu/ml infection). PHASE 4-MC served as controls. Incubation for both growth and phage inhibition studies was at 25 C for 16 h. Fermentation pH at the time of inoculation and at 16 h along with a 16 h titratable acidity were measured. Activity and total cell numbers were determined on the mature starters. A final phage titer was determined for the phage inhibition study.

Effect of initial pH

A fermentation medium consisting of whey, 3.5% (w/v); yeast extract, 0.5% (w/v); $Mg_3(PO_4)_2$, 4.3% (w/v); diammonium citrate, 0.6% (w/v) and trisodium citrate dihydrate, 0.4% (w/v) was pasteurized, cooled to 27 C and aseptically distributed in 100 ml amounts into six sterile Erlenmeyer flasks (250 ml). The initial pH in the six flasks (7.1) was adjusted with sterile 1N HCl or

NaOH to pH 6.4, 6.6, 6.8, 7.0, 7.2 and 7.4. A control made up of 11% (w/w) NDM was steamed for 30 minutes and no pH adjustment made (pH 6.6). Each flask was inoculated with S. cremoris 134 (1%) and incubated at 27 C in a bench-top shaker at 200 rpm. The pH of each flask was measured after 16 h incubation, at which time an activity test (4 h-30 C with 1% inoculation rate) and total cell count were determined. Mature starters were held at ambient temperatures (21-24 C) without agitation for an additional 48 h. Activity and cell counts were determined again.

Effect of fermentation temperature

One vessel of PHASE 4-M was inoculated with S. cremoris 134 (1%) and incubated with continuous agitation at 21 C for 16 h. A similar vessel was incubated at 27 C. Two other fermentations with PHASE 4-M were set with one percent S. cremoris 205 and incubated at 21 and 24 C under the same conditions. A CTP activity test and a total cell count were determined immediately following the 16 h fermentations and again after 24 h storage at ambient (21-24 C) temperatures without agitation.

Effect of agitation

Traditionally, bulk starter media are not agitated during the incubation period (except to initially mix in the inoculum) to prevent excessive air incorporation that could lead to culture

inhibition. Because of the insoluble nature of $Mg_3(PO_4)_2$ in PHASE 4, it was necessary to agitate the fermenting medium to maintain the insoluble buffer in suspension.

Three separate fermentations using PHASE 4-M were run to evaluate different agitation patterns. One vessel was incubated without any agitation, except for five minutes initially and five minutes just before sampling after 16 h. A second vessel was continuously agitated with low speed mixing while the final vessel was briefly agitated to mix in the inoculum followed by 12 h without any agitation. Intermittant agitation, one hour on-one hour off, was then followed during the last four hours of the incubation period. All vessels were inoculated with S. cremoris 134 (1%) and incubated at 21 C for a total of 16 h.

Mature starters were then held at 21 C without agitation for an additional two days. Measurements of CTP activity (0.5%) and cell numbers were made immediately following the 16 h fermentation and again after one and two days holdover.

In a similar experiment, three vessels containing PHASE 4-MC were (a) continuously agitated for 18 h (b) continuously agitated for 6 h following a 12 h period without agitation and (c) agitated for the last 2 h after 16 h with no agitation. All vessels were set with S. cremoris C13 (1%) and incubated at 25 C. PH was monitored during the last 6 h of incubation both before the agitator was engaged and 15 minutes following the onset of mixing. Activity (4 h-32 C at 2.5%) was determined after the 18 h fermentation.

Effect of different 'Complex Nutrient' sources

Complex nutrients are those ingredients supplying carbohydrates, nitrogen sources and miscellaneous growth factors necessary for growth (59). These complex nutrients include such common undefined ingredients as dried sweet whey, NDM and yeast extract.

PHASE 4-MH was used as the basal medium in which five different dried sweet whey sources were incorporated; four from Cheddar cheese manufacture in Oregon and Wisconsin and one Italian cheese whey powder from a plant in New York. A frozen commercial culture (MFS) was used to inoculate (0.062 ml/l) each of the five vessels. Incubation was at 25 C for 18 h after which a CTP activity was determined at 0.5, 1.0 and 2.0% inoculation levels.

Varying concentrations (0.1-1.0% w/v) of autolyzed yeast extract (YEP) were incorporated into the PHASE 4-M formulation. Following a 16 h fermentation at 21 C with S. cremoris 205 (1%), CTP activity at 0.5, 1.0 and 2.0% inoculation rates and total cell numbers were determined for each of the five levels of yeast extract examined.

Six commercially available autolyzed yeast products were compared for their abilities to stimulate growth in PHASE 4-MC. Four lactic culture systems, S. cremoris 205 (1%), a multiple strain starter (1%) consisting of S. cremoris strains $SK_{11}G$, C13, 290-P, 224, 108 and S. lactis BA-1 and frozen bulk sets H99 and MRD (0.062 ml/l) were used as inocula. Fermentations were at 24 C for 16 h after which CTP activity (1 and 2% inoculation rates)

and total cell numbers were determined. Three of the six yeast products were spray-dried, autolyzed yeast extracts made from brewers yeast: Ardamine YEP (Yeast Products Inc., Clifton, NJ); Amberex 1003 (Amber Laboratories, Juneau, WI) and Special Light AYE (Anheuser-Busch, Inc., St. Louis, MO). The AYS (Anheuser-Busch Inc.) yeast product was also spray-dried autolyzed brewers yeast but consisted of the soluble extract and the cell wall material. The final two yeast products, Z113 and Z33 (both from Pure Culture Products Inc., Chicago, IL) were autolyzed primary yeast with the former spray-dried and the latter drum-dried.

Effect of total solids concentration and 'Carryover Buffer'

A commercial blend (Galloway West Co.) of PHASE 4-M (352-1) was used to prepare suspensions of PHASE 4 at the following solids concentrations (w/w): 3.0, 5.0, 6.0, 7.0, 8.0, 8.5, 9.0 and 11.0%. Each vessel was inoculated (0.062 ml/l) with the frozen bulk set H85 and incubated at 24 C for 18 h. A total cell count and CTP activity (0.5, 1.0 and 2.0%) were determined on the mature starter after the 18 h fermentation period.

Commercially manufactured PHASE 4-MC was used to prepare bulk starter (200 gal) at Galloway West using 50, 60, 65 and 75 lb of PHASE 4 mixed with sufficient water to make 100 gallons total volumes. Each tank, made on separate days, was pasteurized at 185 F for 45 minutes with high agitation, cooled to 74-76 F and inoculated with frozen bulk set culture M13. Each tank was incubated for

16-18 h with continuous agitation and then cooled to 40 F. Four 45,000-lb capacity cheese vats were set with sufficient starter (cheesemaker's judgment) from each starter tank to produce Cheddar cheese. Titratable acidity, pH and CTP activity (1%) were determined for each starter before used in the cheese vats.

PHASE 4-MC (249-05) was reconstituted to 8.7% (w/w) ('high solids') and 5.8% (w/w) ('low solids'). Lactic acid was added to lower the pH of each simulated bulk culture to pH 5.5. Each simulated bulk starter was used to inoculate 100 ml amounts of reconstituted NDM (9.0% w/w) at 0, 0.5, 1.0, 2.5 and 5.0% (v/v). Lactic acid (10% w/w) was then added to each beaker of reconstituted NDM to lower the pH to 5.0 to simulate a typical fermentation in a cheese vat. The 10% lactic acid required to lower the pH for each inoculation level at both high and low solids was recorded. In addition, titratable acidity was determined of the acidified reconstituted NDM.

PHASE 4-MC (several different production lots) was used to prepare bulk starter at Galloway West at both high (8.7% w/w) and low (5.8% w/w) solids. A 200-300 gallon volume was prepared for each of 11 commercial cultures used in the starter rotation at both solids levels. The ml of the lactic acid necessary to drop the pH of 20 ml mature starter + 80 ml distilled H_2O to 4.5 was determined for each culture as a direct measure of carryover buffer. Increased starter usage for Cheddar cheese production with the low solids starters was also monitored.

Inhibition of the T189 phage (with 1.25% host S. cremoris 205) was studied in commercial batches of PHASE 4-MC (Galloway West Co.) and laboratory blends at high (8.7% w/w) and low (7.8% w/w) solids. Five samples of 1981 production along with PHASE 4-MC that was assembled in the lab using the GW 4-4 base (Galloway West Co.) and made with the raw ingredients citric acid + $Mg(OH)_2$ were evaluated. Phage infection was at 10^5 pfu/ml with a final phage titer determined after 17 h incubation at 25 C. A CTP activity test was also run on the mature starter (16 h).

Competitive growth by contaminants in PHASE 4

A concern in the use of pH controlled bulk starter media (external or internal) was the potential for enhanced growth by contaminating pathogens because of higher pH maintained in the growth medium. Three separate experiments were performed in which lactic starters were challenged with the gram positive pathogen Staphylococcus aureus, the gram negative pathogen Salmonella jacksonville and the coliform Enterobacter cloacae, while growing in PHASE 4 and other pH control media in order to evaluate competitive growth by contaminants in bulk starter media.

PHASE 4-M and an externally neutralized (EN) medium were inoculated with S. cremoris 134 (1%) and challenged with a trypticase soy broth (BBL) culture (37 C-24 h) of S. aureus 265-1 (1.0×10^5 cfu/ml contamination level). The EN medium was prepared by combining 70.9 g of dried sweet whey with 12.0 g of

Stimulac (Biolac, Logan, UT) and diluting to one liter with water. The initial pH was adjusted from 6.4 to 6.7 with 20% (w/w) NH_4OH. Pasteurization was for 60 minutes at 195 F after which the medium was cooled to 27 C. Incubation of PHASE 4-M medium was at 24 C while for EN the incubation temperature was 27 C. Both vessels were agitated with continuous, low speed stirring. External neutralization was accomplished by addition of 20% (w/w) NH_4OH upon pH demand maintaining pH 6.2 ± .05. Both vessels were incubated for 16 h after which samples were aseptically collected, added to sterile dilution bottles and cooled to 10 C in ice water. The final pH of the EN vessel was raised to 6.7 with NH_4OH before cooling as suggested by the manufacturer (Biolac 1979). Staphylococcus aureus counts were determined at 0, 8, 12 and 16 h of incubation. Samples were diluted and plated on Baird Parker agar (Difco) and counted after 48 h incubation at 35 C. Counts were also determined after 1, 4 and 7 days holdover at 10 C. Noncompetitive trials with S. aureus 265-1 (10^5 cfu/ml) alone were also performed with both PHASE 4-M and EN medium using the same fermentation conditions as previously described. Staphylococcus aureus growth was only monitored during the 16 h incubation period.

A similar experimental design was used for challenge with Salmonella jacksonville. PHASE 4-P and EN (Biolac) were inoculated with frozen bulk set M21 (0.062 ml/l) and contaminated with a 16 h-37 C nutrient broth (Difco) culture of Salmonella

jacksonville (1.1×10^5 cfu/ml). Fermentation conditions were identical to previously described S. aureus challenge except incubation was continued through 18 h and holdover was at ambient room temperature (21-24 C) for an additional 18 h (36 h post inoculation). Samples were plated at 0, 9, 12, 15, 18 and 36 h on XL agar (Difco), incubated 36 h at 37 C, to determine the growth of Salmonella jacksonville in each medium. As before, non-competitive trials in each medium without lactic starter were performed to serve as controls.

The final contaminant challenge was at a lower level (10^2-10^3 cfu/ml) with Enterobacter cloacae. Frozen culture M13 was used as the lactic starter. Four different commercial media were evaluated: PHASE 4-MC (Lot 163, Galloway West Co.), 8.67% (w/w); HBM (Dederich Corp., Germantown, WI) 8.1% (w/w); CFS (Marschall Products, Madison, WI), 8.0% (w/w); and Marstar 1-2-1 (Marschall Products, Madison, WI), 11.5% (w/w). HBM is a highly buffered medium using conventional orthophosphates and not requiring continuous agitation during the incubation period. CFS is an externally neutralized medium (20% w/w NH_4OH) that was maintained at pH 6.0. CFS powder was mixed 1:1 with NDM to make up the 8.0% total solids. One-2-One, requiring no agitation, is a conventional bulk starter medium also combined 1:1 with NDM. Incubation for all media was at 24 C for 16 h. Samples taken at 0, 8, 12 and 16 h were plated on Violet Red Bile agar (Difco) and incubated at 37 C for 24 h to determine coliform counts. After 16 h of

incubation, samples were collected and cooled to 10 C in ice water. Coliform counts at daily intervals were run on holdover samples through 4 days.

Growth of citric acid-fermenting bacteria and Pediococcus cerevisiae in PHASE-4

Streptococcus diacetilactis and some Leuconostoc species are sometimes referred to as flavor or aroma bacteria. They ferment citric acid and produce diacetyl, volatile acids and CO_2. These organisms are incorporated into several commercial culture blends along with S. lactis and S. cremoris strains. Leuconostoc species are generally combined with the acid-producing S. lactis and S. cremoris strains, while the lactose-fermenting ability of S. diacetilactis enables it to be used alone to produce high levels of flavor compounds.

Growth of Leuconostoc cremoris and S. diacetilactis in association with lactic streptococci was evaluated in PHASE 4-MC in a series of laboratory trials. A reconstituted NDM culture (24 h-30 C) of L. cremoris L2 (1% inoculum) was grown alone in PHASE 4-MC for 16.5 h at 26 C.

Using the same conditions, L. cremoris L6 (1%) was combined with S. cremoris C13 (0.5%). Leuconostoc and lactic streptococci cell counts were determined by spread plating on LB Agar (48 h-30 C). Leuconostoc cremoris appeared as large colonies while S.

cremoris colonies were considerably smaller. Representative colonies of each type were confirmed by their litmus milk reaction.

Commercial frozen bulk set culture H52, containing strains of S. cremoris and S. diacetilactis, was inoculated (0.62 ml/l) into PHASE 4-MC and incubated 18 h at 24 C. Differential counts of S. cremoris and S. diacetilactis were determined by spread plating on Reddy's Agar (63). Incubation was anaerobically at 32 C. After 26 h the S. cremoris count was determined and S. diacetilactis was counted after 6 days of incubation.

Streptococcus diacetilactis, isolated from H52 on Kempler and McKay's medium for detection of citrate-fermenting lactic streptococci (35), was used to inoculate PHASE 4-MC along with S. lactis C2-Fast (lac^+, prt^+) and S. lactis C2-slow (lac^+, prt^-; LM0210). All three organisms were grown overnight in reconstituted NDM (16 h-21 C) and combined at a ratio of 5:5:1 (S. diacetilactis: C2 slow: C2 Fast) to provide a total inoculum of 1.4%. The total cell count of the freshly inoculated medium was 5.4×10^6 cfu/ml of which 5% was determined to be S. diacetilactis, 41% S. lactis C2 Fast and 54% S. lactis C2 slow. Incubation was at 25 C for 16 h. Differential cell counts (including the initial cell numbers) were determined by spread plating on FSDA-II[1] and McKay's citrate detection agar. Since S. diacetilactis (H52) appeared as

[1]Plating medium for differentiation of 'fast' and 'slow' acid-producing colonies of lactic streptococci.

'slow' colonies on FSDA-II, the cell count of S. lactis C2 slow was determined by subtracting the S. diacetilactis count on McKay's medium from the total slow count on FSDA-II.

A one percent inoculum of Pediococcus cerevisiae 1220 (24 h-25 C in MRS broth) was inoculated into PHASE 4-MC (7.8% w/w), Italian PHASE 4-MH (9.25% w/w) and Italian PHASE 4-NPC (10.6% w/w). The fermentations were carried out with agitation at 25 C for 20 h. The pH and titratable acidity were measured initially and after 16 and 20 h of incubation. Total cell numbers were determined on MRS agar (24 h at 27 C under anaerobic conditions).

Growth of slow acid-producing variants in PHASE 4-MC

PHASE 4 contains a relatively high concentration of yeast extract. Such a medium provides the necessary stimulation to achieve practical set times (14-18 h) with frozen bulk set cultures. The rich growth environment of PHASE 4 may also enable competitive growth by slow acid-producing variants. Various combinations of S. lactis C2 (fast parent) with the plasmid-characterized S. lactis C2 slow variant (LMO210 strain; lac^+, prt^-) were inoculated into PHASE 4-MC. Incubation was at 25 C for a total of 20 h. Titratable acidity and pH measurements were made initially and at 16 and 20 h. CTP activity was run after 16 h and differential cell counts of the slow and fast colonies were made on FSDA-II (48 h-30 C under anaerobic conditions) at 0, 16 and 20 h.

Commercial cheesemaking trials

Several experimental field trials with PHASE 4-M were performed in Oregon and Washington and by Galloway West Co. in the state of Wisconsin before commercial sales of the product began on January 1, 1980.

The first trial took place at Olympia Cheese Co. (Olympia, WA) in which PHASE 4 (SP-9-178-1) was compared to American Actilac (Galloway West Co.). Fifty gallons of PHASE 4 (8.5% w/w solids) were pasteurized, cooled to 76 F and set with six phage insensitive single strains (grown as intermediates in reconstituted NDM) of S. cremoris (16). After 15 h of incubation (with continuous agitation) the culture was used for manufacture of Cheddar cheese. At 18 h post inoculation, the culture was cooled to 45 F and held for cheese production on the following two days. A total of four vats (20,000-lb. capacity) were made with PHASE 4 starter. Laboratory comparisons for activity (6 h-30 C at 0.5 and 1.0% inoculation rates) and total cell numbers were also made between the two starters.

A second trial was run at Olympia Cheese Co. at a later date soon after the Olympia plant had switched to preparing their bulk starter by external neutralization with ammonia (66). PHASE 4-M (8.0% w/w) was prepared as in the previous trial and was compared to the externally neutralized starter in consecutive vats of Cheddar cheese manufacture. Samples from both starters were also analyzed for activity (4 h-30 C at 0.5 and 1.0%) and cell numbers. In

addition, samples from each starter were centrifuged (10 min - 5000 x g) and filter-sterilized (0.45 um). Dilutions of the filtrates were made in saline and plaqued against the six single strains in the culture to compare phage development in the two bulk starter systems.

PHASE 4-M (8.0% w/w) was compared to skim milk starter in cottage cheese manufacture at Fred Meyer (Portland, OR). PHASE 4 was prepared as in previous trials and set with the frozen bulk set culture H70. Mature starter was cooled after 17 h of incubation and used to inoculate a 14,000-lb capacity vat at 0.95% (w/w) while the other vat was set with 2.5% (w/w) of skim starter. PH was monitored in both vats during the 6.5 h fermentation period.

Bulk Starter Media for Thermophilic Starters

The concept of insoluble, pH-dependent buffering, as used in PHASE 4 formulations, was also applied in developing media for thermophilic starter bacteria. Two formulations were developed for propagating balanced rod-coccus cultures, Italian PHASE 4-MH and -NPC (Table 4). Italian PHASE 4-MH was based on *in situ* generation of $Mg_3(PO_4)_2$ while Italian PHASE 4-NPC was a nonphosphated medium using calcium carbonate as a buffer. Generally, both Italian PHASE 4 formulations incorporated a higher nutritive solids content with addition of NDM solids and increased whey solids while the overall buffering capacity was reduced in comparison to PHASE 4. This allowed the pH to drop below pH 5.0 which apparently is necessary to allow sufficient rod development for balanced growth.

The Swiss PHASE 4 (Table 4) formulation needed only to support growth of *S. thermophilus*. A modified PHASE 4 formulation with slightly higher whey solids and reduced phosphate was developed that maintained the pH well above 5.0 throughout bulk fermentation.

In general, laboratory fermentations with thermophilic starters were run at 40-42 C for 5-9 h of incubation. Activity testing and cell numbers were common criteria for comparison. For balanced culture studies, plating on differential media and microscopic observations were used to determine the rod-coccus ratio of mature starters. Both Italian PHASE 4 media and the Swiss PHASE 4 medium were continuously agitated during fermentation to keep the insoluble buffer suspended and effect maximum neutralization of

Table 4. Formulation modifications made to PHASE 4 necessary to support the growth of the thermophilic starter culture.

	% w/v			
Ingredient	PHASE 4-MC	Swiss PHASE 4	Italian PHASE 4-MH	Italian PHASE 4-NPC
whey	3.50	4.00	5.91	5.91
NDM	-	-	3.19	3.19
YE[a]	0.50	0.50	0.50	0.50
MH[b]	-	-	0.65	-
DAP[c]	2.60	1.50	0.35	-
MAP[d]	-	-	1.63	-
$CaCO_3$	-	-	-	2.00
GW-4-4[e]	1.68	1.68	-	0.25
Total solids (w/w)	7.6	7.1	10.9	10.6

[a] Yeast extract

[b] $Mg(OH)_2$

[c] $(NH_4)_2HPO_4$

[d] $NH_4H_2PO_4$

[e] Magnesium citrate-$Mg(OH)_2$ intermediate produced by combining 42% $Mg(OH)_2$ and 58% citric acid (Galloway West Co., Fond du Lac, WI)

lactic acid produced by starter organisms. Several commercial media were included in these studies, all of which were incubated without agitation following a short period initially to mix in the inoculum.

Studies showing two-stage growth of rod-coccus starters in Italian bulk starter media and their subsequent fate under refrigerated holdover were done. Experiments showing effects of total solids, NDM solids, citric acid, $Mg(OH)_2$ and phosphate on the balanced growth of rod-coccus starter were run.

Growth of several commercial cultures, both rod-coccus and coccus alone, was compared in internally pH-controlled media to performance in other commercial media.

Phage r2 (provided by E. R. Vedamuthu) which caused virulent lysis of a S. thermophilus isolate from frozen commercial culture CR12 (L. bulgaricus CR12 was also isolated from CR12), was used as the primary phage-host system to study phage inhibition.

Several formulation variations of Italian PHASE 4-MH along with commercial media were studied for their abilities to inhibit proliferation of phage r2 in rod-coccus cultures of S. thermophilus CR12 and L. bulgaricus CR12.

Commercial cheesemaking trials with Italian PHASE 4-MH and -NPC media (commercially blended at Galloway West Co.) were done in Washington and Wisconsin at the following cheese plants: Olympia Cheese Co., Olympia, WA; Baker Cheese, St. Cloud, WI; Lone Elm Cheese, Van Dyne, WI; and Tolibia Cheese, Fond du Lac, WI.

$Mg_3(PO_4)_2$-buffered Plating Media

PMP Medium

Trimagnesium phosphate was incorporated into a modified version of M17 agar (81) without disodium glycerophosphate and used as a total count medium for enumeration of lactic streptococci. This medium was designated PMP and was prepared as two separate components. One component consisted of polypeptone (BBL, Cockeysville, MD), 20.0 g; Phytone peptone (BBL), 5.0 g; yeast extract (Ardamine YEP, Yeast Products, Inc., Clifton, NY), 5.0 g; lactose (Sigma Chemical Co., St. Louis, MO), 5.0 g; dipotassium phosphate trihydrate (Baker, reagent grade), 2.5 g; 0.25% (w/v) bromcresol purple (Sigma Chemical Co.), 10.0 ml; Davis agar (Davis Gelatine Ltd., Christchurch, New Zealand), 10.0 g and 900 ml of distilled water. The other component consisted of trimagnesium phosphate (Stauffer Chemical Co., Westport, CT), 2.5 g and 100 ml of distilled water. The two components were autoclaved for 15 min at 121 C. After cooling to approximately 55 C the $Mg_3(PO_4)_2$ suspension was added to the other component. The complete medium (pH 6.9-7.1) was further cooled to approximately 45 C and then asceptically poured into petri dishes. Occasional swirling of the flask during pouring was necessary to keep the insoluble $Mg_3(PO_4)_2$ in suspension. Poured plates were allowed to air dry (48 h) at room temperature before using for total cell counts. Incubation was aerobically or anaerobically at 30 C for 24-48 h.

FSDA-II medium

Trimagnesium phosphate was also substituted for disodium glycerophosphate in a plating medium for differentiation of 'fast' and 'slow' acid-producing colonies of lactic streptococci developed by Limsowtin and Terzaghi (42) and later improved by Huggins and Sandine (30). This medium, entitled FSDA-II (named after Fast Slow Differential Agar developed by Huggins and Sandine), was prepared in three separate components. Component A was made by adding 20.0 ml of a 0.25% (w/v) bromcresol purple solution and 10.0 g Davis agar to 400 ml of distilled water. Three drops of an anti-foaming agent, Pourite (Scientific Products) were also added to this component to reduce entrapped air bubbles in the medium when pouring plates. Incorporation of anti-foaming agent obviated the need to flame plates during pouring. Component B was prepared by dissolving 100 g NDM in 500 ml of distilled water. Component C was made by adding 5.0 g trimagnesium phosphate (Stauffer Chemical Co.) to 100 ml of distilled water. Component C was generally prepared in multiple units and stored at room temperature. Components A and C were autoclaved for 15 min at 121 C. Component B was autoclaved for only 10 min at 121 C. The three components were tempered to approximately 55 C in a water bath at which time components B and C were aseptically added to A. The complete medium was then mixed on a magnetic stirrer and further cooled to 45 C in a water bath. Plates were poured and allowed to air dry for 48 h at room temperature. As with PMP, occasional swirling of the flask

was necessary to maintain $Mg_3(PO_4)_2$ in suspension while pouring plates. Streaked or spread plates of FSDA-II were incubated at 30 C for 48 h under anaerobic conditions.

A comparison between FSDA-II and FSDA was made by spread plating S. lactis C2 (lac^+, prt^+) and the three plasmid-characterized slow variants of S. lactis C2, LMO210 (lac^+, prt^-), LMO220 (lac^-, prt^-) and LMO231 (lac^-, prt^+) (provided by L. L. McKay) on the two media. The C2 parent and the three variants were grown in litmus milk (11% w/w) at 21 C for 24 h. The following mixtures of freshly grown cultures were made: 0.1 ml (lac^+, prt^+) + 0.25 ml (lac^+, prt^-) + 0.5 ml (lac^-, prt^+) + 0.5 ml (lac^-, prt^-); 0.25 ml (lac^+, prt^+) + 0.5 ml (lac^+, prt^-); 0.25 ml (lac^+, prt^-) + 0.5 ml (lac^-, prt^+). Each of the three mixtures was serially diluted and spread-plated on FSDA and FSDA-II.

Streptococcus thermophilus strains 404, 410, 440, 19987, L12, S122 and C3; Lactobacillus bulgaricus strains 404 and 448; and Lactobacillus helveticus strains L112, 15807 and 450 were all streaked from freshly coagulated litmus milk cultures onto FSDA and FSDA-II. The plates were incubated at 37 C for 30 h under anerobic conditions. Qualitative comparisons of growth by these thermophilic strains were made on FSDA and FSDA-II.

CHAPTER IV

RESULTS

EXTERNAL NEUTRALIZATION WITH SODIUM CARBONATE

After 16 h unneutralized Actilac had a final pH of 4.55 with a total cell count of 2.2×10^8 cfu/ml. Externally neutralizing at 12 h with Na_2CO_3 to pH 7.0 allowed the cells to resume dividing which resulted in a cell count of nearly double that of unneutralized control (Table 5). Continuous external neutralization throughout the entire fermentation enabled yet another doubling of cell number between 10 and 14 hours.

Table 5. External neutralization of the commercial bulk starter medium Actilac with Na_2CO_3.

	cfu/ml	
Fermentation hour	Modified external[a]	Continuous external[b]
0	8.3×10^6	-
2	1.6×10^7	-
4	5.6×10^7	3.0×10^7
6	1.3×10^8	8.4×10^7
8	2.2×10^8	2.5×10^8
10	2.0×10^8	4.0×10^8
12	2.2×10^8	5.6×10^8
14	2.7×10^8	7.3×10^8
16	4.0×10^8	7.2×10^8

[a]After 12 h, pH had dropped below 5.0; 5N Na_2CO_3 was added to raise the pH to 7.0 which then resumed dropping to 5.4 by 16 h.

[b]Continuously neutralized with 5N Na_2CO_3 to maintain pH 6.0 ± .05 throughout entire fermentation period.

INTERNAL NEUTRALIZATION WITH CONTROLLED RELEASE ALKALI

Smaller granules with only 50% of coated Na_2CO_3 present in CRA-13 resulted in early release of alkali in CRA-7.5 with insufficient neutralizing capacity to maintain the pH above 5.0 (final pH 4.95). With the larger granules in CRA-13 and twice the neutralizing power, the release was more gradual and pH remained above 5.0 during the entire fermentation period (final pH 5.05) and throughout the 10-day holdover period. CRA-0 contained no controlled release ingredient and the pH dropped to 4.7 by 16 h and to 4.5 by the end of the holdover period. The pH in A-10.4 rapidly rose to 10.1 with addition of uncoated Na_2CO_3 and no further testing was done with this medium.

Cell counts for both CRA-7.5 and CRA-13 remained relatively stable for the entire holdover period, while cell numbers for the control without pH control (CRA-0) rapidly decreased after only two days holdover (Figure 3).

Figure 4 illustrates the immediate loss of activity when no pH control was employed (CRA-0). It also shows the importance of maintaining the pH above 5.0 (CRA-13) compared to CRA-7.5 where the pH of fermenting medium dropped below 5.0 and resulted in a loss of activity after four days of holdover.

Figure 3. Effect of controlled release alkali (Na_2CO_3) on cell numbers during holdover.

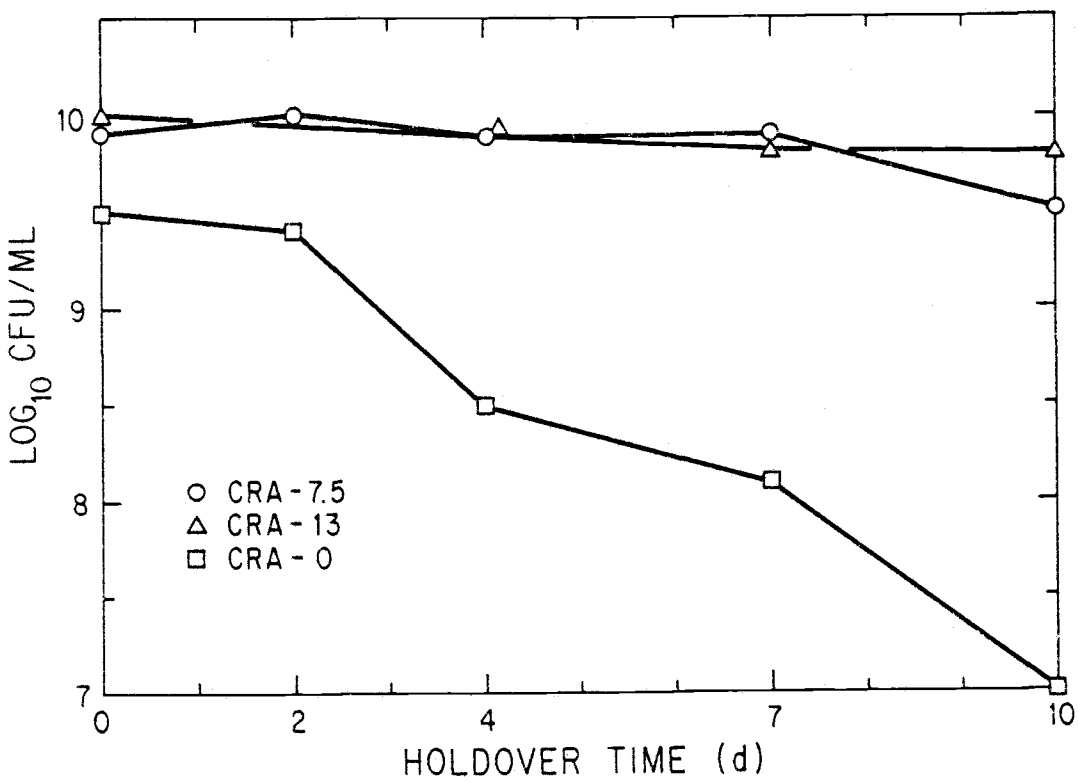


Figure 3.

Figure 4. Effect of controlled release alkali (Na_2CO_3) on activity during holdover.

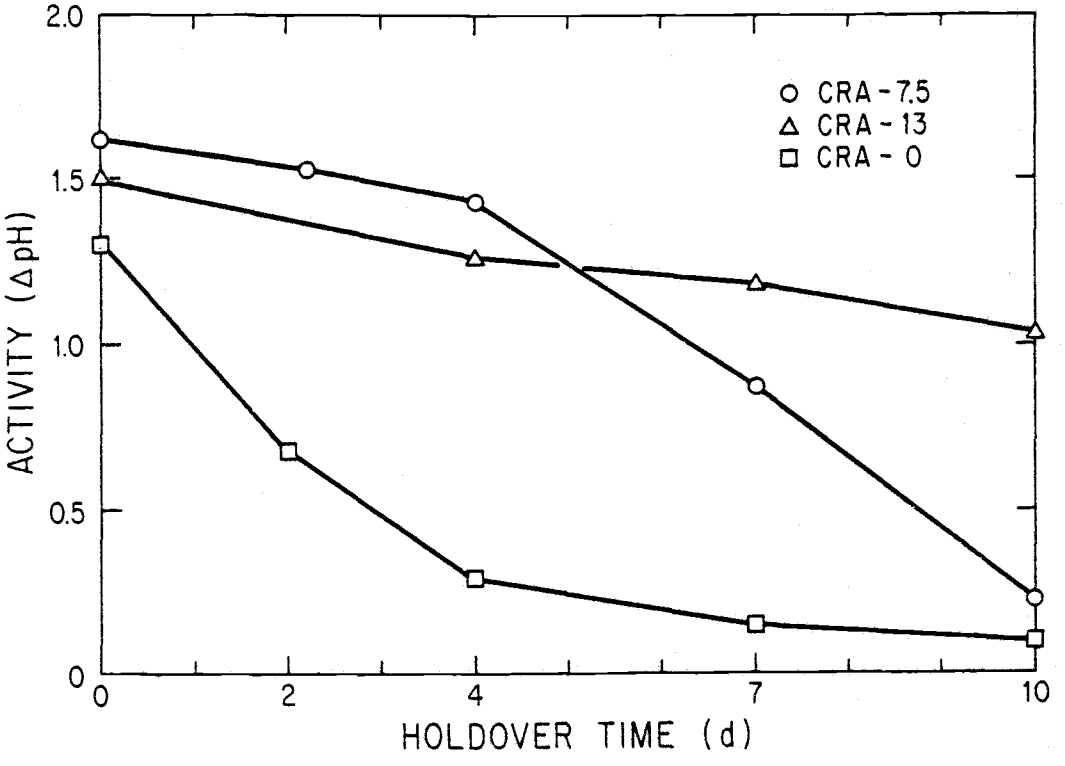

2.0
1.5
1.0
0.5
0
ACTIVITY (ΔpH)
0
2
4
7
10
HOLDOVER TIME (d)
CRA-7.5
CRA-13
CRA-0

Figure 4.

INTERNAL NEUTRALIZATION WITH INSOLUBLE BUFFERS

Bulk Starter Media for Mesophilic Starters

Trimagnesium phosphate and calcium carbonate

From initial screenings of alkaline earth phosphates and carbonates, two compounds, trimagnesium phosphate and calcium carbonate, were selected for further study.

These insoluble buffers did not raise the pH beyond 7.5 when combined with such nutrients as whey, NDM and yeast extract. In addition, both $Mg_3(PO_4)_2$ and $CaCO_3$ were stable to pasteurization temperatures (80-95 C), generally only raising the pH by 0.1-0.2 pH units. Trimagnesium phosphate was stable in the presence of citrate and/or soluble phosphates; however, the pH increased beyond acceptable limits during pasteurization with $CaCO_3$ in the presence of citric acid or ammonium phosphates.

The tremendous buffering capacity of $Mg_3(PO_4)_2$ is illustrated by Figure 5 in comparison to nonfat milk and phosphated whey-yeast extract medium. Instead of a linear drop in pH with increasing additions of lactic acid, the pH was maintained between 6.0 and 5.5 before it sharply dropped below pH 5.0 after 19 ml of added 10% lactic acid.

The activity of S. cremoris 134 is greater when grown in whey-based media buffered with either $Mg_3(PO_4)_2$ or $CaCO_3$ than in nonfat

Figure 5. Buffering capacities of 11% (w/w) NDM (circle), 1% (w/v) $(NH_4)_2HPO_4$ + 3.5% (w/v) whey + 0.5% (w/v) yeast extract (triangle) and 2% (w/v) $Mg_3(PO_4)_2$ + 3.5% (w/v) whey + 0.5% (w/v) yeast extract (square).

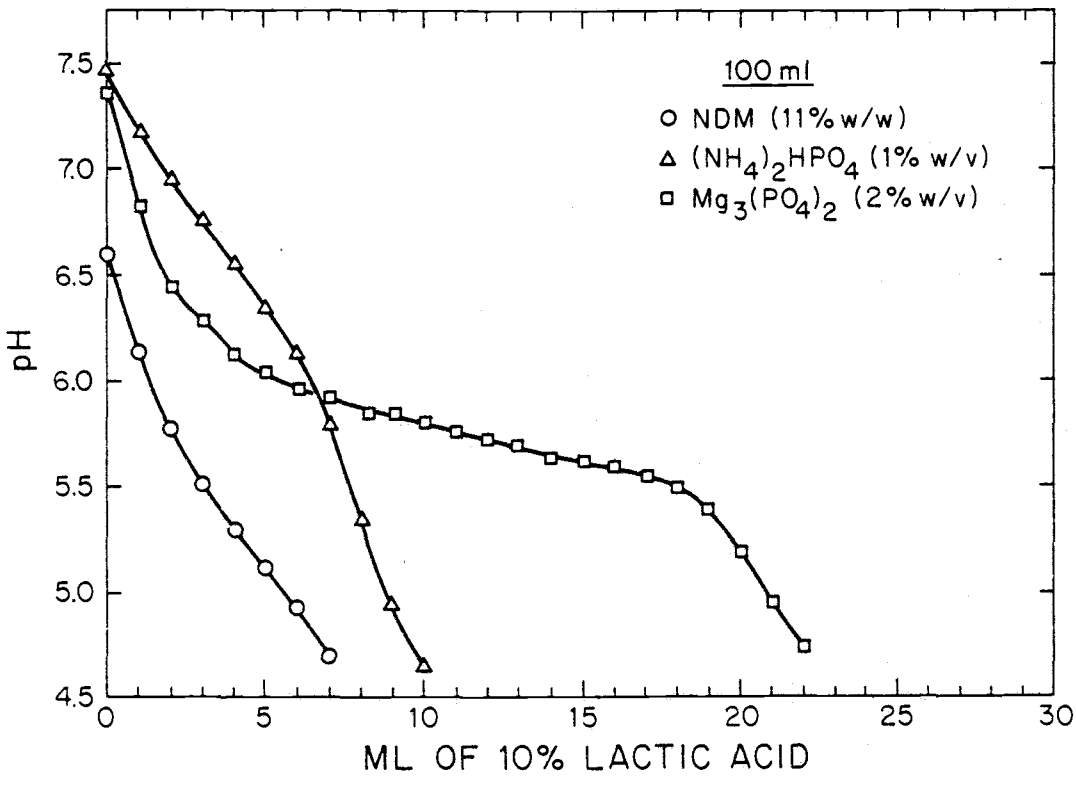

100 ml
NDM (11% w/w)
$(NH_4)_2HPO_4$ (1% w/v)
$Mg_3(PO_4)_2$ (2% w/v)
pH
7.5
7.0
6.5
6.0
5.5
5.0
4.5
0
5
10
15
20
25
30
ML OF 10% LACTIC ACID

Figure 5.

milk (Table 6). In addition, holdover activity is maintained for a longer period of time, especially with the $CaCO_3$ medium.

Neither the $Mg_3(PO_4)_2$ nor $CaCO_3$-buffered media provided any phage-inhibitory properties. When infected with approximately 10^5 pfu/ml of h2 phage, the $Mg_3(PO_4)_2$-buffered medium allowed a three log increase (1.3×10^8 pfu/ml recovered) in phage titer when grown in the presence of its homologous host S. cremoris H2. The $CaCO_3$ medium also was not able to prevent phage replication. The phage titer of hp with its homologous host S. cremoris HP increased from 10^5 pfu/ml to 1.3×10^8 pfu/ml. Nonfat milk controls for both h2/H2 and hp/HP increased from the 10^5 pfu/ml infection level to 3.1 and 4.2×10^8 pfu/ml, respectively.

Effect of $Mg_3(PO_4)_2$ concentration

Essentially no difference was seen in final pH, activity or total cell counts with the three concentrations of $Mg_3(PO_4)_2$. The pH of the medium was maintained above 5.2 at all three levels of $Mg_3(PO_4)_2$ during the 16 h fermentation and through the 48 h holdover period. The pH of the NDM control was 4.5 after 16 h and dropped no further after an additional 48 h. Activities were considerably better than the NDM control (0.5) for $Mg_3(PO_4)_2$-buffered media (1.2) after 16 h of incubation, and a wider spread in activity was seen after the 48 h holdover (0.1 compared to 1.0). Total cell numbers were two times higher after 16 h (1.5 compared to $2.8 - 3.4 \times 10^9$ cfu/ml) for the $Mg_3(PO_4)_2$-buffered starters.

Table 6. Extended holdover activity of S. cremoris 134 grown in media internally neutralized with the insoluble buffers $Mg_3(PO_4)_2$ and $CaCO_3$.

	$Mg_3(PO_4)_2$[a]		$CaCO_3$[b]		NDM[c]	
Holdover (days)[d]	Activity (ΔpH)	Log_{10} cell no.	Activity (ΔpH)	Log_{10} cell no.	Activity (ΔpH)	Log_{10} cell no.
0	1.2	9.0	0.9	8.9	0.7	9.2
1	1.1	9.0	0.8	-	0.5	9.0
2	1.0	9.0	0.8	9.0	0.3	9.1
3	-	-	0.8	-	0.2	-
4	0.3	8.9	0.8	9.0	0.1	8.4

[a] 4.3% (w/v) $Mg_3(PO_4)_2$ in whey-based medium (final fermentation pH of 5.5)

[b] 2.5% (w/v) $CaCO_3$ in whey-based medium (final fermentation pH of 5.05)

[c] 11% (w/w) reconstituted NDM (final fermentation pH of 4.4)

[d] Held at 5 C

The cell count for NDM control dropped to 3.6 x 10^8 cfu/ml after 48 h holdover while $Mg_3(PO_4)_2$-buffered starters retained 100% of their 16 h cell numbers.

Interaction between $Mg_3(PO_4)_2$ and $(NH_4)_2$ HPO_4

Addition of either dibasic sodium or ammonium phosphate to the basal medium without $Mg_3(PO_4)_2$ (WY) increased the pH from 6.1 to 7.6 and 7.5, respectively (Table 7). In the presence of $Mg_3(PO_4)_2$ (WYM), Na_2HPO_4 similarly raised the pH of the medium with increasing additions. However, $(NH_4)_2HPO_4$ caused the pH of the WYM medium to decrease a total of one entire pH unit after the addition of 1.0% $(NH_4)_2HPO_4$. This interaction appeared to occur only between $Mg_3(PO_4)_2$ and $(NH_4)_2HPO_4$ and not the other ingredients as illustrated in Table 7 when $(NH_4)_2HPO_4$ was added to a suspension of $Mg_3(PO_4)_2$ without whey or yeast extract. A similar type of interaction occurred between ammonium citrates and $Mg_3(PO_4)_2$ but not with sodium citrate.

Figure 6 shows the effect of substituting $(NH_4)_2HPO_4$ for some $Mg_3(PO_4)_2$ on buffering capacity. Only 21 ml of 10% lactic acid were required to drop the pH of a medium containing 2.0% (w/v) $Mg_3(PO_4)_2$ to pH 5.0 while with 3.0% (w/v) $Mg_3(PO_4)_2$, 30 ml were needed. When one third of the 3.0% $Mg_3(PO_4)_2$ was substituted with $(NH_4)_2HPO_4$, the resulting buffering capacity was nearly equal to the 3.0% $Mg_3(PO_4)_2$-buffered medium. The $(NH_4)_2HPO_4$-substituted

Table 7. Effect of dibasic phosphate addition on pH of a $Mg_3(PO_4)_2$-buffered medium.

% (w/v) Addition of a Dibasic Phosphate	pH				
	Na_2HPO_4		$(NH_4)_2HPO_4$		
	WY[a]	WYM[b]	WY[a]	WYM[b]	M[c]
0	6.1	7.2	6.1	7.2	9.6
0.25	7.1	7.5	7.1	6.8	6.9
0.50	7.4	7.6	7.4	6.5	6.5
0.75	7.5	7.7	7.5	6.3	6.3
1.00	7.6	7.8	7.5	6.2	6.2

[a]Whey, 3.5% (w/v); yeast extract, 0.5% (w/v)

[b]Whey, 3.5% (w/v); yeast extract, 0.5% (w/v); $Mg_3(PO_4)_2$, 2.0% (w/v)

[c]$Mg_3(PO_4)_2$, 2.0% (w/v)

Figure 6. Buffering capacity of 3.5% (w/v) whey + 0.5% (w/v) yeast extract with 2% $Mg_3(PO_4)_2$ (circle), 2% $Mg_3(PO_4)_2$ + 1% $(NH_4)_2HPO_4$ (triangle) and 3% $Mg_3(PO_4)_2$ (square).

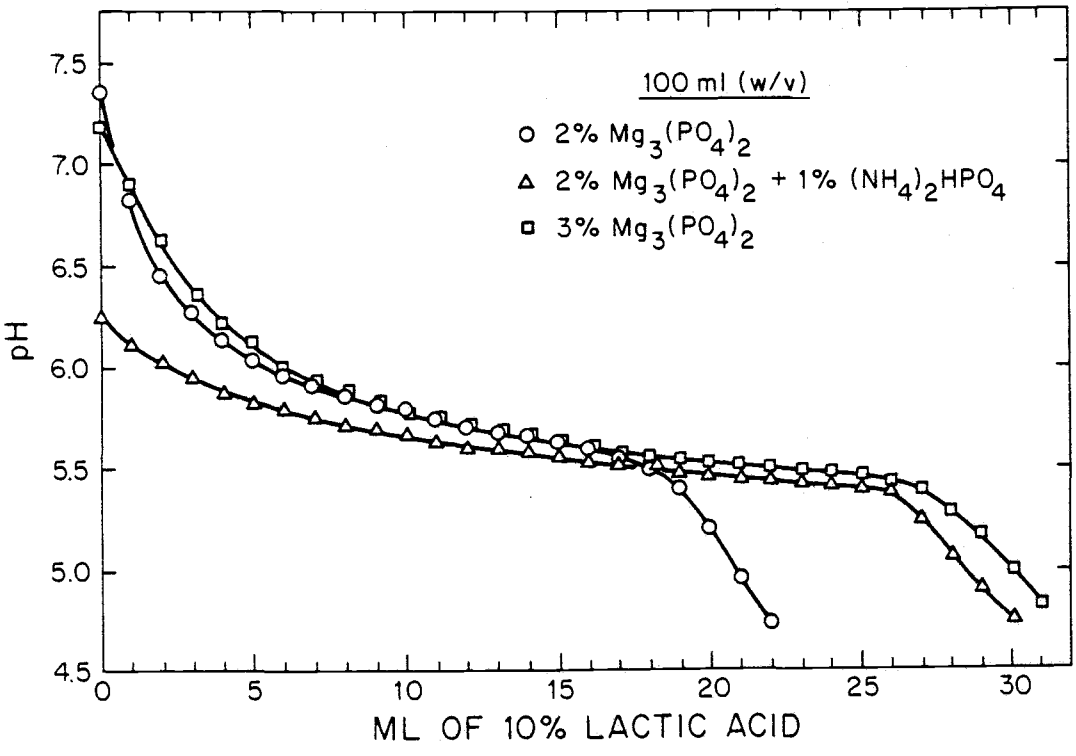

100 ml (w/v)
○ 2% $Mg_3(PO_4)_2$
△ 2% $Mg_3(PO_4)_2$ + 1% $(NH_4)_2HPO_4$
□ 3% $Mg_3(PO_4)_2$
pH
7.5
7.0
6.5
6.0
5.5
5.0
4.5
0
5
10
15
20
25
30
ML OF 10% LACTIC ACID

Figure 6.

medium not only possessed the full buffering capacity of 3.0% $Mg_3(PO_4)_2$-buffered medium but it also had a more physiologically desirable starting pH of 6.3 as compared to 7.2 with the 3.0% $Mg_3(PO_4)_2$ medium.

Addition of citrate for phage inhibition

The formulation containing trimagnesium phosphate, 2.0% (w/v); diammonium phosphate, 1.0% (w/v) and trisodium citrate dihydrate, 1.0% (w/v) when challenged with 5.7×10^4 pfu/ml phage hp, prevented any increase in phage titer (1.0×10^3 pfu/ml recovered) after a 16 h fermentation with S. cremoris HP. However, when challenged with the low calcium-requiring phage T189, the 2M/1P/1C medium allowed nearly a four-log increase in phage titer (Figure 7). When the trimagnesium phosphate concentration was reduced to 1.75% (w/v) coupled with increased amounts of phosphate and citrate, the phage inhibition improved, but it was not until the $Mg_3(PO_4)_2$ was dropped to 1.5% (w/v) with increased phosphate (1.5% w/v) and 1.5% (w/v) citrate that complete inhibition of T189 phage was possible. Addition of disodium tartrate dihydrate was also found to have an additive effect in aiding phage inhibition with phosphate and citrate. Further challenge of the 1.5M/1.5P/1.5C (PHASE 4-M) formulation with other phage/host combinations showed the medium to be inhibitory for all those phages (Table 8) also. Recently the S. cremoris C13 phage, mC13, (provided by D. L. Wallace of

Figure 7. Effect of different formulations on the inhibition of phage T189.

Phage inhibition = $\log_{10} \frac{\text{phage added}}{\text{phage recovered}}$; ≥ 0 indicates complete inhibition.

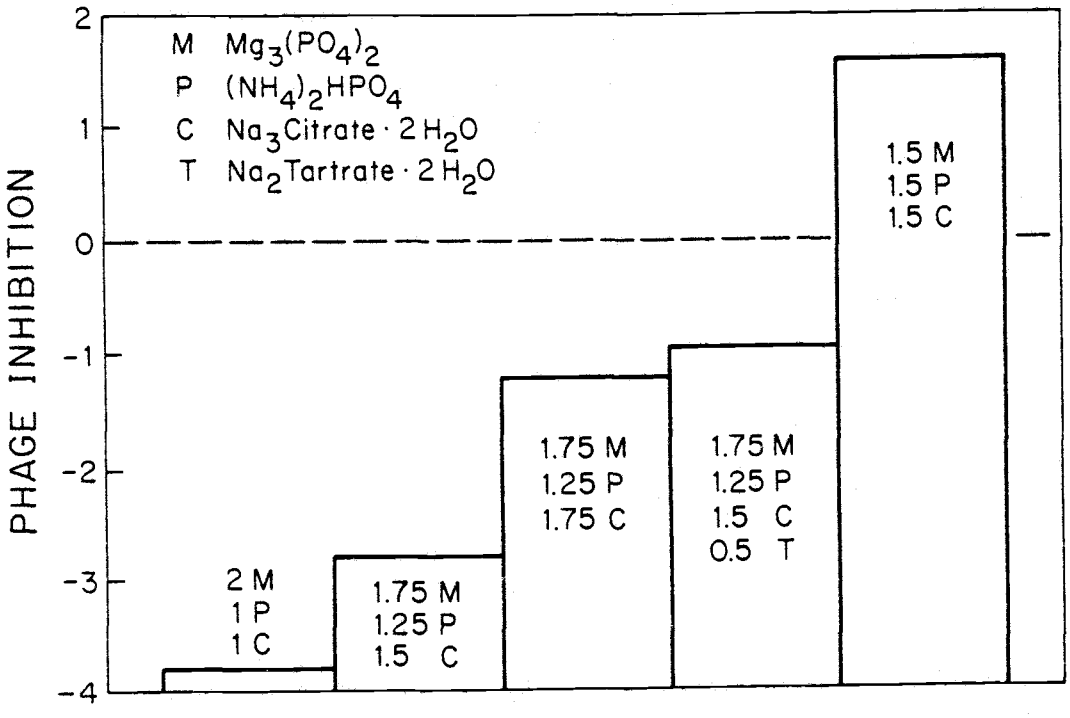

PHAGE INHIBITION
2
1
0
-1
-2
-3
-4
M $Mg_3(PO_4)_2$
P $(NH_4)_2HPO_4$
C $Na_3Citrate \cdot 2H_2O$
T $Na_2Tartrate \cdot 2H_2O$
2 M
1 P
1 C
1.75 M
1.25 P
1.5 C
1.75 M
1.25 P
1.75 C
1.75 M
1.25 P
1.5 C
0.5 T
1.5 M
1.5 P
1.5 C

Figure 7.

Table 8. Phage inhibition of medium containing 1.5% (w/v) $Mg_3(PO_4)_2$, 1.5% (w/v) $(NH_4)_2HPO_4$ and 1.5% (w/v) trisodium citrate dihydrate (PHASE 4-M).

Phage/host	Phage added/ml	Phage recovered/ml	Phage inhibition[a]
T189/205	8.7×10^4	2.3×10^3	1.6
ml_8/ML_8	3.1×10^4	4.3×10^3	0.9
hp/HP	3.1×10^4	1.0×10^3	1.6
h2/H2	3.7×10^4	1.0×10^3	1.6
c2/C2	5.9×10^4	1.0×10^3	1.7

[a] $\log_{10} \frac{\text{phage added/ml}}{\text{phage recovered/ml}}$; ≥ 0 indicates complete inhibition

Marschall Products), also was shown to be inhibited while the host grew well.

In situ generation of $Mg_3(PO_4)_2$ and $MgNH_4PO_4$

We have already shown that a considerable amount of the $Mg_3(PO_4)_2$ can be replaced with $(NH_4)_2HPO_4$. However, further reductions or total elimination of the $Mg_3(PO_4)_2$ ingredient were desired to increase economic feasibility for commercial production.

Magnesium hydroxide (127.5 mMoles) was reacted with citric acid (85 mMoles) and trisodium phosphate (85 mMoles) to theoretically generate the same amount of $Mg_3(PO_4)_2$ (42.5 mMoles) present in PHASE 4-M. An in vivo trial using such a medium (containing in addition to the above, whey, 3.5% w/v; yeast extract, 0.5% w/v and $(NH_4)_2HPO_4$, 1.5% w/v) was found inhibitory to S. cremoris 205.

The citric acid content was reduced to 51 mMoles (equivalent to citrate in PHASE 4-M). Other compounds were reduced in accordance with the limiting citrate level (76.5 mMoles $Mg(OH)_2$ and 51 mMoles Na_3PO_4). This combination generated 25.5 mMoles $Mg_3(PO_4)_2$. Addition of the remaining 17 mMoles as preformed $Mg_3(PO_4)_2 \cdot 5H_2O$ (0.6% w/v) produced a medium with slightly greater buffering capacity than PHASE 4-M and it supported growth of S. cremoris 205 comparable to PHASE 4-M.

Titrations with lactic acid were then performed to determine the minimum amount of $Mg_3(PO_4)_2 \cdot 5H_2O$ that had to be added to this formulation (PHASE 4-RM) to match the buffering capacity of PHASE

4-M. It was found that only 0.5% (w/v) $Mg_3(PO_4)_2 \cdot 5H_2O$ (14.5 mMoles) was required to supplement the buffering instead of the calculated 0.6%. An additional fermentation with S. cremoris 205 in PHASE 4-RM containing 0.5% (w/v) added $Mg_3(PO_4)_2 \cdot 5H_2O$ showed the same activity as with PHASE 4-M after a 16 h-21 C incubation period. Phage inhibition was also retained as evidenced by a two log reduction (following 10^7 pfu/ml infection) of recoverable phage (T189) after a 16 h-21 C fermentation with the homologous host S. cremoris 205.

The total elimination of the preformed $Mg_3(PO_4)_2 \cdot 5H_2O$ was accomplished by in situ generation of the required $Mg_3(PO_4)_2$ by either of the two following methods: (1) combination of 120 mMoles $Mg(OH)_2$ (0.7% w/v) with 80 mMoles of H_3PO_4 (0.92% of 85% w/w H_3PO_4) to form the required 40 mMoles of $Mg_3(PO_4)_2$ or (2) 120 mMoles of $Mg(OH)_2$ with 80 mMoles of $NH_4H_2PO_4$ to form 40 mMoles of $Mg_3(PO_4)_2$. Method 1 (PHASE 4-P) was further combined with $(NH_4)_2HPO_4$, 1.5% (w/v); trisodium citrate dihydrate, 1.5% (w/v); whey, 3.5% (w/v) and yeast extract, 0.5% (w/v) to make it compositionally the same as PHASE 4-M. Method 2 (PHASE 4-MH) was combined with whey, 3.5% (w/v); yeast extract, 0.5% (w/v); trisodium citrate dihydrate, 1.5% (w/v); $NH_4H_2PO_4$, 1.8% (w/v) and $(NH_4)_2HPO_4$, 0.5% (w/v). The ratio of monobasic : dibasic ammonium phosphates was adjusted so as to get a pH of 6.7-7.0 for the combined medium.

Both PHASE 4-P and -MH were compared to PHASE 4-M in their abilities to support growth of lactic cultures and to prevent phage

replication when infected with the T189 phage (Table 9). Both *in situ* formulations were comparable to PHASE 4-M for activity produced with the three commercial cultures. PHASE 4-P also was able to prevent phage replication at both infection levels; however, at 10^7 pfu/ml PHASE 4-MH allowed a slight increase in titer of T189 phage which was not seen with either of the other two formulations.

Uniform commercial blending

Figure 8 illustrates the nonuniformity of sodium citrate content in a batch of dry blended PHASE 4-MH after 10 minutes mixing time. Increased mixing times of 20 and 30 minutes did not improve the distribution of citrate in the PHASE 4-MH blend (unpublished data).

In an attempt to overcome the abrasive nature of magnesium hydroxide, it was determined that by reacting with citric acid to form magnesium citrate, which was much more soluble, the wear on the spray drying equipment could be reduced. This intermediate, along with the remaining unreacted magnesium hydroxide, would then be able to combine *in situ* with soluble phosphates to form the desired magnesium phosphates in the bulk starter tank.

The commercially dry blended PHASE 4-MC proved to be vastly improved in uniformity as evidenced by the more consistent citric acid content (Figure 9). The greater solubility of the magnesium citrate intermediate also enabled increased production with fewer valve and nozzle replacements.

Table 9. Activity and phage inhibition of different PHASE 4 formulations using three commercial cultures and the T189/205 phage/host system.

	Activity (ΔpH)[a]			T189 phage/ml recovered[b]	
	M13[c,d]	M21[d]	H4[d]	10^5 Ø /ml added	10^7 Ø /ml added
PHASE 4-M	1.3	1.3	1.3	1.0×10^3	1.5×10^5
PHASE 4-P	1.3	1.2	1.2	1.0×10^3	4.7×10^4
PHASE 4-MH	1.3	1.3	1.2	1.3×10^3	5.3×10^7

[a]CTP activity test (0.5% inoculum)

[b]Recovered after 16 h-24 C incubation

[c]All three media had approx 7×10^9 cfu/ml total cell counts at the end of the 16 h-24 C incubation period

[d]Commercial frozen bulk set cultures

Figure 8. Nonuniformity of sodium citrate content in a dry blended batch (40 bags) of PHASE 4-MH.

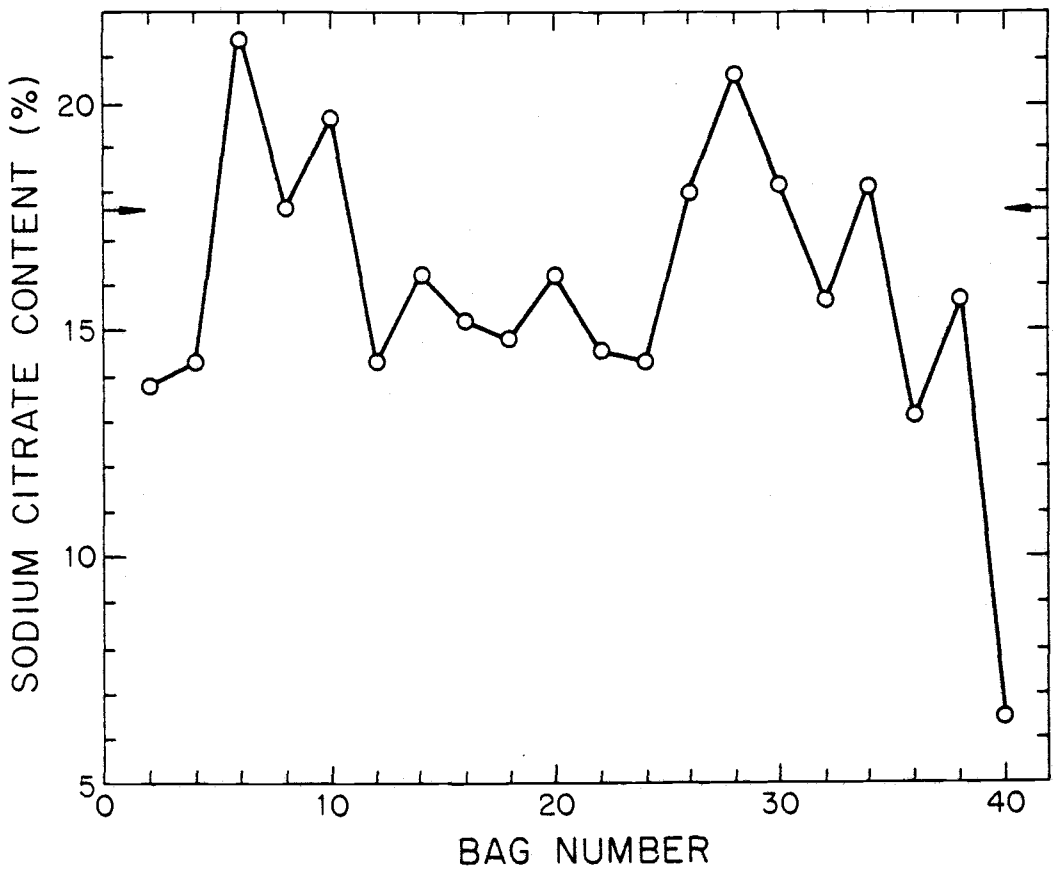


Figure 8.

Figure 9. Citric acid content of ten successive batches (1st and last bag from each batch) of PHASE 4-MC.

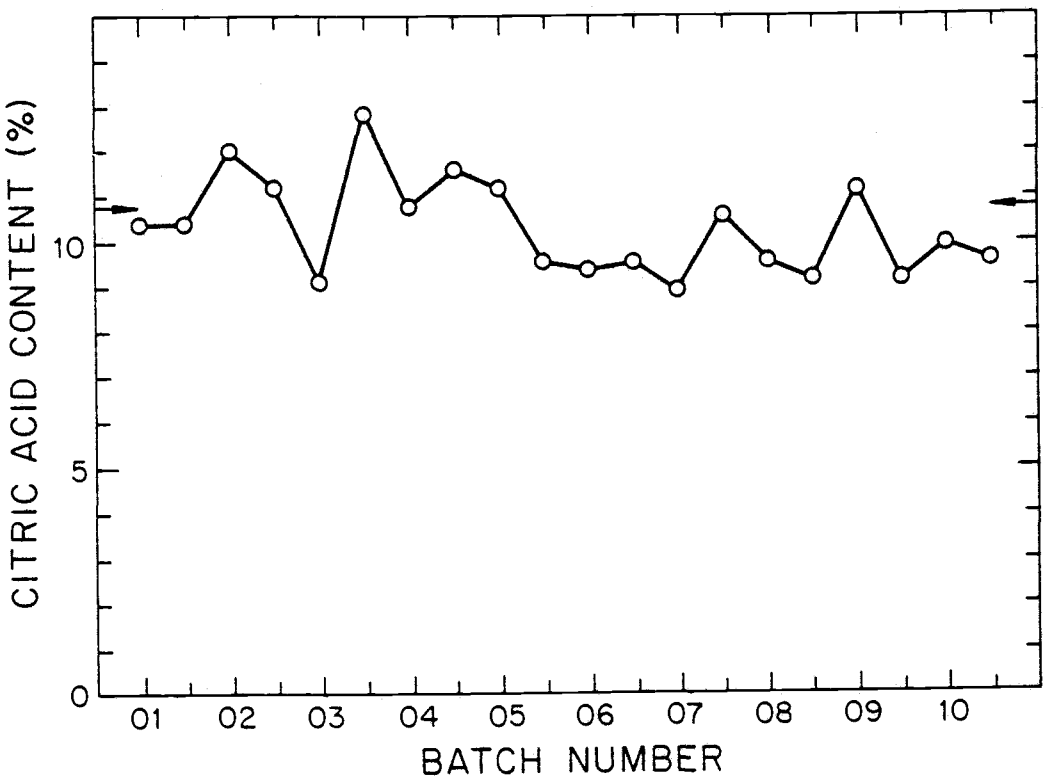

CITRIC ACID CONTENT (%)
0
5
10
01
02
03
04
05
06
07
08
09
10
BATCH NUMBER

Figure 9.

Using commercial culture H86, activity was identical whether grown in PHASE 4-MH or PHASE 4-MC (CTP activity of 1.3). As with previous PHASE 4 formulations, PHASE 4-MC retained the ability to suppress replication of the T189 phage (with host S. cremoris 205) when infected with 2.3×10^5 pfu/ml (final titer of 1.5×10^5 pfu/ml).

Milk-based PHASE 4 and adaptation to HTST pasteurization

Nonfat dry milk contains approximately twice the calcium of dried sweet whey (88). Therefore, direct replacement of whey solids in PHASE 4-MH with NDM resulted in a loss of phage inhibition against T189 phage (unpublished data). The NDM + whey was reduced, maintaining the recommended 3:1 NDM to whey ratio, to a combined level of 1.5% (w/v); and enough lactose was added to give a total lactose content of 2.5% (w/v) (including the lactose in the NDM and whey). When infected with approximately 10^5 pfu/ml T189 phage with homologous host S. cremoris 205, PHASE 4-MBØ was capable of preventing phage replication during a 16 h fermentation at 25 C (1.2×10^5 pfu/ml recovered). Table 10 shows that no activity was lost from the PHASE 4-MBØ reformulation.

Many large volume plants pasteurize their bulk starter media through a HTST pasteurizer at temperatures in excess of 200 F instead of the more common batch pasteurization at 185 F in the bulk tank. Experimental trials by Galloway West showed that PHASE 4-MH when run through a HTST at such temperatures left a heavy precipitate on the plates making pasteurization in this

Table 10. Growth of four commercial cultures in a reduced-whey PHASE 4 formulation containing NDM solids (PHASE 4-MBØ).

	Activity[a] (ΔpH)	
Culture	PHASE 4-MH	PHASE 4-MBØ
HH70[b]	1.2	1.3
H86[b]	1.5	1.5
H92[b]	1.0	0.9
VT6[b]	1.5	1.5

[a]CTP activity (1% inoc rate)

[b]Commercial frozen bulk set culture

manner and at this temperature undesirable (Galloway West personnel-personal communication). To make PHASE 4 more amenable to HTST pasteurization, a two-step procedure was developed that split the PHASE 4-MC formulation into two separate parts which were separately HTST-pasteurized and then recombined to the whole. Part A combined magnesium hydroxide with citric acid to form the partially soluble magnesium citrate/magnesium hydroxide suspension which could be successfully run through the HTST pasteurizer at temperatures exceeding 200 F. The remaining soluble ingredients were combined in part B and HTST pasteurized. Parts A and B were then combined which allowed the magnesium citrate/magnesium hydroxide to react with soluble phosphate and produce insoluble buffering complex in the bulk tank.

Only the whey-based A + B variation of PHASE 4-MC was experimentally evaluated by Galloway West. The milk-based formulation was developed in a further attempt to meet the needs of a milk-based PHASE 4, even though phage inhibition was decreased because of inclusion of NDM solids. Both formulations successfully supported the growth of H86 compared to PHASE 4-MH (CTP activity of 1.2 with 1% inoculum). Later field studies by Galloway West have shown that PHASE 4-MC is capable of passing through a HTST pasteurizer at temperatures $\leq$ 200 F with minimal 'burn on' the plates and such a procedure is being used by a few cheese plants using PHASE 4.

Improved PHASE 4 formulation using $MgSO_4$: PHASE 4-MS

PHASE 4-MS was found equivalent to PHASE 4-MC as a growth medium and in its ability to inhibit T189 phage replication (Table 11).

Table 12 compares the chemical composition of PHASE 4-MH, -MC and -MS. Most notable is the lower Mg^{+2} and total PO_4^{-3} content of PHASE 4-MS. Even with this reduction in buffering, the final fermentation pH remained above 5.0 after a 16 h fermentation with S. cremoris 205 (Table 11). A return to PHASE 4-MH nutrient concentration (whey + yeast extract) was achieved with the PHASE 4-MS formulation (using $MgSO_4 \cdot 7H_2O$) without changing the commercially-recommended reconstitution rate of 8.7% w/w (75 lb per 100 gal). Use of anhydrous $MgSO_4$ would require a reduction in total solids usage to 70 lb per 100 gal (8.0% w/w) by the cheesemaker. However, reconstitution of that 70 lb (with anhydrous $MgSO_4$) would provide the same functional ingredients as when adding 75 lb of solids containing heptahydrated $MgSO_4$.

Effect of initial pH

The initial pH (6.4-7.4) of $Mg_3(PO_4)_2$-buffered medium had very little effect on the final fermentation pH, activity or total cell number. Regardless of starting pH, final pH after 16 h was 5.5-5.7 (NDM control pH 4.5). Activity of NDM starter dropped from 0.5 at 16 h to a total depletion of activity after 48 h holdover.

Table 11. Comparison of PHASE 4-MC and PHASE 4-MS for supporting growth of S. cremoris 205 and ability to suppress phage proliferation.

Medium	Fermentation pH		Final (16 h) TA	Activity (ΔpH)[a]	Final (16 h) cfu/ml	pfu/ml[b]
	Initial	Final (16 h)				
PHASE 4-MC	6.8	5.4	2.62%	1.35	5.3×10^9	5.0×10^3
PHASE 4-MS	6.8	5.2	2.52%	1.3	7.6×10^9	2.8×10^3

[a]Controlled temperature profile activity (1% inoc rate)

[b]T189 phage recovered after 16 h-25 C fermentation with host S. cremoris 205 (initial infection of 10^5 pfu/ml)

Table 12. Comparison of the chemical composition of PHASE 4 formulations.

Medium component	PHASE 4-MH (7.8% w/w)	Commercial PHASE 4-MC (8.7% w/w)	PHASE 4-MS (8.7% w/w)
Whey	3.5% (w/v)	4.0% (w/v)	3.5% (w/v)
Yeast extract	0.5% (w/v)	0.57 (w/v)	0.5% (w/v)
Mg^{+2}	120 mM	147 mM	87 mM
Citrate	51 mM	62 mM	52 mM
PO_4^{-3}-combined[a]	80 mM	98 mM	58 mM
PO_4^{-3}-free[b]	114 mM	124 mM	127 mM
PO_4^{-3}-total	194 mM	222 mM	185 mM

[a] Combined with Mg^{+2} as $Mg_3(PO_4)_2$

[b] Free or soluble PO_4^{-3} not combined with Mg^{+2}

Activities for $Mg_3(PO_4)_2$-buffered starters were essentially the same (1.1-1.2) and considerably greater than the control at 16 h. Very little activity was lost during the 48 h holdover period (0.9-1.1). Total cell numbers were two to three times higher (ranging 2.7-3.8 x 10^9 cfu/ml) than NDM control (1.2 x 10^9 cfu/ml) after 16 h of incubation. The cell count dropped to an estimated 3.6 x 10^7 cfu/ml in NDM control, while no decrease in cell numbers was observed with any buffered starters after 48 h.

Effect of fermentation temperature

With both strains of S. cremoris 205 and 134, fermentation at 21 C produced starter with greater activity and higher cell numbers than at 24 and 27 C (Table 13). The pH of mature starter remained above 5.2 in all four fermentations through the holdover period, yet activity was better maintained in the 21 C fermentations for both strains.

Effect of agitation

A nonagitated vessel of PHASE 4 developed a pH gradient from top to bottom during fermentation with a lactic starter. PH dropped relatively quickly near the surface while localized neutralization of produced lactic acid occurred on the bottom of the vessel. Likewise, growth reached higher numbers near the bottom than the upper portion where lack of sufficient buffering allowed the pH to rapidly drop below 5.0 at which point growth rapidly slowed down.

Table 13. Effect of fermentation temperature on activity and cell numbers of S. cremoris.

Strain	Fermentation Temp (C)	Activity[a]		cfu (x 10^9/ml)	
		16 h	Holdover[b]	16 h	Holdover[b]
Sc 134	21	2.9	2.5	8.8	8.8
Sc 134	27	2.7	2.1	6.6	6.2
Sc 205	21	2.4	1.9	6.9	8.9
Sc 205	24	2.0	1.6	6.1	7.0

[a]Controlled temperature profile activity (0.5 + 1.0% inoc rate)

[b]Held for 24 h at 21-24 C

Mixing of the vessel at the end of the incubation period produced a starter with intermediate activity and cell numbers as a result of combining the top and bottom portions (unpublished data).

The maximum potential of activity and cell numbers was not reached in the vessel without agitation (Table 14). However, intermittant agitation during the last 4 h of the fermentation produced a starter that was comparable in activity and cell numbers to the continuously-agitated vessel.

Delayed agitation as late as for just the last two hours of a 18 h fermentation produced starter with comparable activity to those continuously agitated and continuously agitated after a 12 h delay (Table 15). Slight culture inhibition may have occurred in the continuously agitated vessel as evidenced by the slower decrease in pH compared to the last 6 h of the 12 h delayed vessel.

Effect of different 'Complex Nutrients' sources

Minor differences in supporting starter growth were found between the five whey sources when substituted into PHASE 4-MH (Table 16). The dried sweet Italian whey enabled the highest level of activity by the MFS culture and was selected as the whey source to be used in commercial production of PHASE 4 by Galloway West.

Table 17 illustrates the stimulatory effect of increasing concentrations of the yeast extract YEP on the growth of S. cremoris 205. At 0.1% (w/v), activity was poor and the cell number was only

Table 14. Effect of different agitation patterns on the growth of S. cremoris 134 in PHASE 4-M.

	Activity[b] (ΔpH)			cfu/ml (x 10^9)		
Agitation	16 h	1 day	2 day	16 h	1 day	2 day
NO AGITATION	1.0	1.0	0.8	3.9	5.2	4.4
INTERMITTANT[a]	1.3	---	1.1	5.2	---	5.6
CONTINUOUS	1.3	1.2	1.0	6.7	5.3	6.5

[a]12 h off followed by every other hour on

[b]Controlled temperature profile activity (0.5%)

Table 15. Effect of delayed agitation on the growth of S. cremoris C13 in PHASE 4-MC.

Agitation On After	Fermentation pH								Activity[c]
	0 h	12 h		14 h		16 h		18 h	
		B[a]	A[b]	B[a]	A[b]	B[a]	A[b]		
CONTINUOUS	6.8	---	6.4	---	6.2	---	5.6	5.4	1.5
12 h	6.8	5.1	5.7	---	5.4	---	5.3	5.2	1.6
16 h	6.8	---	---	---	---	4.8	5.3	5.3	1.55

[a] pH from upper third of vessel before agitation turned on

[b] pH after 15 minutes agitation

[c] Activity is measured as change in pH following 4 h-32 C incubation (25% inoc rate); sampled at 18 h

Table 16. Effect of whey source on the growth of a commercial frozen bulk set culture (MFS) in PHASE 4-MH.

Dried Sweet Whey	Source[a]	Activity[b] (ΔpH) 0.5%	1.0%	2.0%
A	Cheddar-OR	1.0	1.4	1.7
B	Cheddar-WI	1.1	1.45	1.7
C	Italian-NY	1.2	1.55	1.8
D	Cheddar-WI	1.15	1.5	1.7
E	Cheddar-WI	1.15	1.5	1.7

[a]From type of cheese manufactured and state in which produced

[b]Controlled temperature profile activity

Table 17. Effect of yeast extract concentration on the growth of S. cremoris 205 in PHASE 4-M.

% Yeast Extract (w/v)	Final Fermentation pH	Activity[a] (ΔpH) 0.5%[b]	1.0%[b]	2.0%[b]	cfu/ml ($x10^9$)
0.1	6.2	0.5	0.6	0.8	1.6
0.3	5.6	1.0	1.2	1.5	5.2
0.4	5.6	0.9	1.2	1.5	8.3
0.5	5.4	1.1	1.4	1.6	8.6
1.0	5.3	1.1	1.5	1.6	9.0

[a]Controlled temperature profile activity

[b]Inoculation rate

one-fifth of the cell count attained with 0.4-1.0% (w/v) YEP. The high final fermentation pH of 6.2 after 16 h also suggested incomplete development by the starter grown in the presence of 0.1% (w/v) yeast extract. The optimum concentration of YEP appeared to be 0.4-0.5% (w/v). Very little additional stimulation was seen when the concentration was doubled to 1.0% (w/v).

Many commercial yeast products are available. Most of the yeast products are designed as flavor enhancers and differ widely in their abilities to stimulate the growth of lactic starter bacteria. Table 18 compares six yeast products used as fermentation stimulants. The results show the extracts (YEP, AYE and 1003) are the most stimulatory; this stimulatory effect is probably related to their higher protein content. The superiority of AYS over the two primary yeast products (Z113 and Z33) suggests that brewers yeast may be more stimulatory than the primary grown Torula yeast. The three whole yeast products (AYS, Z113 and Z33) were also noticeably deficient in something needed to stimulate growth of the two frozen cultures H99 and MRD. The OSU multiple strain starter culture appeared the least affected by the varying quality of the six yeast products.

Long bulk culture set times (>18 h) with frozen commercial cultures are generally associated with a poor quality yeast extract in the medium. By incorporating a 'good' whey source into the medium, the necessary stimulation was provided in order that an

Table 18. Evaluation of six commercially available autolyzed yeast products for their abilities to stimulate growth of four different lactic culture systems in PHASE 4-MC.

Yeast Extract	Approx[a] Protein Content	Activity[b] (ΔpH)				
		Sc 205	OSU-MSS	H99	MRD	Overall Average
YEP	65%	3.2	3.2	2.7	2.9	3.0
AYE	65%	3.2	3.2	2.6	2.9	3.0
1003	65%	3.0	3.2	2.6	2.9	2.9
AYS	50%	2.9	3.0	2.1	2.4	2.6
Z113	50%	2.9	3.2	1.7	1.8	2.4
Z33	50%	2.4	2.8	1.6	1.8	2.2

[a]Manufacturer's specifications

[b]Controlled temperature profile activity (1% + 2% inoc rates)

Table 18. Continued

Yeast Extract	cfu/ml (X 10^9)				
	Sc 205	OSU-MSS	H99	MRD	Overall Average
YEP	7.1	7.2	1.7	0.55	4.1
AYE	6.3	5.6	2.1	0.47	3.6
1003	5.0	7.2	1.4	0.63	3.6
AYS	4.3	5.1	0.78	0.48	2.7
Z113	4.5	3.7	1.3	0.10	2.4
Z33	3.2	3.0	0.42	0.39	1.8

acceptable set time could be achieved. When a high quality yeast extract was coupled with a good whey source, the stimulatory effects were cumulative (unpublished data).

Effect of total solids concentration and 'Carryover Buffer'

Maximum culture activity was achieved between 7.0 and 9.0% (w/w) solids with the commercial culture H85 when grown in PHASE 4-M, and the cell number peaked and plateaued at 7.0% solids (Figure 10). The initial pH was inversely related to the percent solids concentration (unpublished data).

On a commercial scale, starter propagated in PHASE 4-MC at 7.5% (w/w) solids (65 lb/100 gallons) was as active as starter grown in PHASE 4 at 8.7% (w/w) solids (Table 19). At 7.0% (w/w) solids, slightly more starter was required for Cheddar cheese production and at only 5.8% (w/w) solids approximately 30% more starter was required for an equivalent performance in the cheese vat.

PHASE 4-M, the original PHASE 4 formulation, was formulated at 7.8% (w/w) solids. Subsequent PHASE 4 formulations were also designed for use at 7.8%. However, when PHASE 4 was commercially transferred to Galloway West Co., the decision was made on their part to recommend usage of PHASE 4 at 75 lb (made up to total volume of 100 gallons) or approximately 8.7% (w/w) solids. Early feedback from the field identified a problem of inconsistent and irregular performance in the cheese vat by several users of PHASE 4.

Figure 10. Effect of total solids concentration on the activity (open) and cell number (closed) for H85 (commercial frozen bulk set culture) in PHASE 4-M.

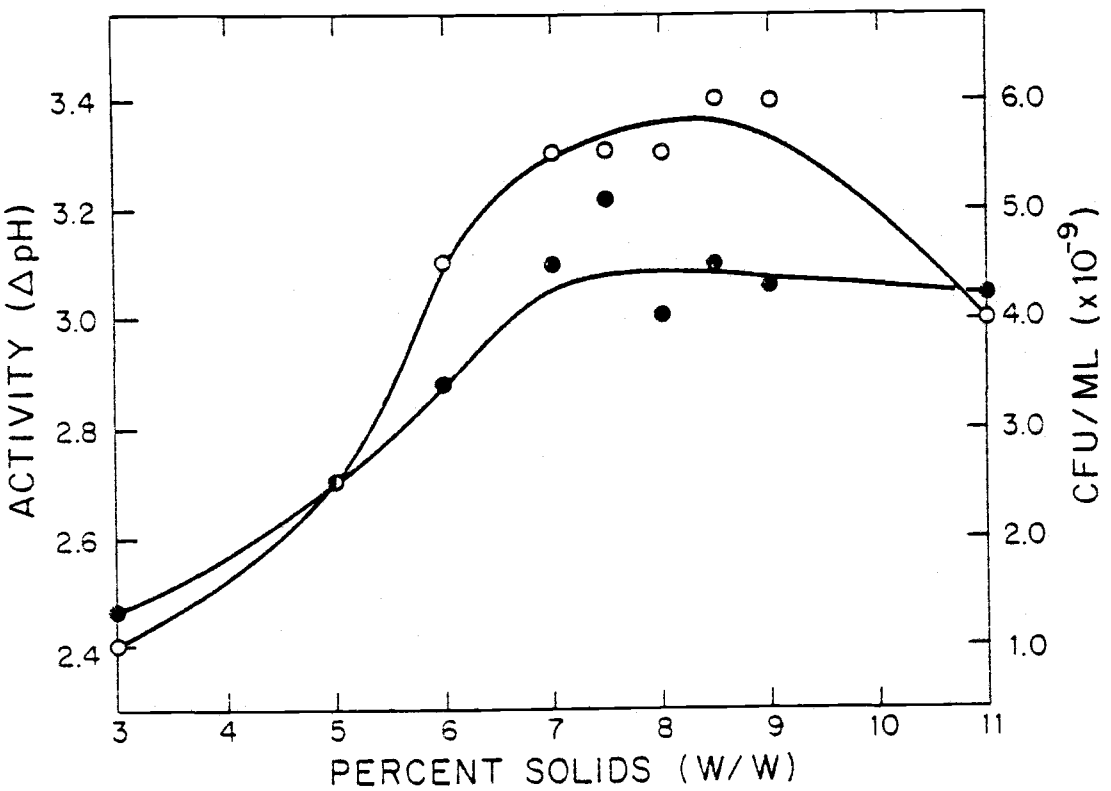

ACTIVITY (ΔpH)
3.4
3.2
3.0
2.8
2.6
2.4
CFU/ML (x10⁻⁹)
6.0
5.0
4.0
3.0
2.0
1.0
3
4
5
6
7
8
9
10
11
PERCENT SOLIDS (W/W)

Figure 10.

Table 19. Effect of PHASE 4 solids concentration on the development of MI3[a] in the bulk starter tank and in the amount used for Cheddar cheese production.

% Solids	lb/100 gal	lb/cheese vat (45,000 lb)	Bulk Starter TA	Bulk Starter pH	Activity (ΔpH)[b]
5.8	50	500	2.0	5.4	1.5
7.0	60	400-500	2.6	5.35	1.5
7.5	65	380	2.9	5.2	1.6
8.7	75	370-400	2.9	5.35	1.6

[a]Commercial frozen bulk set culture

[b]Controlled temperature profile activity (1%)

This problem increased in frequency and was commonly associated with a high pH in the finished cheese; often several tenths of pH unit higher at grading than when milled. As a result of the tremendous buffering capacity of PHASE 4, we speculated that if a significant portion of unused buffer were carried over into the cheese vat, the buffering capacity of the cheese milk and the subsequent milling pH of the cheese curd would be altered. PHASE 4 used at a higher than necessary solids level (8.7% w/w) would only compound this problem.

Table 20 shows the increased lactic acid that is required in a simulated system to lower the pH to 5.0 with high (8.7%) solids compared to low (5.8%) solids. This is especially evident at the higher inoculation levels.

A comparison between high and low solids, made on a commercial level at Galloway West, showed that an average of 11% less carryover buffer was transferred to the cheese vat when low solids were used to grow commercial starters (Table 21). Note also that the greatest reduction in carryover buffer resulted with the most active cultures which are generally the most widely used by the cheesemaker. This reduced carryover buffer most likely resulted because even though approximately 30% more starter was required with low solids, the total was less still than the 50% additional solids used with high solids.

Data were obtained from a 10-week period of Cheddar cheese production at Galloway West in which M13 was grown in both high

Table 20. Simulated fermentation in reconstituted NDM with high and low solids PHASE 4 that had been adjusted to pH 5.5 with lactic acid (LA).

Inoc[a] Rate	High Solids (8.7% w/w)		Low Solids (5.8% w/w)		% Additional LA Required with High Solids
	ml LA[b]	TA[c]	ml LA[b]	TA[c]	
0	4.8	.49	4.8	.49	0
0.5	4.9	.51	4.8	.50	2
1.0	5.0	.53	4.8	.51	4
2.5	5.4	.57	5.1	.55	6
5.0	5.9	.66	5.4	.59	8

[a]Percent (v/v) addition of pH 5.5 high or low solids PHASE 4

[b]ml of 10% (w/w) lactic acid necessary to lower pH of 100 ml reconstituted NDM (9.0% w/w) to pH 5.0

[c]Titratable acidity of acidified (pH 5.0) reconstituted NDM

Table 21. Reduced 'carryover buffer' (CB) with low solids PHASE 4 using commercial cultures in a Cheddar cheese plant.

Culture[a]	Relative[b] Activity	CB-High[c]	CB-Low[d]	ICF[e]	% Less CB with Low Solids
MRD	1	3.1	2.6	1.25	16
SG1	1	3.5	2.9	1.27	17
MI3	1	3.3	2.9	1.25	12
WP	1	3.4	2.8	1.32	18
MI1	1	3.5	3.0	1.26	14
OS	2	3.4	2.8	1.32	18
EV	2	3.2	2.9	1.28	9
C5	3	3.6	3.3	1.33	8
CS24	3	3.3	3.1	1.25	6
CC1	3	3.2	3.1	1.33	3
VT7	3	3.4	3.4	1.40	0
	$\bar{X}$	3.35	3.0	1.30	11

[a]Frozen bulk set cultures (Marschall Products)

[b]1; most active

[c]Carryover buffer from high solids (8.7% w/w) PHASE 4; ml lactic acid required to titrate 20 ml (plus 80 ml distilled H_2O) of mature starter to pH 4.5

[d]Carryover buffer from low solids (5.8% w/w) PHASE 4; determined as with high solids and then multiplied times ICF

[e]Inoculation correction factor = # $\frac{\text{starter used with low solids}}{\text{starter used with high solids}}$

and low solids PHASE 4 on several occasions. Comparing 21 vats (45,000-lb capacity) made with high solids M13 starter to 12 vats made with low solids (approx 30% more starter usage) the mean ($\bar{x}$) titratable acidity at milling for both solids levels was 0.56%. However, the milling pH for the 21 vats made with high solids starter ($\bar{x}$ = 5.13) was significantly ($P<.001$) higher than the milling pH for the low solids vats ($\bar{x}$ = 5.07). The effects of carryover buffer on the final cheese curd are demonstrated by these data.

Better phage inhibition was achieved with PHASE 4-MC at high (8.7% w/w) solids than at low (7.8% w/w) solids (Table 22). Only one sample (156-11) allowed an increase in titer when used at high solids, whereas phage proliferation resulted in four of the seven samples at low solids. The results of the five commercial batches indicate a general inconsistency in the ability of PHASE 4-MC to inhibit phage replication.

Batches 118-19 and 118-20 were successive batches blended on the same day. Common ingredients were used in both batches except for the GW 4-4 base which was from two separate production runs. The laboratory blend made with citric acid + $Mg(OH)_2$ was found phage inhibitory at both solids levels. However, substitution of the citric acid + $Mg(OH)_2$ with GW 4-4 resulted in partial loss of phage inhibition at low solids. These results suggest that variability in the GW 4-4 might have been responsible for the

Table 22. Inhibition of T189 phage (host S. cremoris 205) in commercial production batches and laboratory blends of PHASE 4-MC at high and low solids.

	High Solids (8.7% w/w)		Low Solids (7.8% w/w)	
PHASE 4-MC	Activity (ΔpH)[a]	pfu/ml[b]	Activity (ΔpH)[a]	pfu/ml[b]
1-118-19[c]	1.5	3.0×10^3	1.5	5.0×10^3
1-118-20[c]	1.5	3.9×10^3	0.7	6.9×10^6
1-147-5[c]	1.4	5.9×10^3	1.4	5.1×10^5
1-156-11[c]	1.4	1.1×10^6	0.3	2.3×10^9
1-171-5[c]	1.4	1.1×10^3	1.4	2.5×10^3
Commercial[d] Formulation w/GW 4-4	1.4	2.8×10^2	1.3	1.0×10^6
Laboratory[e,f] Formulation	1.4	1.3×10^3	1.4	2.8×10^3

[a] Controlled temperature profile activity (1% inoc rate)

[b] Phage recovered following 10^5 pfu/ml infection

[c] Commercial production batches of PHASE 4-MC

[d] Whey, yeast extract, diammonium phosphate and GW4-4 individually weighed and combined

[e] Whey, yeast extract, diammonium phosphate, citric acid and magnesium hydroxide individually weighed and combined

[f] Low solids at 7.6% (w/w)

inconsistent phage inhibition with the commercially blended PHASE 4-MC samples.

Competitive growth by contaminants in PHASE 4

Figure 11 illustrates the unrestricted growth of S. aureus 265-1 in both PHASE 4-M and the externally neutralized (EN) medium when grown without the lactic starter. However, in competition with S. cremoris 134, the growth of S. aureus 265-1 was effectively suppressed in both media. Growth of S. cremoris 134 in both media reached cell numbers of nearly 5×10^9 cfu/ml after 16 h of incubation with the S. aureus contaminant.

Salmonella jacksonville increased four logs when grown alone and over three logs when in competition with the commercial starter M21 in both pH-controlled media (Figure 12). Further growth by S. jacksonville (with M21) was suppressed only during the latter hours of the 18 h incubation period. Holdover at ambient temperatures for an additional 18 h did result in a considerable loss of viable Salmonella in the PHASE 4-P starter (with lactic culture); while in the externally neutralized (EN) medium the Salmonella count remained stable through the 36 h period. Growth by the M21 starter was unaffected by the growth of S. jacksonville in both media; cell counts of 5.0×10^9 cfu/ml in PHASE 4-P and 6.0×10^9 cfu/ml in EN were reached in fermentations with and without competition by S. jacksonville.

Figure 11. Growth of S. aureus 265-1 in PHASE 4-M (open circle) and in externally neutralized whey (open triangle); and in competition with S. cremoris 134 (closed symbols).

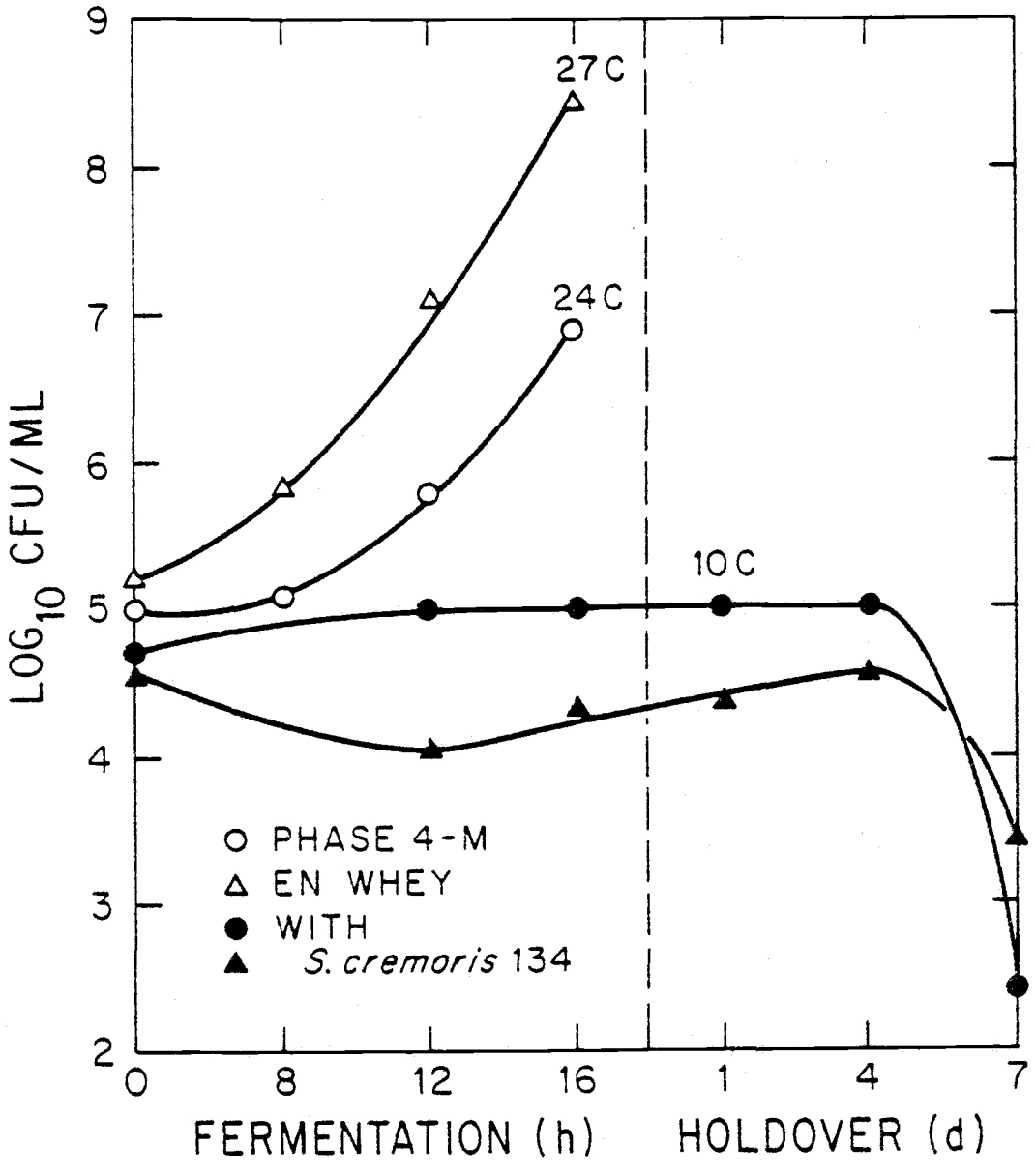

9
8
7
6
5
4
3
2
LOG10 CFU/ML
27C
24C
10C
PHASE 4-M
EN WHEY
WITH
S. cremoris 134
0
8
12
16
1
4
7
FERMENTATION (h)
HOLDOVER (d)

Figure 11.

Figure 12. Growth of Salmonella jacksonville in PHASE 4-P (open circle) and in externally neutralized whey (open triangle); and in competition with the commercial culture M21 (closed symbols).

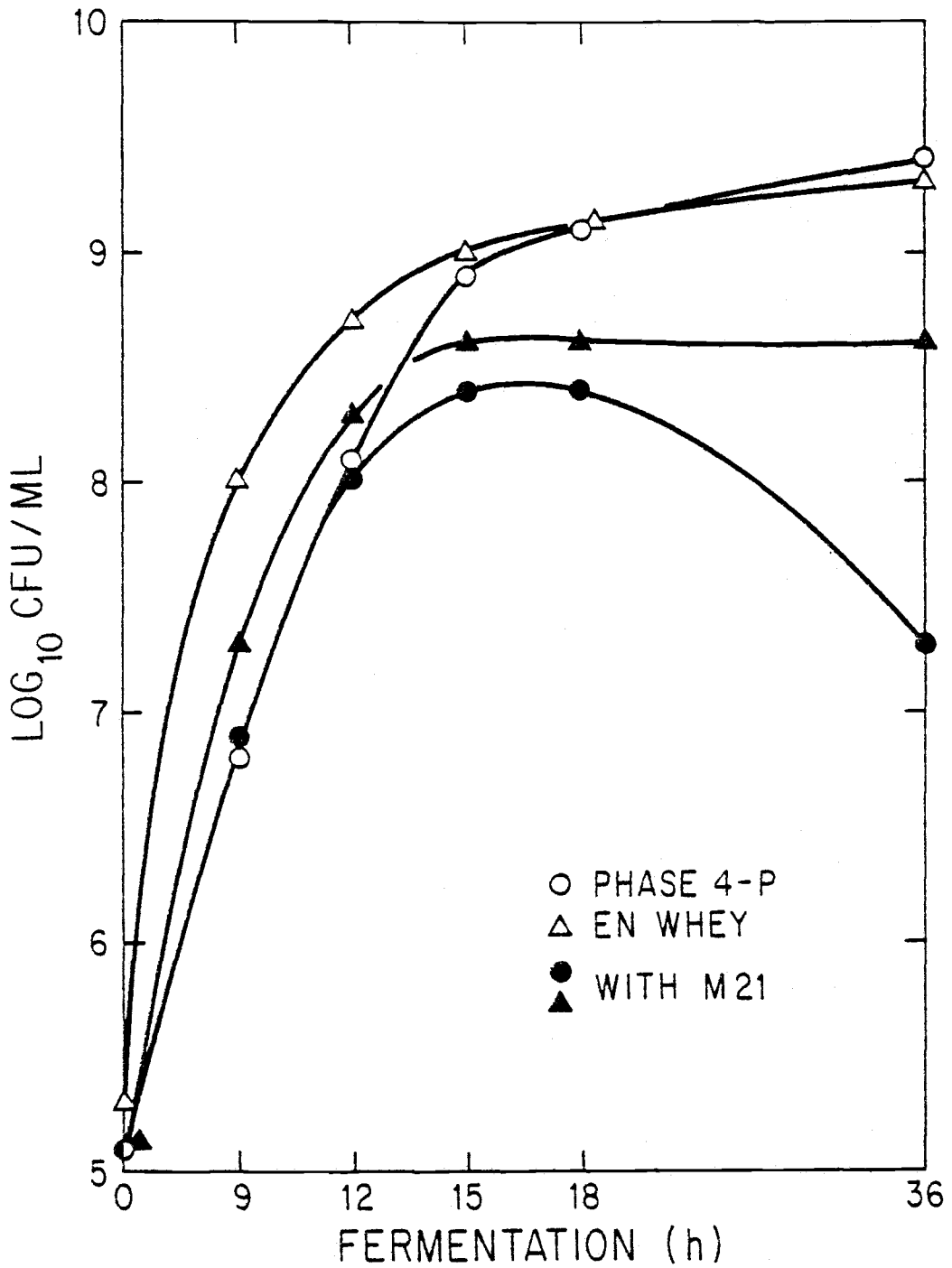

10
9
8
7
6
5
LOG10 CFU/ML
0
9
12
15
18
36
FERMENTATION (h)
PHASE 4-P
EN WHEY
WITH M21

Figure 12.

Growth of the coliform Enterobacter cloacae (in competition with M13) was unrestricted in the externally neutralized CFS medium and HBM, whereas coliform growth levelled off after 12 h of fermentation in PHASE 4-MC and Marstar 1-2-1 (Figure 13). No additional growth of coliforms resulted in any of the media during the four day holdover at 10 C. Data did suggest that growth of the lactic culture (M13) was somewhat suppressed when competitively grown with E. cloacae in PHASE 4-MC but not in the other three media (unpublished data). When the cooled (10 C), mature starters were contaminated with 1.6×10^3 cfu/ml E. cloacae (post fermentation contamination), the coliform numbers rapidly declined to an undetectable level in all four media.

Growth of citric acid-fermenting bacteria and Pediococcus cerevisiae in PHASE 4

Conventional phage-inhibitory media have been shown to restrict the growth of some strains of citric acid-fermenting starter bacteria, especially Leuconostoc (29). In PHASE 4-MC, even though very little acid was produced by L. cremoris L2 (final pH of 6.5) after 16.5 h incubation, a cell count of 1.2×10^9 cfu/ml was achieved. In addition, PHASE 4-MC not only supported excellent growth of L. cremoris L6 and S. cremoris C13; when grown associatively, the two organisms were still reasonably balanced after the 16.5 h fermentation (Table 23).

The relatively high concentrations of citrate in PHASE 4 coupled with the active citrate metabolism by S. diacetilactis

Figure 13. Growth of Enterobacter cloacae in competition with the commercial culture M13 in PHASE 4-MC (open circle), HBM (open triangle), Marstar 1-2-1 (open square) and CFS (open diamond).

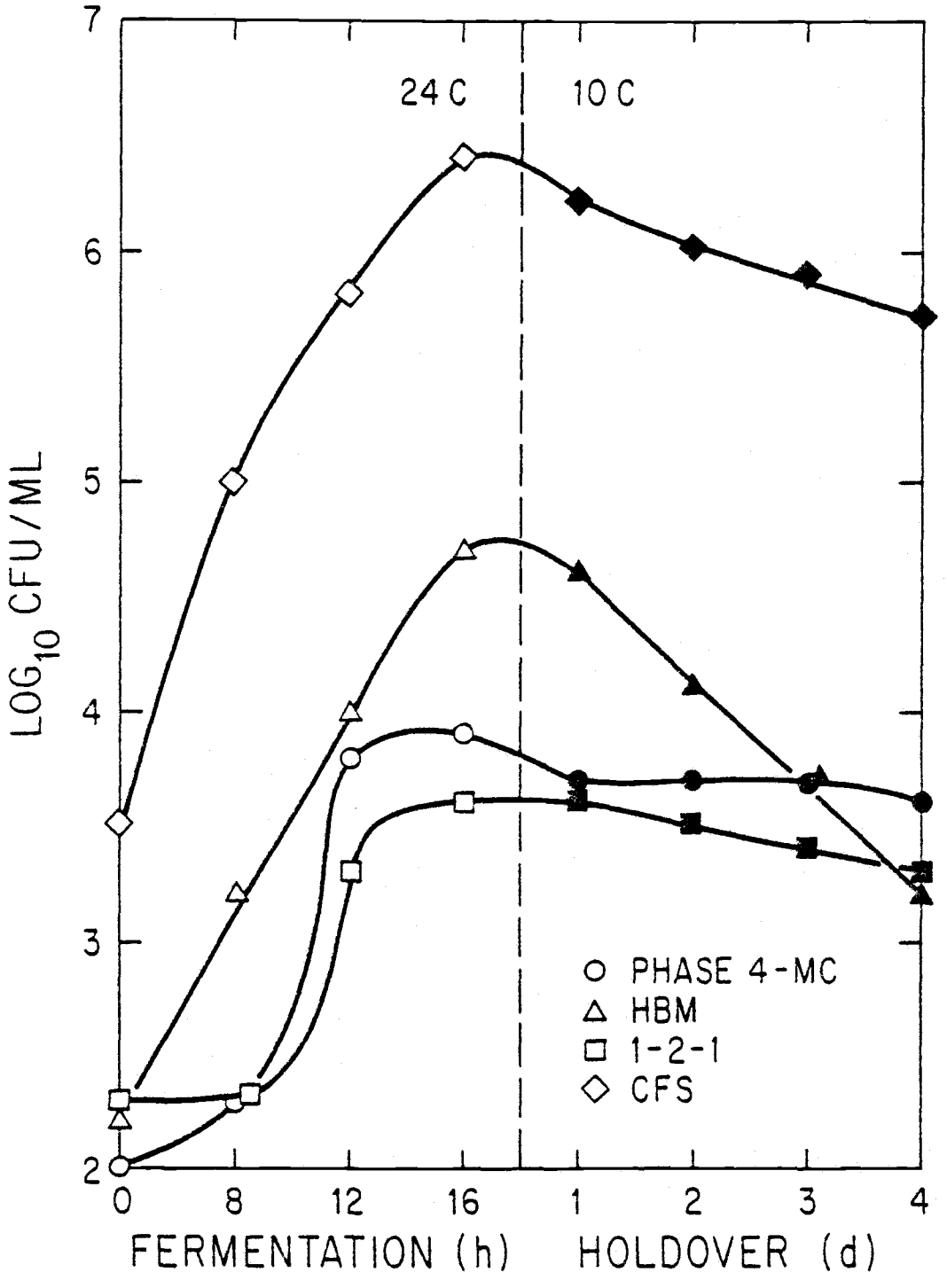

24 C
10 C
LOG10 CFU/ML
7
6
5
4
3
2
0
8
12
16
1
2
3
4
FERMENTATION (h)
HOLDOVER (d)
PHASE 4-MC
HBM
1-2-1
CFS

Figure 13.

Table 23. Growth of Leuconostoc cremoris and Streptococcus diacetilactis in PHASE 4-MC.

Culture Description	Lactic Strep (cfu/ml)	Citric Acid-Fermenting Bacteria (CAF)	% CAF Bacteria
L. cremoris L2	--	1.2×10^{9}[e]	--
L. cremoris L6 (2:1) +S. cremoris C13	2.3×10^{9}[e]	1.2×10^{9}[e]	34%
H52[a]	2.9×10^{9}[f]	1.6×10^{9}[f]	36%
S. diacetilactis H52 (5%)[b] +S. lactis C2-F (41%)[c] +S. lactis C2-S (54%)[d]	6.6×10^{9} (C2-F)[g] 9.3×10^{8} (C2-S)	1.8×10^{9}[h]	19%)

[a] Commercial culture consisting of multiple strains of S. diacetilactis and S. cremoris

[b] Isolated from H52 (McKay's citrate agar)

[c] Parent (lac^+ prt^+)

[d] Slow mutant (lac^+ prt^-)

[e] Plated on LB Agar-large colonies determined to be Leuconostoc and confirmed by litmus milk rxn

[f] Differential counts on Reddy's Agar

[g] Fast-Slow differentiation on FSDA-II (slow = total slow colonies minus S. diacetilactis colonies) on McKay's citrate agar

[h] McKay's citrate agar

might conceivably allow this organism to become dominant in a mixed strain fermentation in PHASE 4. The data presented in Table 23 suggest such a dominance does not result with S. diacetilactis grown in association with fast lactic acid-producing lactic streptococci. Apparently, S. diacetilactis utilizes citrate only in the presence of a fermentable carbohydrate and when the limiting concentration of lactose in PHASE 4 is exhausted, citrate metabolism stops and the growth of S. diacetilactis stops along with the other lactic streptococci in the culture.

Final cell counts of P. cerevisiae 1220 were 0.74, 1.6 and 3.0×10^9 cfu/ml after 16 h of incubation in PHASE 4-MC, Italian PHASE 4-MH and Italian PHASE 4-NPC, respectively. Good growth resulted even though the developed acidity and pH change were small in all three media compared to growth by lactic streptococci. Specific reformulation directed to meet the specific needs of P. cerevisiae could provide a better growth environment for this organism. The nonphosphated $CaCO_3$ buffering appears to be the most promising pH control mechanism for such a medium.

Growth of slow acid-producing variants in PHASE 4-MC

Growth after 16 h in PHASE 4-MC by all fast-slow mixtures, including 100% slow, appeared normal according to the fermentation parameters of pH and titratable acidity (Table 24). According to field use recommendations, the mature starter in all four cases would have been used for cheese manufacture. Activity test results

Table 24. Growth of S. lactis C2 'Fast' and 'Slow' Acid-Producers in PHASE 4-MC (25 C).

Culture	0 h pH	0 h TA	16 h pH	16 h TA	16 h Activity[a] (ΔpH)	20 h pH	20 h TA
Fast[b] (1%)	6.8	0.53	5.4	2.70	1.35	5.3	2.94
Fast[b] (0.5%) +slow[c] (0.5%)	6.8	0.53	5.4	2.70	1.3	5.3	2.92
Fast[b] (.05%) +slow[c] (0.95%)	6.8	0.53	5.45	2.54	0.95	5.4	2.76
slow[c] (1%)	6.8	0.53	5.5	2.42	0.5	5.4	2.72

Culture	0 h Total cfu/ml	0 h % S[d]	16 h Total cfu/ml	16 h % S[d]	20 h Total cfu/ml	20 h % S[d]
Fast[b] (1%)	2.1×10^7	18	8.7×10^9	24	1.4×10^{10}	32
Fast[b] (0.5%) +slow[c] (0.5%)	2.0×10^7	49	1.2×10^{10}	52	9.9×10^9	51
Fast[b] (.05%) +slow[c] (0.95%)	2.4×10^7	94	1.4×10^{10}	93	1.2×10^{10}	96
slow[c] (1%)	3.0×10^7	100	1.5×10^{10}	100	1.9×10^{10}	100

[a] Controlled temperature profile activity (1% inoc rate)

[b] S. lactis C2 (lac^+ prt^+)

[c] S. lactis LM 0210 (lac^+ prt^- variant of C2)

[d] % slows

reflect the probable outcomes in the cheese vats. Only the starter with all fast and the 50/50 mixture would have made cheese with acceptable make times. These data illustrate the importance of performing an activity test on all bulk starters before use in the cheese vat. Unfortunately, activity testing seems to be the exception instead of the rule in the cheese industry.

The second part of Table 24 shows that the S. lactis C2 slow variant (lac^+, prt^-) is able to successfully compete with the parent cells and maintain its original population ratio when growing in mixed populations in PHASE 4-MC. A slight increase of slows was seen with the S. lactis C2 parent fermentation, possibly as a result of increased plasmid loss in the high phosphate-citrate medium, PHASE 4-MC.

When equal inocula (0.5%) of the same fast and slow cultures were grown in NDM (11% w/w) + 0.5% yeast extract, the slow population decreased from an initial 54% to 31% after 16 h incubation at 21 C. Apparently, the yeast extract does not account for all of the nitrogenous compounds necessary for the slows to compete with the fast parent cells. The available amino acids and peptides present in the whey component of PHASE 4 may also contribute to supporting the growth of slows in PHASE 4.

Commercial cheesemaking trials

An estimated 650 million pounds of cheese, including American cheese varieties and cottage cheese, have been manufactured using PHASE 4 since it was first commercialized 20 months ago. Overall, the introduction of PHASE 4 to the cheese industry has been very successful.

Early commercial trials were directed towards Cheddar cheese manufacture. PHASE 4-M (the first commercially blended formulation) was found to be superior to both the conventional bulk starter medium American Actilac (Galloway West Co.) and the relatively new externally neutralized whey-based medium (Table 25). In comparison to Actilac, PHASE 4-M produced bulk starter with more than twice the activity and nearly four times the cell number. The externally neutralized bulk culture had equivalent cell numbers but the PHASE 4-M starter was more active.

Table 26 shows the reduction in starter inoculation to the cheese vat that was possible with PHASE 4, even after two days of holdover.

Table 27 presents Cheddar cheese manufacturing results from consecutive vats under the same conditions comparing PHASE 4-M (experimental blend) with an externally-neutralized bulk culture (continuous neutralization). Used at the same inoculation rate (0.5% w/w), the PHASE 4-M starter was slightly faster in the vat and could be used at lower inoculation levels to achieve comparable activity with the externally-neutralized bulk. When these two

Table 25. Commercial comparison of PHASE 4-M with bulk cultures of Actilac and External Neutralization used for the manufacture of Cheddar cheese.

Bulk Starter Medium	Activity (ΔpH) 0.5%	Activity (ΔpH) 1.0%	cfu/ml	Cell Number Ratio[c]
American Actilac	0.8[a]	1.2[a]	1.3×10^9	0.26:1
PHASE 4-M	1.4[a]	1.6[a]	4.9×10^9	---
	0.8[b]	1.2[b]	5.0×10^9	
External Neutralization	0.6[b]	0.9[b]	4.9×10^9	1:1

[a] 6 h-30 C activity

[b] 4 h-30 C activity

[c] The cell number of PHASE 4-M equals 1

Table 26. Cheddar cheese make results using bulk starter prepared with PHASE 4-M.

Starter Age (days)	Inoc. Rate	Acidity at Milling	Make Time	Comments
0	0.75%[a]	0.58	3 h 25 min	Fast
0	0.60%	0.58	3 h 25 min	Fast
1	0.40%	0.43	3 h 45 min	Excellent
2	0.40%	0.43	3 h 45 min	Excellent

[a]Normal inoculation rate using bulk starter prepared with conventional bulk starter media such as Actilac.

Table 27. Comparative Cheddar cheese make results.

	Continuous Neutralization	Experimental Blend
Milk	same source	
Inoculation Rate	0.5%	
Ripening Time	60 minutes	
Set Temperature	89 F	
Cook Temperature	100 F	
T.A.[a] Set	0.175	0.18
T.A. Cut	0.10	0.12
T.A. Draw	0.13	0.145
T.A. Pack	0.18	0.22
T.A. Mill	0.43	0.50
Make Time	3 h 55 min	3 h 20 min
	normal	fast

[a]T.A. = titratable acidity

same bulk cultures were tested for the presence of phage against the five strains present in the culture used, the externally neutralized bulk showed a relatively high level of phage for one of the strains (S. cremoris 224) while the PHASE 4-M bulk had low to undetectable levels of phage for all five strains (Table 28).

PHASE 4-M was also evaluated in a plant manufacturing cottage cheese. Used at 2.5 times the level of the PHASE 4-M starter, the skim milk starter was still not as active, as illustrated by Figure 14. The rapid decrease in vat pH with the PHASE 4-M starter supports the reasoning that uninjured cells (pH injury) are in a better physiological state to allow earlier acid production because of a reduced lag time for repair.

Two other commercial trials were run at Mayflower Farms (Portland, OR) where PHASE 4-M culture was used for four consecutive days for the manufacture of Cheddar and Monterey Jack cheese, and at Darigold (Chehalis, WA) in which a PHASE 4-grown culture was again found to be more active than an externally-neutralized bulk culture (unpublished data).

Common to all the early experimental trials was a noticeable increase in the incubation temperature of the PHASE 4 bulk cultures (2-5 F) by the end of the fermentation. The temperature increase was most dramatic when growth was greatest (10-14 h after inoculation). This phenomenon was not observed in the laboratory development of PHASE 4 and has been attributed to the heat of

Table 28. Phage detection in EN and PHASE 4-M bulk starters.

Host (*S. cremoris*)	pfu[a]/ml	
	EN	PHASE 4-M
C13	<40	<40
108	<40	<40
224	27,000	280
290	400	<40
SK11G	<40	80

[a]pfu/ml - plaque forming units per ml.

Figure 14. Comparison of the change in pH between two vats of cottage cheese; one inoculated with 2.5% skim starter (open) and the other with 0.95% PHASE 4-M grown starter (closed). Both bulk cultures were set with the commercial culture H70.

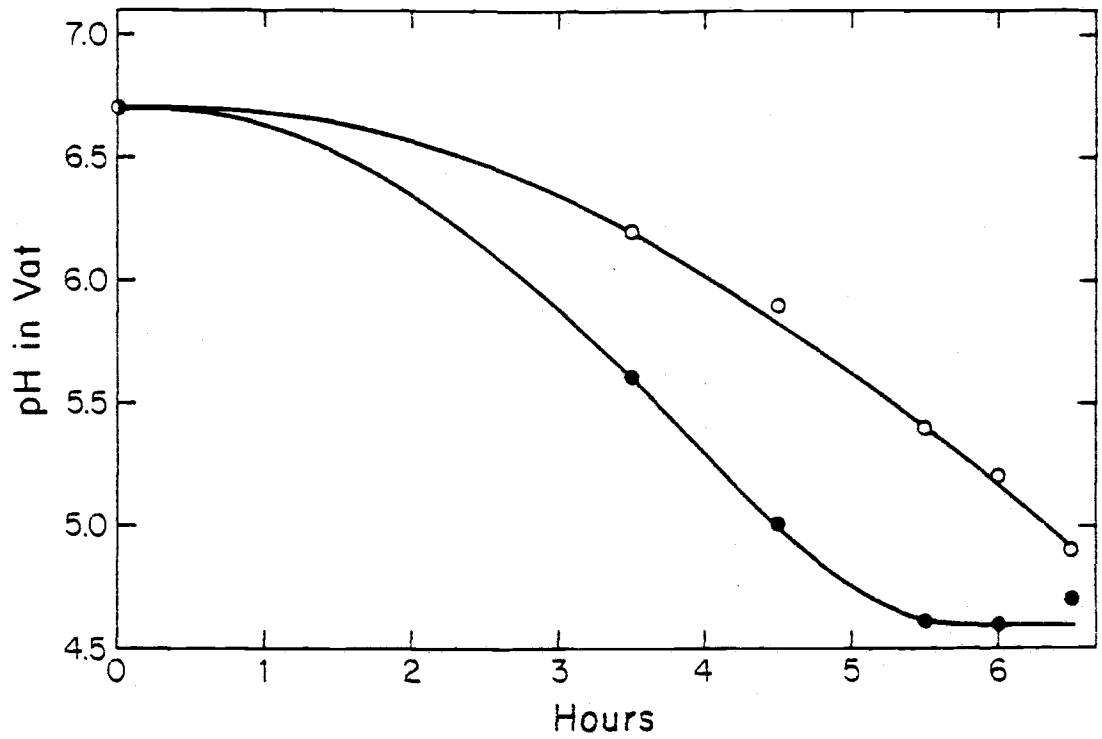


Figure 14.

fermentation as a result of the rapid production of lactic acid by the starter cells and the subsequent neutralization by the internal buffering of PHASE 4.

Bulk Starter Media for Thermophilic Starters

Formulation development

The pH-dependent, insoluble buffers, $Mg_3(PO_4)_2$ and $CaCO_3$, were added to a milk-whey-yeast extract basal medium. More active starter and better rod-coccus balance with a frozen inoculum were achieved with the internally pH-controlled media than with the commercial medium Phage-Stat (Table 29). Reduced buffering capacities, compared to PHASE 4, were necessary to allow the pH to drop below 5.0 for good rod growth. Increased levels of $Mg_3(PO_4)_2$ increased activity but only increased S. thermophilus cell numbers (unpublished data).

PHASE 4-MC used as a medium to propagate the growth of the rod-coccus starters was unacceptable. Table 30 shows complete rod inhibition in PHASE 4-MC with considerably less activity compared to the commercial medium Thermostar and the $Mg_3(PO_4)_2$-buffered medium, Italian PHASE 4-MH.

Concentrations of citrate as high as 0.95% (w/w) did not affect the activity or the total cell numbers in Italian PHASE 4-MH (Table 31). However, at citric acid additions of 0.67% (w/w) or higher, the rod population was markedly suppressed. PHASE 4-MC contains a relatively high citrate level which probably accounts for a large portion of the rod inhibition with thermophilic starters.

Increased amounts of $Mg(OH)_2$ in Italian PHASE 4 resulted in greater activity with higher cell numbers and increasingly better

Table 29. Growth of S. thermophilus-L. bulgaricus intermediate culture and a commercially combined frozen culture in internally pH-controlled media and the commercial medium, Phage-Stat (6 h-40 C).

Medium	S. thermophilus CH2 + Lb CH2[a]			CH2[b]		
	Activity[c] (ΔpH)	Total cfu/ml	MO[d] % Rods	Activity[c] (ΔpH)	Total cfu/ml	% Rods[e]
Phage-Stat (10.75% w/w)	2.3	9.3×10^8	50	2.0	1.0×10^9	<1
Nutrient base[f] + 2.0% (w/v) $Mg_3(PO_4)_2$	4.1	2.5×10^9	40	4.2	1.9×10^9	14
Nutrient base[f] + 2.0% (w/v) $CaCO_3$	3.6	1.7×10^9	67	4.0	8.2×10^8	43

[a] S. thermophilus and L. bulgaricus isolates from CH2, prepared as intermediates in reconstituted NDM (8 h-37 C), inoculated at 1% rate of each organism into media

[b] Frozen commercial bulk set culture (0.125 ml/l)

[c] 5 h-35 C (and 2% inoc rate)

[d] Estimate of L. bulgaricus (rods) content from microscopic observation of methylene blue-stained smear

[e] Differential plate count on Lee's Agar

[f] Consisting of NDM, 3.19% (w/v); whey, 5.91% (w/v); yeast extract 0.5% (w/v)

Table 30. Growth of commercial frozen cultures used for the production of Italian cheeses in PHASE 4 and the modified formulation for thermophilic starters, Italian PHASE 4-MH (41 C-8 h).

Medium	CR4[a]			CR14[b]		
	Activity[c] (ΔpH)	Total cfu/ml	% Rods[d]	Activity[c] (ΔpH)	Total cfu/ml	% Rods[d]
PHASE 4-MC	2.2	1.9×10^9	<1	1.5	5.8×10^8	<1
Thermostar	3.0	1.4×10^9	37	2.7	1.7×10^9	42
Italian PHASE 4-MH[e]	3.9	2.9×10^9	38	3.6	3.1×10^9	9

[a] Frozen commercial culture containing 38% rods (0.25 ml/l)

[b] Frozen commercial culture containing 26% rods (0.25 ml/l)

[c] Thermophilic Activity Test (1 and 2% inoc rate)

[d] Determined by differential plate count on LB agar

[e] NDM, 3.19% (w/v); whey, 5.91% (w/v); yeast extract, 0.5% (w/v); $(NH_4)_2HPO_4$, 0.35% (w/v); $NH_4H_2PO_4$, 1.63% (w/v); $Mg(OH)_2$, 0.65% (w/v)

Table 31. Effect of citric acid on the growth of CR12[a] in Italian PHASE 4-MH[b].

% Citric Acid . H_2O (w/w)	% Total Solids (w/w)	% PO_4^{-3} (w/w)	Activity (ΔpH)[d]	Total cfu/ml	% Rods
0	9.4	1.3	1.9	3.1×10^9	19
0.23	9.6	1.3	1.9	2.4×10^9	12
0.45	9.8	1.3	1.9	3.6×10^9	15
0.67	10.1	1.3	1.8	3.6×10^9	4
0.95	10.2	1.1	1.8	3.1×10^9	4
Control[c]	9.25	1.2	1.8	3.9×10^9	26

[a] CR12 (0.25 ml/l) - 53% R initial rods

[b] Including whey, 6.89% (w/v); NDM, 0.63% (w/v); yeast extract, 0.45% (w/v); $Mg(OH)_2$, 0.61% (w/v) plus $NH_4H_2PO_4$ - $(NH_4)_2HPO_4$ resulting in an initial pH of 6.5 - 6.6

[c] Italian PHASE 4-MH with whey, 4.94% (w/v); NDM, 2.67% (w/v); yeast extract, 0.54% (w/v) plus $NH_4H_2PO_4$ - $(NH_4)_2HPO_4$ resulting in initial pH of 6.5

[d] Thermophilic activity test (1% inoc rate)

rod populations (Table 32). The higher cell numbers most probably occurred as a result of increased buffering capacity provided by $Mg(OH)_2$. Even at 0.56% (w/w) $Mg(OH)_2$, the pH of the medium dropped below 5.0 (4.85) after 6 h at 41 C, providing excellent growth conditions for the rods.

Table 33 shows that the addition of 1.3% (w/w) phosphate to a nutrient basal medium resulted in complete inhibition of the R1 rods. Buffering with $CaCO_3$ in place of the in situ generated $Mg_3(PO_4)_2$ did, however, produce a more active starter with good rod growth compared to the nutrient basal medium. Apparently, some L. bulgaricus strains are sensitive to phosphate and a non-phosphated medium is necessary for balanced rod-coccus growth.

Six to ten percent of the total solids as NDM solids is necessary to support the growth of the rods (R. L. Sellars - personal communication). Preliminary results showed a ratio of 65 parts whey to 35 parts NDM (26% w/w of total solids) was optimum for activity (unpublished data). The results presented in Table 34 show little difference in activity at different NDM levels, however a minimum concentration of 6% (w/w) NDM appears necessary for optimum rod growth in Italian PHASE 4-MH. The difference in final rod populations was more pronounced in the nonphosphated Italian PHASE 4-NPC when the NDM level decreased from 27 to 10%. Growth in 100% NDM did not stimulate greater rod development than with Italian PHASE 4-NPC (27% w/w NDM), suggesting that levels of NDM higher than 27% (w/w) would not be needed for maximum rod growth.

Table 32. Effect of $Mg(OH)_2$ on the growth of S. thermophilus[a] CR12 and L. bulgaricus[a] CR12 in Italian PHASE 4 (6 h-41 C).

% $Mg(OH)_2$[b,c] (w/w)	% Total Solids (w/w)	Activity[d] (ΔpH)	Total cfu/ml	% Rods
0	9.8	1.4	1.4×10^9	<1
0.11	9.8	1.55	2.9×10^9	4
0.23	9.9	1.6	4.1×10^9	8
0.34	10.0	1.7	5.6×10^9	10
0.45	10.1	1.8	3.9×10^9	12
0.56	10.2	1.8	4.6×10^9	20

[a]Isolated from CR12; NDM-grown cultures inoc at 1.0% (Sth) and 0.75% (Lb); Initial 40% R

[b]Combined with whey, 6.89% (w/v); sodium caseinate, 0.63% (w/v); yeast extract, 0.45% (w/v) and 2.0% (w/w) PO_4^{-3} (combinations of $NH_4H_2PO_4$ + $(NH_4)_2HPO_4$ resulting in initial pH of 6.1-6.3)

[c]All fermentations resulted in a final pH of less than 5.0

[d]Thermophilic activity test (1% inoc rate); sampled at pH 4.5-4.8

Table 33. Inhibition of R1[a] rods when grown in phosphated Italian PHASE 4.

Medium Description	Activity (ΔpH)	Total cfu/ml	Initial - % Rods - Final
Nutrient base[b] (NB)	1.3	5.5×10^8	16 10
NB + 0.59% (w/v) $MH^c_{-3\ d}$ + 1.3% (w/w) PO_4	1.2	3.1×10^9	19 <1
NB + 1.06% (w/v) $CaCO_3$	1.5	1.1×10^9	16 24

[a]Frozen commercial culture (0.125 ml/l)

[b]Consists of NDM, 2.90% (w/v); whey, 5.38% (w/v); yeast extract, 0.45% (w/v)

[c]$Mg(OH)_2$

[d]Combination of $NH_4H_2PO_4$ (130 mM) + $(NH_4)_2HPO_4$ (24 mM)

[e]Thermophilic activity test (1% inoc rate)

Table 34. Effect of NDM solids in Italian PHASE 4-MH[a] and Italian PHASE 4-NPC[b].

% NDM (w/w) of Total Solids	% Total Solids (w/w)	Activity[c] (ΔpH)	Total cfu/ml	Initial - % Rods - Final
0[a]	9.2	1.9	1.9×10^9	34 - 17
6[a]	9.4	1.9	2.6×10^9	34 - 26
12[a]	9.5	1.7	2.9×10^9	34 - 26
17[a]	9.7	1.7	2.9×10^9	34 - 28
23[a]	9.9	1.9	3.2×10^9	34 - 26
26[a]	10.0	1.9	4.7×10^9	34 - 23
10[b]	10.6	1.7	2.0×10^9	27 - 28
27[b]	10.6	1.6	1.6×10^9	27 - 40
100	11.0	1.4	1.4×10^9	27 - 39

[a] CR15 (0.25 ml/l) - Yeast extract, 0.45% (w/v); $NH_4H_2PO_4$, 1.48% (w/v); $(NH_4)_2HPO_4$, 0.31% (w/v); $Mg(OH)_2$, 0.59% (w/v) with remaining solids as whey

[b] 408 (0.125 ml/l) - Yeast extract, 0.5% (w/v); $CaCO_3$, 2.0% (w/v); GW 4-4, 0.25% (w/v) with remainder solids as whey

[c] Thermophilic activity test (1% inoc rate)

In preparing rod-coccus bulk starter, two fine-tuning adjustments are commonly used to achieve a higher rod population: (a) increase of incubation temperature and (b) decrease of total solids. The results in Table 35 support the latter of the two fine-tuning measures using Italian PHASE 4-MH. Reduction in total solids from 12 to 8% (w/w) markedly stimulated higher rod growth without a major loss of activity. As a result of these data and similar field results, commercial use of the Italian PHASE 4-MH was recommended at 9.25% (w/w) (40 lb per 50 gallons).

Growth of commercial cultures in Italian PHASE 4

The two-stage growth pattern involving early cocci growth followed by later development of rods is evident in the data in Table 36 with Phage-Stat, Thermostar and Italian PHASE 4-MH. The balanced population of CR4 rods and cocci rapidly became cocci-dominanted during the first 4 h of growth. Between 4 and 6 h, cell numbers increased to 10^9 cfu/ml as the rods rapidly divided and made up a larger part of the mixed culture. At 6 h, growth in Phage-Stat had nearly matured with a total cell number of approximately 2×10^9 cfu/ml and nearly 40% rods. Rod development in both Thermostar and Italian PHASE 4-MH was still immature, even though the total cell count of Italian PHASE 4-MH was already greater than Phage-Stat at 6 h. From 6 to 10 h the total cell number nearly doubled in Thermostar (matching Phage-Stat) with the rod population maturing at 42%; while in Italian PHASE 4-MH,

Table 35. Effect of total solids on the growth of commercial cultures R5 and CR12 in Italian PHASE 4-MH[a].

% Total Solids (w/w)	R5[b]			CR12[c]		
	Activity[d] (ΔpH)	Total cfu/ml	% Rods	Activity[d] (ΔpH)	Total cfu/ml	% Rods
8	1.85	3.4×10^9	38	1.75	3.1×10^9	32
10	1.9	9.2×10^9	8	1.8	3.6×10^9	21
12	2.0	1.0×10^{10}	14	1.8	5.0×10^9	9

[a]Commercial blend (0329-01) containing whey, 26.08%; NDM, 48.36%; yeast extract, 4.09%; $NH_4H_2PO_4$, 13.29%; $(NH_4)_2HPO_4$, 2.86%; $Mg(OH)_2$, 5.32% all on % (w/w) dry wt basis

[b]0.2 ml/l with 20% rods

[c]0.25 ml/l with 47% rods

[d]Thermophilic activity test (1% inoc rate)

Table 36. Growth of CR4[a] in two commercial media and Italian PHASE 4-MH.

	Phage-Stat[b]		Thermostar[c]		ItPh4-MH[d]	
Fermentation (h)	Total cfu/ml	% Rods[e]	Total cfu/ml	% Rods[e]	Total cfu/ml	% Rods[e]
0	1.4×10^6	50	1.4×10^6	50	1.4×10^6	50
2	2.2×10^6	2	1.7×10^6	6	2.5×10^6	16
4	2.7×10^8	<2	2.3×10^8	2	1.3×10^8	<4
6	1.8×10^9	33	1.1×10^9	15	2.4×10^9	19
8	1.5×10^9	45	1.2×10^9	32	3.7×10^9	41
10	2.3×10^9	38	2.0×10^9	42	3.9×10^9	39
Activity[f] (ΔpH)	3.3[g]		2.9[h]		3.9[i]	

[a] 0.25 m/l

[b] 10.75% (w/w) solids

[c] 11.0% (w/w) solids

[d] 10.9% (w/w) solids

[e] Differential plating on LB agar

[f] 5 h-35 C (1 + 2% inoc rate)

[g] Sampled at 5.5 h

[h] Sampled at 7 h

[i] Sampled at 8 h

the total cell number continued to increase to nearly 4 x 10^9 cfu/ml (2X Thermostar and Phage-Stat) yet maintained a balanced culture. The higher cell number in the Italian PHASE 4-MH starter probably accounted for its superior activity.

Refrigerated holdover of CR4 grown in Phage-Stat, Thermostar and Italian PHASE 4-MH was excellent through three days at 7 C. Only the Phage-Stat culture showed an appreciable loss of activity and rods after three days of storage (Table 37).

Figure 15 illustrates the superior activity generated by five frozen commercial rod-coccus cultures grown in Italian PHASE 4-MH in comparison to Thermostar and Phage-Stat. Of the two commercial media, Phage-Stat appears to have an edge even though these particular cultures were designed for growth in Thermostar.

Tables 38 and 39 compare the growth of many commercial cultures in Italian PHASE 4-MH, the nonphosphated Italian PHASE 4-NPC and Thermostar.

The CR culture series (Marschall Products, Madison, WI) are generally phosphate tolerant strains of S. thermophilus and L. bulgaricus that were designed for growth in phosphated media such as Thermostar. These cultures are widely used by the Italian cheese industry and in combination with Thermostar represent the most common bulk starter system used for the manufacture of Italian cheeses. Table 38 shows both Italian PHASE 4 media produced higher mean activities than with Thermostar. Thermostar and -NPC produced nearly the same cell numbers while Italian

Table 37. Refrigerated (7 C) holdover of CR4[a] grown in two commercial media and Italian PHASE 4-MH.

	Phage-Stat[b]		Thermostar[c]		ItpH4-MH[d]	
Holdover (d)	Total cfu/ml	% Rods[e]	Total cfu/ml	% Rods[e]	Total cfu/ml	% Rods[e]
0	1.5×10^9	38	1.1×10^9	31	3.5×10^9	27
1	1.2×10^9	50	1.3×10^9	52	3.0×10^9	28
2	1.0×10^9	31	1.0×10^9	54	3.5×10^9	30
3	1.7×10^9	48	1.4×10^9	63	2.7×10^9	34
4	1.4×10^9	17	1.0×10^9	46	2.8×10^9	26
5	1.5×10^9	21	1.3×10^9	35	2.5×10^9	33
LOA[f] (ΔpH)	0.7		0.4		0.5	

[a] 0.25 ml/l

[b] 10.75% (w/w) cooled after 5 h-42 C fermentation

[c] 11.0% (w/w) cooled after 7 h-42 C fermentation

[d] 11.2% (w/w) cooled after 8 h-42 C fermentation

[e] Differential plating on LB agar

[f] Loss of Activity - calculated as difference in 5 h-35 C (1 + 2% inoc rate) activity from 0-5 days

Figure 15. Comparison of activity (5 h-35 C) for five commercial cultures (0.25 ml/l) grown in Italian PHASE 4-MH (Exp Blend), Phage-Stat (CM-A) and Thermostar (CM-B). Each medium was sampled for activity following 6-8 h incubation at 41 C.

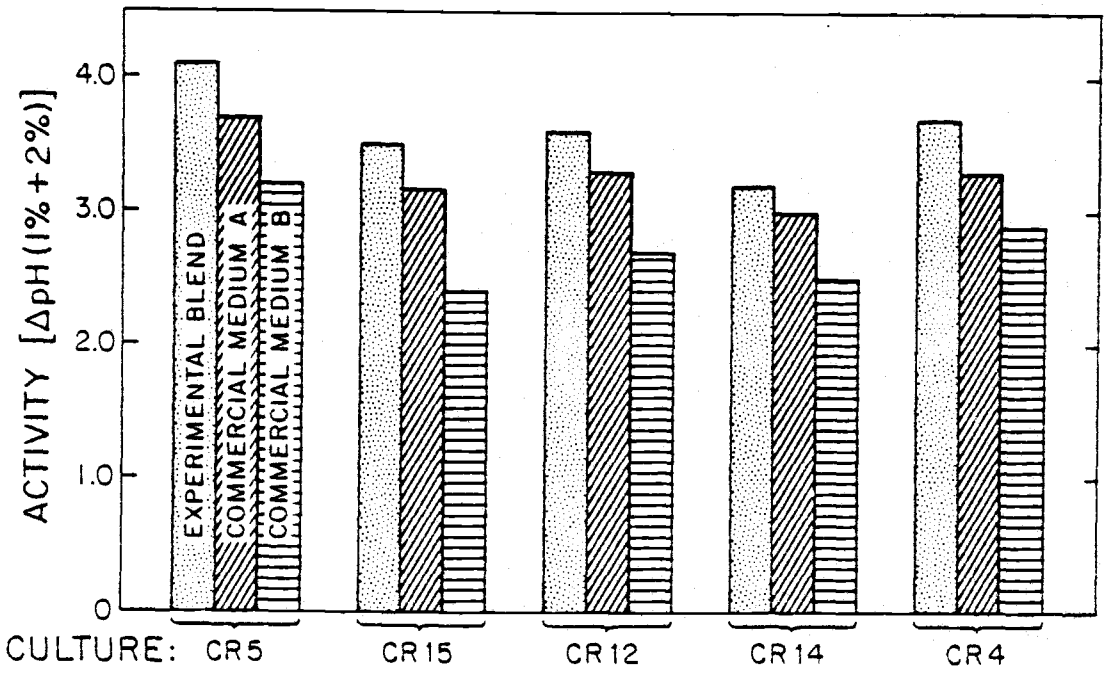

ACTIVITY [ΔpH(1% + 2%)]
4.0
3.0
2.0
1.0
0
EXPERIMENTAL BLEND
COMMERCIAL MEDIUM A
COMMERCIAL MEDIUM B
CULTURE: CR5
CR15
CR12
CR14
CR4

Figure 15.

Table 38. Growth of Marschall rod-coccus cultures in Italian PHASE 4-MH, -NPC and Thermostar.

	Italian PHASE 4-MH				Italian PHASE 4-NPC				Thermostar			
Culture[b]	Act[a]	cfu/ml	Int	-%Rods- Final	Act[a]	cfu/ml	Int	-%Rods- Final	Act[a]	cfu/ml	Int	-%Rods- Final
CR3	1.7	5.5×10^9	<1	4	-	-	-	-	1.1	9.4×10^8	<1	7
CR4	1.9	2.5×10^9	50	36	1.6	1.6×10^9	50	69	1.4	1.4×10^9	38	37
CR5	1.9	3.3×10^9	<1	2	1.8	1.2×10^9	<1	33	1.6	3.3×10^8	2	2
CR7	1.8	8.0×10^9	33	16	-	-	-	-	1.3	9.8×10^8	33	31
CR9	1.8	2.8×10^9	54	30	-	-	-	-	1.4	1.0×10^9	54	60
CR12	1.8	4.9×10^9	47	12	-	-	-	-	-	-	-	-
CR14	1.7	3.1×10^9	26	9	-	-	-	-	1.2	1.7×10^9	26	42
$\bar{X}$	1.8	4.3×10^9	30	16	1.7	1.4×10^9	25	51	1.3	1.1×10^9	26	30

[a] Thermophilic activity test (ΔpH-1% inoc rate)

[b] Sampled between 6 and 8 h-41 C incubation when the pH of Italian PHASE 4-media and Thermostar was 4.8 and 4.5, respectively

Table 39. Growth of Hansen and Microlife rod-coccus cultures in Italian PHASE 4-MH, -NPC and Thermostar.

	Italian PHASE 4-MH					Italian PHASE 4-NPC					Thermostar				
Culture[c]	Act[a]	cfu/ml	Int	-%Rods-	Final	Act[a]	cfu/ml	Int	-%Rods-	Final	Act[a]	cfu/ml	Int	-%Rods-	Final
R1	1.1	3.1×10^9	19		<1	1.6	1.7×10^9	12		17	0.5	$<5.0 \times 10^6$	35		<1
R2	1.4	2.0×10^9	21		25	1.5	1.2×10^9	12		9(7)[b]	-	-	-		-
R5	1.9	3.7×10^9	63		30	1.4	1.6×10^9	63		73(56)[b]	-	-	-		-
R6	1.9	3.9×10^9	69		21	1.5	2.6×10^9	69		69	1.2	1.3×10^9	53		39
R39	1.7	3.4×10^9	50		4	1.6	1.7×10^9	52		47	-	-	-		-
CH1	1.5	2.8×10^9	30		5	-	-	-		-	0.5	2.2×10^8	30		14
CH2	1.7	2.9×10^9	2		2	1.7	8.2×10^8	2		6(4)[b]	0.4	5.0×10^7	9		90
CH3	1.5	3.8×10^9	3		<1	1.7	1.0×10^9	3		4	0	4.0×10^7	17		38
408	1.0	3.9×10^9	27		<1	1.6	1.6×10^9	27		22	-	-	-		-
410	1.2	3.2×10^9	54		3	-	-	-		-	-	-	-		-
$\bar{X}$	1.5	3.3×10^9	34		9	1.6	1.5×10^9	30		31	0.5	3.2×10^8	29		36

[a]Thermophilic activity test (ΔpH-1% inoc rate)

[b]6 h sampling

[c]Sampled between 6 and 8 h-41 C incubation when the pH of Italian PHASE 4 media and Thermostar was 4.8 and 4.5, respectively

PHASE 4-MH generated much higher cell counts. Slightly lower rod populations resulted in -MH compared to Thermostar, but the -NPC medium appeared to stimulate better rod growth than either medium.

The rod-coccus cultures manufactured by Chr. Hansen Laboratories (Milwaukee, WI) and Microlife Technics (Sarasota, FL), with the exception of R5 and R6, apparently contain strains that are more phosphate sensitive, especially L. bulgaricus. Growth of these cultures was best in the nonphosphated Italian PHASE 4-NPC medium (Table 39). Very little growth resulted in Thermostar except with R6, one of the more phosphate-tolerant cultures. Total cell numbers were on the average two times higher in Italian PHASE 4-MH than with the -NPC medium, but slightly lower activities and inferior rod growth (except R5 and R6) were prevalent with these cultures in Italian PHASE 4-MH. Cultures R5 and R6 which apparently contain 'hearty' L. bulgaricus strains generated high rod populations in the -NPC medium and growth was easily dominated by rods upon extended incubation.

Commercial cheesemaking trials with Italian PHASE 4

Seven trials at four different plants located in the states of Washington and Wisconsin were run with Italian PHASE 4-MH and -NPC. The results of these trials are presented in Table 40. CR series cultures were used in all the Italian PHASE 4-MH trials and R1 was used with the nonphosphated -NPC medium. The trials

Table 40. Results of commercial cheesemaking trials with Italian PHASE 4-MH and -NPC.

Starter	O-1	B-1	O-2	B-2	LE	T	B-3[h]
Medium	ItpH4-MH	ItpH4-MH	ItpH4-NPC	ItpH4-MH	ItpH4-MH	ItpH4-MH	ItpH4-MH
% Solids (w/w)	10.5	10.9	10.6	9.4	9.4	9.4	9.25
Culture	CR4	CR14	R1	CR12	CR15	CR15	CR12/CR5
Activity (ΔpH)	1.7[a]	1.0[d]	1.5[a]	2.0[d]	1.9[d]	1.9[d]	0.7[d]
% Rods	26[b]	70[e]	14[b]	25[e]	40[e]	40[e]	40[e]
Cheese							
Location (State)	WA	WI	WA	WI	WI	WI	WI
Type of cheese	MOZ	MOZ	MOZ	STRING	MOZ	PROV	ND
% less starter usage	0	12	0	20	10	25	ND
Cheesemaker comments	EX[c]	SL[f]	EX[c]	EX[c]-LS[g]	EX[c]-LS[g]	EX[c]	ND

[a] 4 h-40 C (1% inoc rate)
[b] Differential plating
[c] Excellent
[d] Thermophilic activity test (1% inoc rate)
[e] Estimate from microscopic observation
[f] Slow acid development
[g] Less starter needed
[h] Phage detected in bulk starter

at Olympia Cheese Co. (O-1 and O-2) were run by weighing the individual ingredients separately and combining in the bulk starter tank at the cheese plant. The other -MH trials were run with commercial blends prepared by Galloway West Co. The first commercial blending of Italian PHASE 4-MH, used at Baker Cheese (B-1), proved to be unacceptable in quality and uniformity. All cultures grown in this blend were rod-dominated with long (10-12 h) bulk set times. Subsequent blends of -MH resulted in bulk cultures more typical of laboratory studies.

Starter usage was unnecessarily high. This can be attributed to a resistance on the part of the cheesemakers to use less starter on their first exposure of the medium. Starter reductions were also found to vary considerably, dependent on the particular variety of Italian cheese being produced and the style of manufacture.

The bulk culture prepared at Baker Cheese (B-3) was not used for cheese manufacture because of poor activity. Further laboratory analysis of that bulk culture detected the presence of an inhibitory (suspected phage) towards the S. thermophilus strains in the starter (unpublished data).

Phage inhibition of Italian PHASE 4-MH

Table 41 presents a summary of the reformulation done with Italian PHASE 4-MH to make it more phage inhibitory. Two general approaches were taken to improve phage inhibition: (a) lower the

Table 41. Variations of Italian PHASE 4-MH.

	% w/v				
Ingredient	MH-1	MH-2	MH-3	MH-4	MH-5
Whey	5.91	6.89	6.89	6.89	6.89
NDM	3.19	0.63	0.63	0.63	0.63
YE[a]	0.50	0.45	0.45	0.45	0.45
MH[b]	0.65	0.61	0.61	0.54	0.54
DAP[c]	0.35	1.05	1.75	0.50	1.88
MAP[d]	1.63	0.83	-	2.33	1.13
CA[e]	-	0.50	1.06	-	0.50
$MnSO_4$	-	0.01	0.06	-	0.06
Total Solids (w/w)	10.9	9.9	10.3	10.2	10.8
% MH (w/w)	0.58	0.55	0.55	0.48	0.48
% PO_4^{-3} (w/w)	1.4	1.3	1.1	2.0	2.0
% CA (w/w)	0	0.45	0.96	0	0.45

[a]Yeast extract, [b]$Mg(OH)_2$, [c]$(NH_4)_2 HPO_4$, [d]$NH_4H_2PO_4$, [e]Citric acid monohydrate

total divalent cation concentration and (b) increase total chelating capacity of the medium. Since dried sweet whey contains approximately one-half the Ca^{+2} of NDM (88), the NDM solids were decreased to approximately 6% (w/w) of the total solids and whey solids added as replacement solids. The Mg^{+2} concentration was reduced by using less $Mg(OH)_2$. Additions of citrate and/or more phosphate were used to increase the total chelating capacity.

Complete inhibition of r2 phage replication did not result with any of the Italian PHASE 4-MH formulations, including the commercial medium Thermostar (Table 42). Formulations 2, 3 and 4 did reduce the final phage titers, but growth in these formula without phage resulted in a 50% loss of rods compared to the original Italian PHASE 4-MH (MH-1) and Thermostar. Italian PHASE 4-MH-5, which incorporated both citrate and higher phosphate, was most effective in suppressing the r2 phage but was inhibitory to rod development.

Table 43 compares several commercial media along with Italian PHASE 4-MH-4 in their abilities to contain the proliferation of the r2 phage. Only RCT-164 was effective in propagating the rod-coccus starter while restricting phage replication to a relatively low final phage titer. Italian PHASE 4-MH-4 and NDM provided no phage protection and the other three commercial media were only moderately effective. Phage infection was at a relatively low level of 50 pfu/ml.

Table 42. Ability of Italian PHASE 4-MH variations to support growth and inhibit phage (r2) replication with S. thermophilus[a] CR12 and L. bulgaricus[a] CR12 (6 h-41 C).

	w/phage r2		w/o phage r2		
Formulation	Activity (ΔpH)[b]	pfu/ml[c]	Activity (ΔpH)[b]	Total cfu/ml	% Rods[d]
ItpH4-MH-1[e]	0.9	1.7×10^{10} [f]	1.8	4.0×10^{9}	43
ItpH4-MH-2	0.5	1.4×10^{8} [g]	1.9	2.8×10^{9}	21
ItpH4-MH-3	0.3	9.3×10^{7} [g]	1.7	2.5×10^{9}	26
ItpH4-MH-4	0.4	1.1×10^{8} [h]	1.9	3.7×10^{9}	23
ItpH4-MH-5	0.1	6.0×10^{6} [h]	1.8	2.9×10^{9}	2
Thermostar	0.4	1.1×10^{8} [h]	1.2	1.4×10^{9}	52

[a] Isolates from CR12, S. thermophilus CR12 was attacked by phage r2

[b] Thermophilic activity test (1% inoc rate)

[c] Phage recovered

[d] Initial 40-50% rods

[e] 9.25% (w/w) solids

[f] Infected with 10^{2} pfu/ml

[g] Infected with 10^{5} pfu/ml

[h] Infected with 10^{4} pfu/ml

Table 43. Proliferation of phage r2 in Italian PHASE 4-MH and several commercial media with S. thermophilus CR12 and L. bulgaricus CR12 (5 h-41 C).

Commercial Medium	Activity (ΔpH)[a]	Total cfu/ml	% Rods[b]	pfu/ml[c]
NDM (11.0% w/w)	0.9	9.6×10^8	100	4.0×10^9
ItpH4-MH-4 (9.25% w/w)[d,e]	1.0	1.1×10^9	100	6.5×10^9
Italian Actilac (11.0% w/w)	0.9	1.4×10^9	86	1.3×10^6
Phage-Stat (11.0% w/w)	1.0	1.4×10^9	79	6.5×10^6
Thermostar (11.0% w/w)	0.8	1.7×10^9	47	2.1×10^6
RCT-164 (11.0% w/w)	0.8	1.6×10^9	63	3.5×10^3

[a] Thermophilic activity test (2% inoc rate)

[b] Initial rods; 51%

[c] Phage recovered; infection level at 50 pfu/ml

[d] 6 h-41 C incubation

[e] $(NH_4)_2\ HPO_4$, 0.63% (w/v); $NH_4H_2PO_4$, 2.13% (w/v) at 10.1% (w/w) solids

Swiss PHASE 4

A modification of the PHASE 4 formulation with lower phosphate and higher whey solids successfully supported the growth of four commercial S. thermophilus cultures (Table 44). Two of the cultures also grew well in the Italian PHASE 4-NPC medium. PHASE 4-MH did not support acceptable growth of the S. thermophilus cultures, apparently because of phosphate inhibition.

Table 44. Growth of commercial S. thermophilus cultures in Swiss PHASE 4 and several other bulk starter media (6 h-40 C).

MEDIUM	Activity (ΔpH)[a]			
	LAM[b]	ST 135[b]	ST[c]	ST4[c]
Thermostar[d]	1.5	0.6	-	-
Phage-Stat[d]	1.9	1.9	-	-
PHASE 4-MH	0.3	0.4	-	-
Swiss PH4	2.6	2.2	2.4	2.1
Italian PHASE 4-NPC	-	-	2.1	1.9

[a] 5 h-35 C (1 and 2% inoc rate)

[b] Frozen commercial culture (0.125 ml/l)

[c] Frozen commercial culture (0.5 ml/l)

[d] Sampled after 9 h-40 C incubation

$Mg_3(PO_4)_2$-buffered Plating Media

PMP medium

PMP was used as a plating medium to enumerate total lactic cell numbers during PHASE 4 research. Lactic streptococci produced bright yellow colonies surrounded by halos of clearing contrasted against a purple medium. The clear halos resulted from the acid-solubilization of the suspended $Mg_3(PO_4)_2$ in the medium. The insoluble $Mg_3(PO_4)_2$ served to localize the acid produced by the individual colonies. On crowded plates or regions of several closely associated colonies, clearing of the medium would overlap and form large halos.

PMP was also useful in differentiating acid-producing colonies from non acid-producers. This enabled easy detection of non acid-producing contaminants incurred during plating.

FSDA-II medium

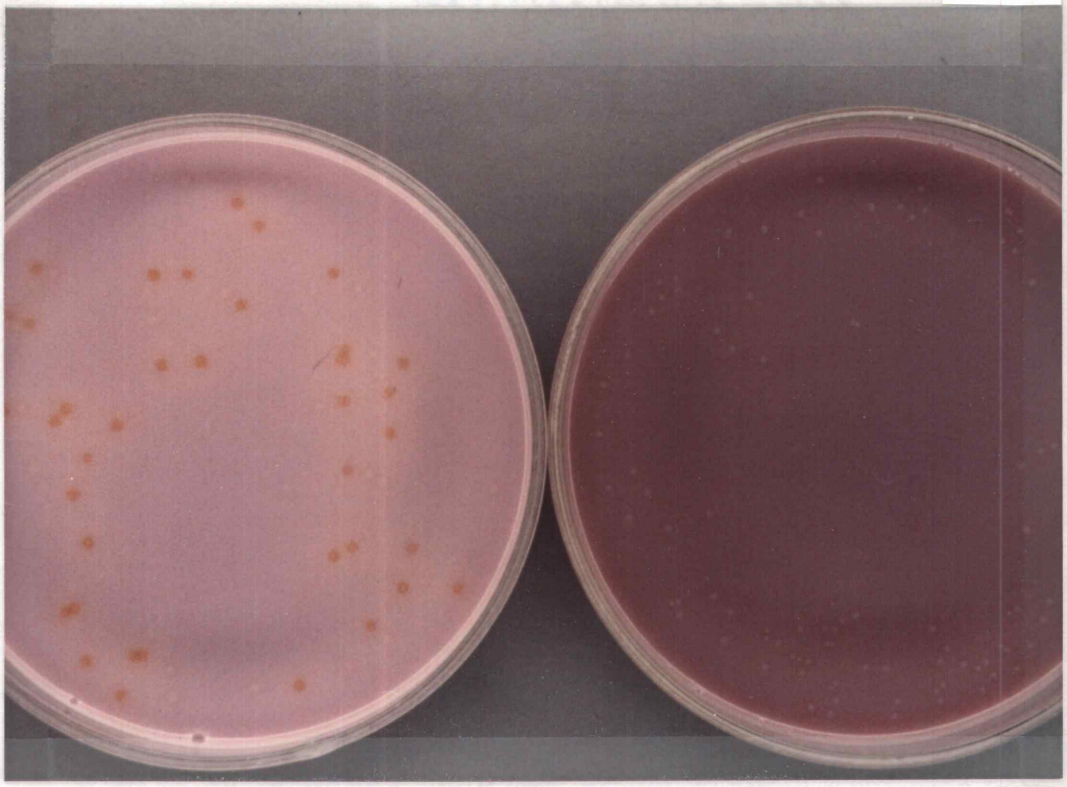
Halos resulting from solubilized $Mg_3(PO_4)_2$ were not detectable on FSDA-II because of the opaqueness contributed by the NDM solids. However, as with FSDA, differentiation between fast and slow acid-producers was possible on FSDA-II. Differentiation between lac^-, prt^+ and lac^-, prt^- was not possible on FSDA-II as with FSDA. Three distinct colony types were evident on FSDA-II. The *S. lactis* C2 parent appeared as a full-colored yellow colony against an opaque baby-blue background. The lac^+, prt^- colonies were less

brightly colored and resembled a yellow doughnut. Colonies of lac^-, prt^+ and lac^-, prt^- were indistinguishable from each other but clearly differentiated from the other two colony types. They appeared as colorless, translucent colonies; best visualized by observing their projection above the surface of the plating medium.

Six color photographs (Figures 16, 17 and 18) illustrate the differences between FSDA and FSDA-II. Figure 16 shows comparative plating of lac^+, prt^- and lac^-, prt^+ on both media and an enlargement of the FSDA-II plating. Visual observation of the FSDA plate reveals only a single colony type. A precise measurement of colony size would enable differentiation between the two colony types (lac^+, prt^- being slightly larger than lac^-, prt^+). However, with FSDA-II it is visually apparent that two distinct colony types are present. This is more clearly illustrated by the enlargement with arrow (a) pointing out a lac^+, prt^- colony and arrow (b) a lac^-, prt^+ colony.

Figure 17 is a comparative plating of all four phenotypes mixed together. On FSDA, two distinctly different colony types are evident, and again precise measurement would allow differentiation of an intermediate-sized colony representing the lac^+, prt^-, variant. On FSDA-II, three distinctly different colony types are clearly distinguishable without any measurement. The lac^+, prt^+, indicated by arrow P in the FSDA-II enlargement is a bright, fully-colored yellow colony with a darkened halo; the lac^+, prt^- designated by arrow (a) is not as brightly colored and is without a

Figure 16. (Top) Lac^+, prt^- and lac^-, prt^+ colonies on FSDA (right) and FSDA-II (left). Lac^+, prt^- (a) and lac^-, prt^+ (b) colonies on FSDA-II (bottom).



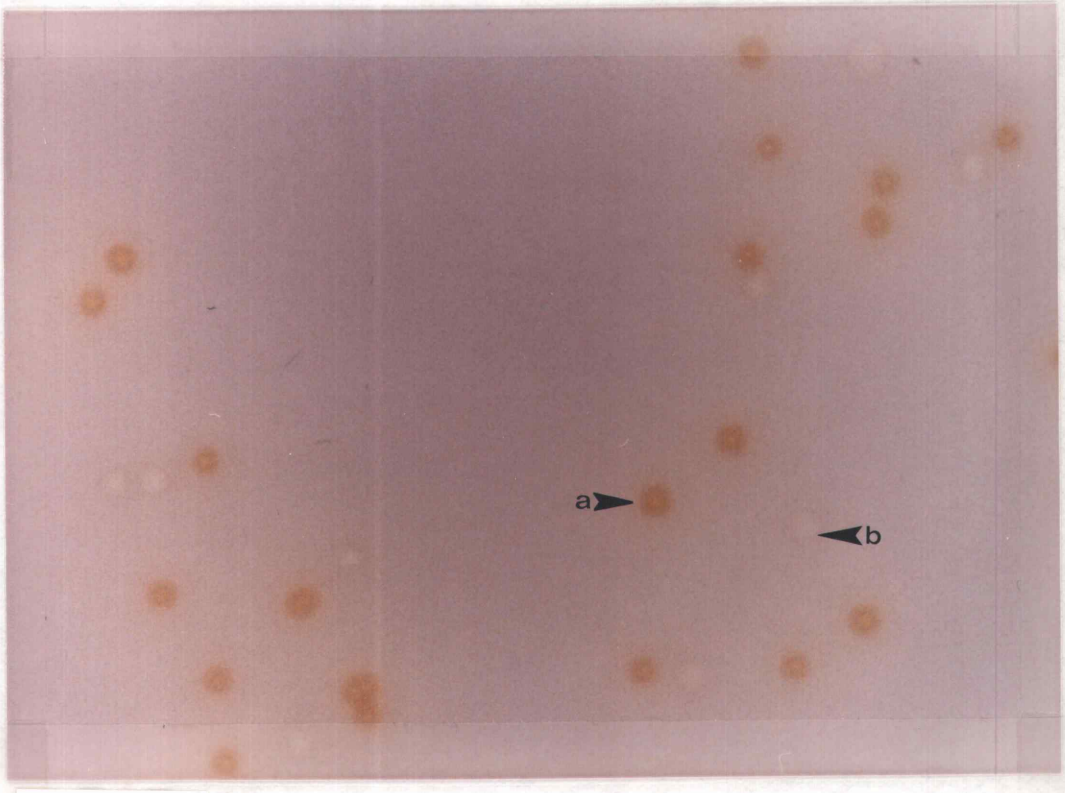


Figure 16.

Figure 17. (Top) Lac^+, prt^+; +, -; -, +; -, - colonies on FSDA (right) and FSDA-II (left). Lac^+, prt^+ (P); +, - (a) and -, + or -, - (b) colonies on FSDA-II (bottom).

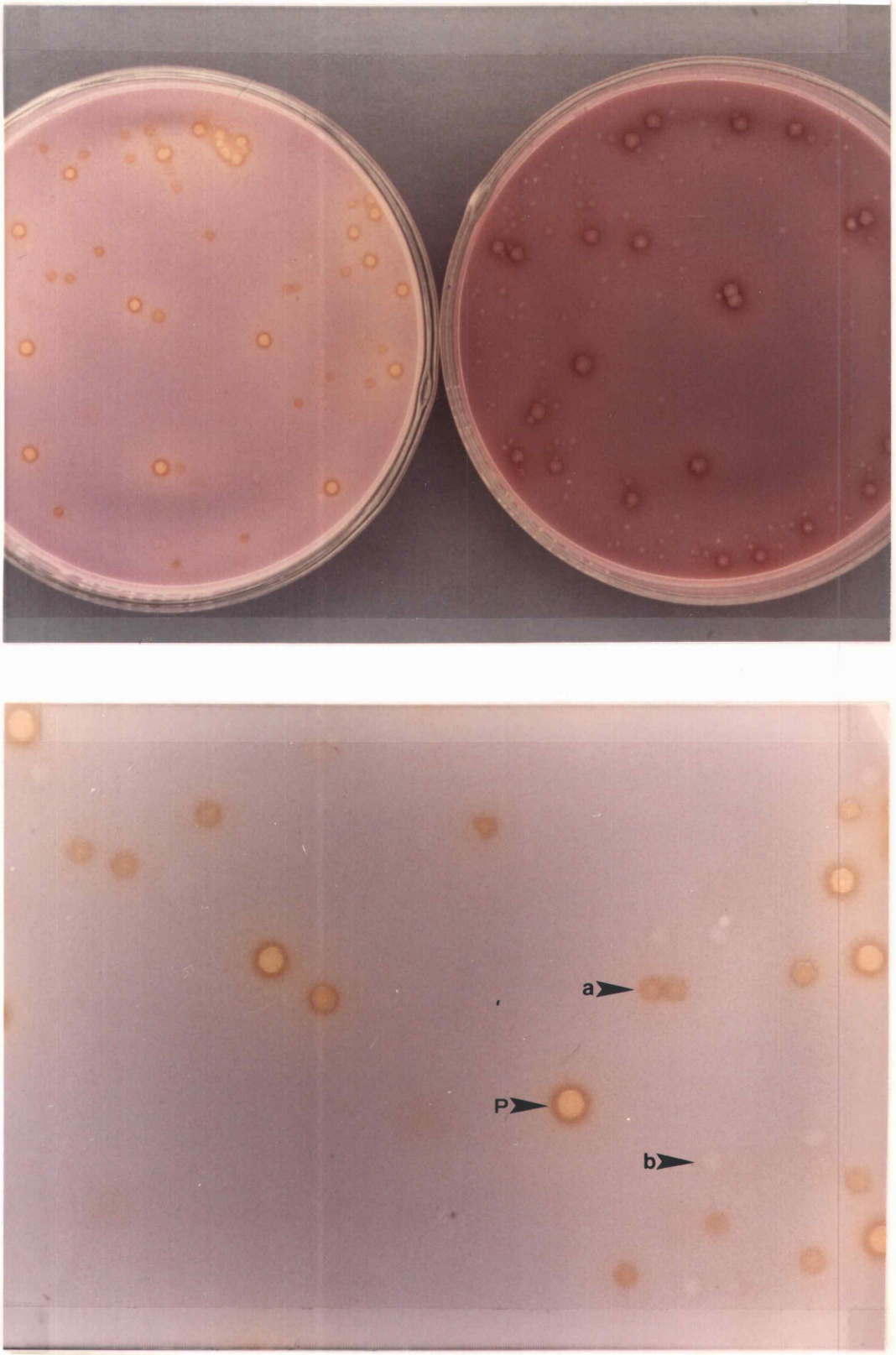


Figure 17.

Figure 18. (Top) Lac^+, prt^+ and lac^+, prt^- colonies on FSDA (right) and FSDA-II (left). Lac^+, prt^+ (P) and Lac^+, prt^- (a) colonies on FSDA-II (bottom).

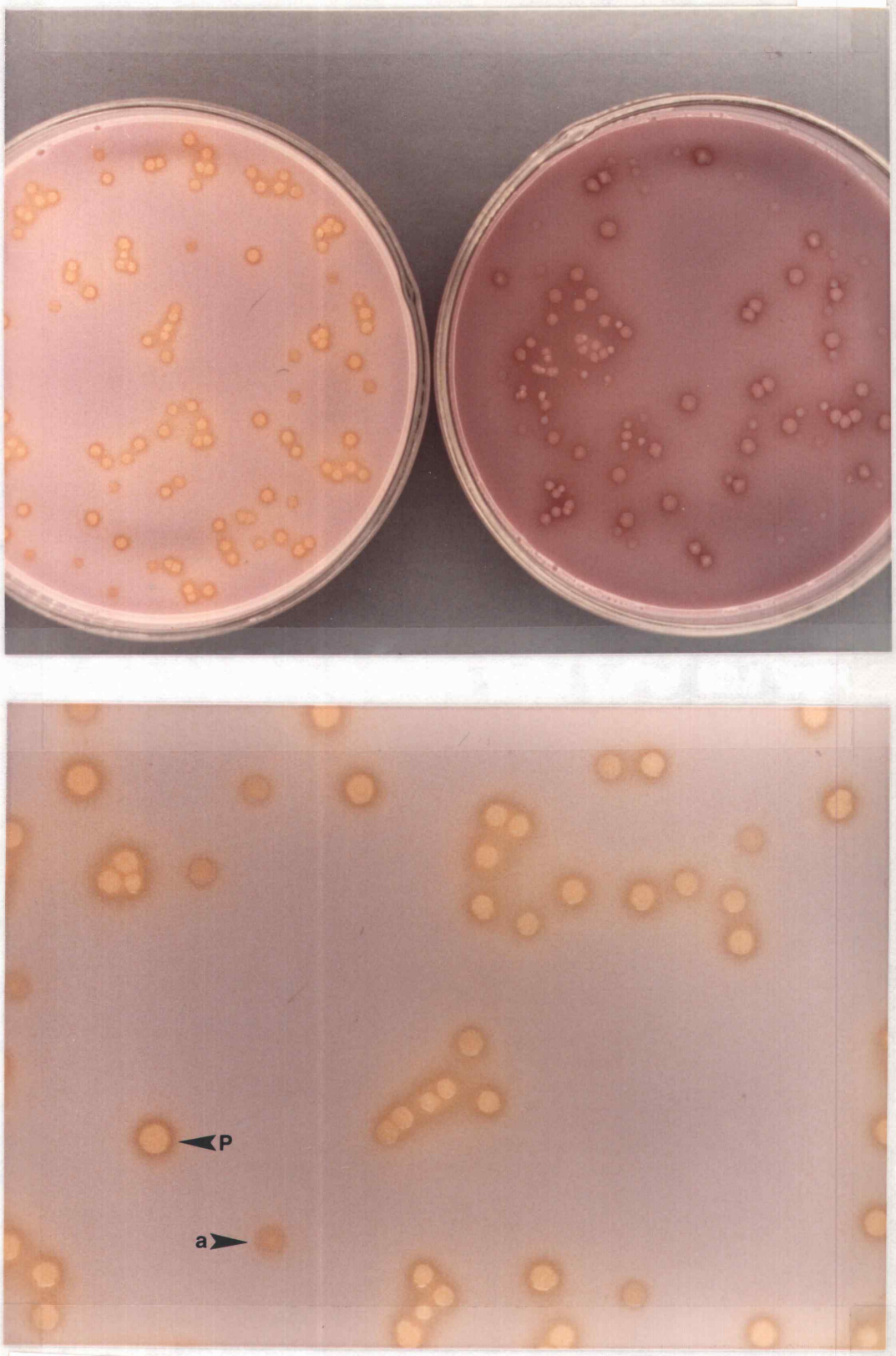


Figure 18.

halo. The lac^-, prt^+ and lac^-, prt^-, indicated by arrow (b) appear as colorless colonies.

Figure 18 depicts a plating of lac^+, prt^+ and lac^+, prt^- on both media. Two colony types are discernible on both FSDA and FSDA-II. The FSDA-II enlargement shows typical lac^+, prt^+ (P) and lac^+, prt^- (a) colonies.

Fast-slow differentiation on FSDA is dependent on size differences. Crowded plates or dehydrated plates (surface concentration of agar) make size differentiation difficult. Injured cells also appear initially as slow colonies on FSDA but with additional incubation time they slowly transform into typical fast colony types (John J. Wulf, M.S. Thesis, Oregon State University, 1981).

Differentiation on FSDA-II is based primarily on color differences and not size. This enables visual differentiation without the need for colony size measurement. Because of the lower phosphate concentration in FSDA-II (0.5% w/v) compared to nearly 2% (w/v) in FSDA, injured cells are more likely to recover and develop their true colony types on FSDA-II within the 48 h incubation period.

All the S. thermophilus strains examined grew equally well on FSDA or FSDA-II. However, L. bulgaricus 404 and 448 and L. helveticus 450 did not grow nearly as well on FSDA as on FSDA-II and MRS agar (control medium, Difco). Lactobacillus helveticus L112 and 15807, while growing poorly on FSDA-II, were completely inhibited on FSDA.

CHAPTER V

DISCUSSION

Increased cell populations for lactic streptococci result when the pH of the growth medium is maintained above pH 5.0 by neutralizing the metabolic end product, lactic acid (57). This principle is utilized by commercial starter culture manufacturers in preparing frozen culture concentrates by externally neutralizing the fermentation medium with concentrated alkali (59). External neutralization of bulk starter cultures in the individual cheese plant has also been adopted by the dairy fermentation industry to propagate highly active starter cultures for cheese manufacture (66).

We found single-shot external neutralization to be a simplified but workable approach to generating a more concentrated starter culture using external pH control. This approach has been recently used in three New Zealand cheese plants to produce bulk starter with greater activity per unit volume (41). The cell numbers approximately double during the additional growth period following neutralization which effectively increased the plants' bulk starter capacities without having to install additional bulk starter tanks. However, extreme care must be exercised to prevent phage contamination of the bulk culture when adding the alkali. The period of additional cell multiplication provides an opportunity for further phage proliferation if the medium is not phage inhibitory.

An internally neutralized medium obviates the need for mechanical addition of an external source of alkali. Internal neutralization requires that the alkali or buffer be discontinuous from the other soluble ingredients by either a physical barrier or by being temporarily insoluble; otherwise the high initial pH and/or high concentration of soluble buffer salts resulting from the presence of sufficient neutralizing power would inhibit culture growth.

Controlled release of Na_2CO_3 into the growth medium from encapsulated tablets was found effective in neutralizing much of the lactic acid and producing good holdover activity. From a commercial standpoint, disadvantages include the variable release rate dependent on the degree of agitation, nonuniform distribution of the macro-size tablets in the final dry blend and preventing the leftover encapsulation coatings from entering the cheesemaking process.

Timed-release from wax coated gelatin capsules in a static medium is used to transform nonselective enrichment media into selective media for the enumeration and detection of coliforms and Salmonella, respectively (38,80). The delayed release of the selective ingredients allows the injured cells to repair before the media become inhibitory. The principle of delayed release is also used to stimulate mushroom growth (8). Increases in mushroom yield are obtained when mushroom compost is supplemented with micro-droplets of vegetable oil within a denatured protein coat. Micro-

encapsulation of cheese ripening enzymes within a milk fat coating is also capable of being used as a valuable tool to effectively modify cheese flavor (43).

Microencapsulation of alkali for internal pH control is especially attractive because the small particle size would enable coated alkali to be uniformly blended into the final blend of ingredients. Attempts at microencapsulation of Na_2CO_3 were unsuccessful. It was especially difficult to produce a sufficiently resistant coating to prevent early release during the initial pasteurization period and during the lag phase of growth before lactic acid was actively produced. The potential for controlled release alkali would be greatly improved if a pH-dependent encapsulation material (maximum release at pH 5.5-6.0), meeting food grade requirements, could be found.

Trimagnesium phosphate and calcium carbonate are practically insoluble in water, hot or cold, but both compounds are soluble in dilute acid (91). When included in a growth medium for propagating lactic acid bacteria, $Mg_3(PO_4)_2$ and $CaCO_3$ remain insoluble until the pH begins to drop from the lactic acid that is produced by the growing cells. As the pH falls below 6.0, these pH-dependent compounds become increasingly soluble and neutralize the lactic acid, thus controlling the pH of the medium. Trimagnesium phosphate buffers most effectively around pH 5.5 while $CaCO_3$ buffers closer to 5.0. Both of these insoluble buffers are commercially available as food-grade powders and can be dry-blended with other common bulk

starter media ingredients. When such blends are reconstituted with water an excellent growth environment is provided in which the pH is internally controlled, allowing optimum yields of uninjured cells to be produced.

Dried sweet whey was selected as the principle nutrient source and source of lactose over more complete nutrient sources such as NDM, primarily because of economics. Skim milk solids cost approximately five times more than dried whey solids and contain only two-thirds the lactose on a weight basis. In addition, dried sweet whey contains only one-half the calcium of NDM (88) which is important in making the medium phage inhibitory. By formulating PHASE 4 to be lactose-limiting and coupled with sufficient buffering capacity, the final fermentation pH was controlled to stop at approximately pH 5.2-5.4. Since the accumulation of lactate to 2-3% eventually slows the growth rate of a neutralized lactic culture (52,59), the lactose concentration was set at approximately 2.5% by using 3.5% (w/v) dried sweet whey.

Our results showed very little difference between different sources of whey; however, subsequent field experience has shown a generally superior medium results from using whey obtained from Italian cheese production. Porubcan and Sellars (59) recommended the use of Swiss or mozzarella whey over Cheddar or cottage cheese wheys in media for propagating optimum yields of lactic streptococci. Both Swiss and Italian cheeses are generally manufactured with the thermophilic starters S. thermophilus and L. bulgaricus.

Streptococcus thermophilus is much more sensitive to penicillin in milk (37) than the lactic streptococci, and such increased sensitivity would help prevent antibiotic-contaminated whey from getting to the media supplier. Another possible advantage for using Swiss or Italian whey might be the unrelatedness of phage in the whey to lactic streptococci. Finally, the growth of L. bulgaricus or L. helviticus may also provide stimulation to the growth of S. cremoris and S. lactis as they do to S. thermophilus (28).

Optimum growth was attained when the PHASE 4 media were supplemented with 0.5% (w/v) yeast extract. This is in agreement with Richter et al. (68) who found 0.5% to provide the highest stimulation to S. lactis C_2F in lactose-limited skim milk. As with our results, they also observed that concentrations above 0.5% yeast extract did not further stimulate acid production.

The stimulatory properties of the yeast extract varied considerably between commercial suppliers and especially with the source of yeast used to prepare the extract. Porubcan and Sellars (59) recommended using two or more different suppliers simultaneously to minimize the lot-to-lot variations of any one yeast extract.

Yeast extract stimulates the growth of lactic streptococci primarily by providing a source of amino-acid material. Other stimulatory substances present in yeast extract include inorganic constituents and the bases adenine, guanine, uracil and xanthine

(75). Porubcan and Sellars (59) also found yeast extract to increase the resistance to ammonium lactate, produced with pH control, by exerting a therapeutic effect on the cells.

The incorporation of air into the bulk starter as a result of high agitation can lead to culture inhibition from the inhibitory levels of hydrogen peroxide that are produced by lactic streptococci (34). Addition of catalase or yeast extract (0.5%) can reverse the effects of stirring (34,58). To effect maximum neutralization with the insoluble buffering complex in PHASE 4, continuous agitation was recommended to keep the buffer in suspension during the bulk fermentation. Less starter usage by the cheesemaker coupled with the need to rotate several commercial cultures often resulted in making too small a volume for the bulk tank capacity. This problem led to excessive aeration during the starter fermentation. A high quality yeast extract to overcome this potentially inhibitory situation was required in PHASE 4. Intermittent agitation of PHASE 4 in such situations may also relieve some of the stress put on the pseudocatalase activity provided by the yeast extract without sacrificing significant activity of the bulk culture.

The relatively high level of yeast extract in PHASE 4 furnished a growth environment rich in amino acids and small peptides to allow 'slow' acid-producing cells to compete with the 'fast' parent cells. The lactic streptococci spontaneously generate slow acid-producing variants at a frequency of approximately 1-2%

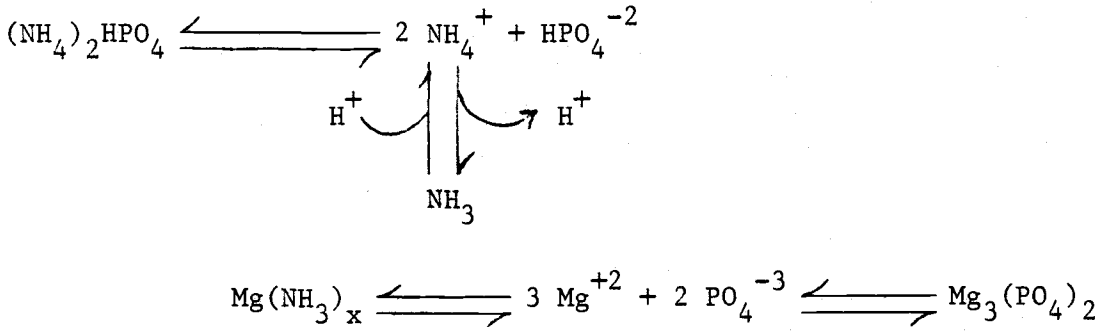
(13,20,56,89). Slowness results from a deficiency in proteinase activity and/or lactose fermentation, both of which have been shown to be plasmid-linked (45,46). Plasmids are relatively unstable and physical stresses such as pH, freezing, drying and growth at elevated temperatures may play a role in promoting their cellular loss. Ledford and Speck (39) speculated that culturing lactic starters in bulk starter media containing high concentrations of phosphate might promote the loss of a plasmid responsible for proteinase activity.

In milk the proteinase-deficient variants grow at low densities at the same growth rate as the parent; however, these slow variants become growth-limited at approximately 25% of the maximum cell populations reached by the parent culture because of the depletion of amino acids and peptides initially present. When available nitrogen compounds are added to milk, the growth and acid production of the 'slow' cells are markedly stimulated and resemble that of the 'fast' parent cells from which they can no longer be readily distinguished (13).

The stimulation provided by the yeast extract in PHASE 4 is essential for its commercial success. It appears important to minimize the concentration of 'slows' being inoculated into media such as PHASE 4 to prevent a slow dominated bulk culture from being used for cheese manufacture. As long as the proportion of proteinase-deficient 'slows' does not exceed 50% of the starter population, cheesemaking is unaffected (56). Control of this

factor would best be handled by the culture house in supplying 'fast' cultures and by the individual cheese plants in routinely performing activity tests on bulk starters before they are used in the cheese vat. In addition, to minimize exposure of proteinase-deficient 'slows' to the rich growth environment of PHASE 4, it is not recommended to use PHASE 4 in place of reconstituted NDM as a growth medium to propagate intermediate cultures for bulk culture inoculation.

As an ingredient for commercially-manufactured bulk starter media, trimagnesium phosphate is relatively expensive and available from only a few suppliers. A bulk starter medium entirely buffered with pre-formed $Mg_3(PO_4)_2$ would increase the raw ingredient cost of PHASE 4 by approximately 50%. When attempts were made to substitute soluble phosphates for part of the buffering capacity provided by $Mg_3(PO_4)_2$, the beneficial interaction between diammonium phosphate and $Mg_3(PO_4)_2$ was uncovered. This interaction allowed for partial substitution of the expensive $Mg_3(PO_4)_2$ with the widely available and reasonably priced diammonium phosphate without compromising the overall buffering capacity of the medium and in addition resulted in a physiologically more desirable starting pH. The following chemical relationship is proposed as a possible explanation for such an interaction:



As the diammonium phosphate disassociates in solution, the ammonium ion reacts with the free Mg^{+2} releasing a hydrogen ion and forming the $Mg(NH_3)_x$ complex. The ammonium ion shifts the $Mg_3(PO_4)_2$ equilibrium to further disassociation. The liberated H^+ accounts for the decrease in pH when the two compounds are combined. However, when lactic acid is produced by the cells during growth, the process is reversed and excess H^+ are absorbed. In addition, the medium is buffered by the HPO_4^{-2}, PO_4^{-3} and untapped $Mg_3(PO_4)_2$. Essentially, the buffering capacity provided by the dibasic ammonium phosphate is held in reserve by interacting with Mg^{+2} and released when needed as the equilibrium is shifted back with the production of more hydrogen ion.

Further reductions and finally the complete elimination of pre-formed $Mg_3(PO_4)_2$ was achieved by generating this compound or related chemical moieties <u>in situ</u>. These formulations were compositionally very similar, in theory, to the original PHASE 4-M formulation; however, physical differences were evident in their performances. With PHASE 4-M the $Mg_3(PO_4)_2$ was added preformed and blended with the other ingredients. Upon reconstitution with

water in the bulk tank, no additional chemical reactions took place, aside from the interaction with diammonium phosphate. PHASE 4-P would be expected to behave similarly in the starter tank since the only difference was that $Mg_3(PO_4)_2$ was prepared instead of purchased premade. PHASE 4-MH, even though compositionally very similar to PHASE 4-M and P, was a dry-blended mixture of the raw ingredients before generation of magnesium phosphate compounds. Not until this blend was reconstituted did the chemical conversions to magnesium phosphates occur in the bulk tank. Serious uniformity problems resulted with the blending of this formulation because of the differences in particle size and the densities of the ingredients. In addition, serious problems of excessive settling of insoluble solids on the bottom of bulk tanks occurred in numerous cheese plants. Often, these settlings would cement together on the tank bottom into a solid mass making for very difficult clean-up for the cheese plants. It is now believed that the insoluble nature of $Mg(OH)_2$ in PHASE 4-MH made it difficult for the complete chemical reactions to take place in the bulk tank before the high temperature from bulk pasteurization began precipitating the whey proteins, which then in combination with some of the unreacted $Mg(OH)_2$ formed the cemented mass on the bottom.

The settling problem was accentuated in many individual cheese plants due to bulk starter tank design. A wide variety of tanks are used to prepare bulk starter and many are not equipped to properly agitate a suspension such as PHASE 4. Some tank bottoms

are domed resulting in a ring of settling around the outer rim where the agitator does not effectively reach. Others are square, resulting in dead spots in the corners. Even many of the newer swept-surface agitating tanks have dead spots immediately under the agitator shaft.

PHASE 4-MC not only improved the uniformity of blending, but now much of the Mg^{+2} was present in a more soluble form as magnesium citrate and a more complete conversion to magnesium phosphates in the bulk tank resulted. This resulted in disappearance of the settling problem in commercial usage. Insoluble solids remained in the bulk tank, but they no longer fused together to form the hard settlings found with PHASE 4-MH.

PHASE 4-MS, the newest generation of PHASE 4 reformulation, is expected to allow even greater conversion to magnesium phosphates because the Mg^{+2} is completely soluble as $MgSO_4$. In addition, it is speculated that this formulation can be completely dry blended with uniformity. This would simplify the production and eliminate the need to make the GW-4-4 base, which has proven most recently to be a difficult ingredient to produce consistently and accounts for the majority of quality problems that exist with the existing PHASE 4 product.

Another problem that went undetected in the early laboratory studies with PHASE 4 but was eventually realized in the field was the adverse effects from carryover buffer. Because of the potent buffering of PHASE 4, unused buffer in the ripened culture when

added to the cheese vat would tend to buffer the pH above the normal cutting or milling pH. This problem was most acute in cottage cheese manufacture using unusually high inoculation rates or in sweet acid cheeses such as Colby and Monterey Jack. The final result in the American cheeses was undergrade cheese because of too high a pH. Corrections in make procedure such as milling at higher titratable acidities were necessary to overcome this problem. In cottage cheese, time for cutting the cheese based only on titratable acidity often resulted in a premature cut with fragile curd. Cutting on pH was necessary to protect from such happenings. In general, conversion to PHASE 4 from conventional bulk starter media or reconstituted NDM required subtle adjustments in cheese manufacturing procedures by the individual cheesemakers.

The effect of the carryover $Mg_3(PO_4)_2$ into the cheese milk is much like the effects of the natural buffering in cheese by calcium phosphate. Calcium phosphate is important in maintaining a satisfactory pH after salting. Too much 'wet acid' during Cheddar cheese manufacture results in much of the insoluble calcium phosphate solubilizing and being lost in the whey which can result in a low pH at press (37).

Magnesium phosphate will also help maintain a desirable press pH, except when excessive amounts are trapped in the cheese and the pH is held too high because of the higher pK_a for $Mg_3(PO_4)_2$ than $Ca_3(PO_4)_2$. The presence of $Mg_3(PO_4)_2$ in the cheese milk can also be viewed as a built-in safety factor in those cases where

too much acid is produced too early by an overly active starter or use of too much starter. The additional buffering capacity of the milk prevents the pH from dropping too low and resulting in an intense acid flavored cheese.

Based on our research findings we strongly recommend the commercial use rate of PHASE 4 be lowered from 8.7 to 7.5-8.0% (w/w) solids. However, economic pressure dictated that such a change not be made. Recent reformulation as PHASE 4-MS has provided the opportunity to reduce the amount of carryover buffer without altering the commercial use rate.

Ever since the findings by Reiter (65) in 1956 that phage replication was inhibited without calcium and by Hargrove et al. (25) who demonstrated that a phosphated milk medium effectively tied up the free calcium and prevented phage development, most commercial bulk starter media have been formulated with the intent to make them phage inhibitory. This approach by the dairy industry has been heavily relied upon in the United States to help control phage (71).

PHASE 4 was made phage-inhibitory by using whey instead of NDM solids, reducing the $Mg_3(PO_4)_2$ by substitution with diammonium phosphate and using citrates to bind the remaining Ca^{+2}. Phage inhibition in PHASE 4 appears to be critically affected by the balance of Ca^{+2}-Mg^{+2} to PO_4-citrate. Potter and Nelson (61) showed that Mg^{+2} alone failed to replace Ca^{+2} in lactic streptococcal phage proliferation; however, Mg^{+2} apparently functioned in a

sparing action at sub-optimum concentrations of Ca^{+2}. This critical balance of the cations Mg^{+2} and Ca^{+2} with PO_4-citrate makes precision blending of PHASE 4 a critical factor.

Our results indicated phage inhibition in commercially blended PHASE 4 was marginal when the solids usage was dropped from 8.7 to 7.8% (w/w). This may appear as a strong reason to use PHASE 4 at the higher solids level regardless of the consequences of carryover buffer; however, phage inhibition in bulk starter media is overrated as a phage control measure. A phage inhibitory medium will only prevent phage proliferation in the bulk starter tank, not in the cheese vat. Effective sanitation practices, sufficient bulk pasteurization and careful strain selection should prevent significant phage proliferation in the bulk starter tank. Phage inhibitory media can lead to a false security by the cheesemaker that often results in a breakdown of good sanitation practices. More effective control of phage in bulk starters can be accomplished by using lactic cultures that are insensitive or resistant to phage; with in-plant monitoring of the phage present against those cultures, phage inhibition provided by the bulk starter medium is no longer necessary (84).

Extensive reformulation of Italian PHASE 4-MH was not effective in establishing a phage inhibitory medium for the propagation of the thermophilic starter bacteria. Nurmikko and Karha (50) found calcium was necessary for growth in 30 of 124 strains of S. thermophilus. Citrate and phosphate inhibited many of these S.

thermophilus strains, but this inhibition was reversed by the addition of calcium. In addition, only two amino acids are required for growth by S. thermophilus; however, in a calcium-free medium, five additional amino acids are required for growth (51). Apparently calcium plays a role in the amino acid metabolism of S. thermophilus. In contrast, we found elevated levels of citrate and/or phosphate effected most obvious inhibition of rod development.

From the standpoint of both organisms, it appears impractical to formulate a calcium-free thermophilic bulk starter medium to inhibit all phage replication for the thermophilic starters. Phage inhibition and optimum growth conditions for the rod-coccus starters appear mutually exclusive. Attempts should be made to maximize growth, and phage inhibition might be best handled at the level of the cultures in selecting phage insensitive strains, much like is done in some regions with lactic streptococcal starters.

Italian cheese varieties are typically manufactured with 'balanced' bulk starter cultures containing both S. thermophilus (coccus) and L. bulgaricus (rod). These two organisms have a complementary relationship when grown together. The streptococci initiate growth, removing excess oxygen and producing stimulatory compounds such as formic acid (19) for growth of the lactobacilli. In return, the lactobacilli liberate peptides and amino acids which stimulate further growth by the streptococci. Moon and Reinbold (48) showed that the growth of S. thermophilus and L. bulgaricus

together stimulated greater acid production than when either organism was grown alone. A properly-formulated Italian bulk starter medium should allow these two organisms to grow together in approximately equal proportions. A culture dominated by either of the organisms results in irregular cheese manufacture.

Swiss cheese (USA), on the other hand, is more commonly made with separate bulk cultures of S. thermophilus and L. bulgaricus, L. helveticus or L. lactis, with the coccus culture making up the majority of the inoculum. Bulk starter media are generally formulated to best suit S. thermophilus growth whereas the rod culture is generally grown in milk.

A major difference between PHASE 4 and the Italian PHASE 4 formulations was the degree of pH control. PHASE 4 was formulated to be lactose-limiting and, coupled with sufficient buffering capacity, the pH was maintained well above 5.0. The Italian PHASE 4 products were well-buffered with insoluble, pH-dependent buffers but the pH was designed to drop below 5.0 to promote better rod growth. This lower final pH did not seem to affect the holdover activity since S. thermophilus and L. bulgaricus are more acid tolerant than the lactic streptococci.

Two distinct types of commercial rod-coccus cultures were found during the development of Italian PHASE 4, phosphate tolerant and phosphate sensitive. The phosphate tolerant cultures were generally manufactured by Marschall Products, which was not really a surprise since they reportedly screen their cultures in the phosphated

medium Thermostar before releasing for use by the industry. The phosphate sensitive cultures were produced by the other two major suppliers, Chr. Hansen and Microlife Technics. Phosphated Italian PHASE 4-MH was found most compatible with the CR cultures from Marschall's, and it was necessary to develop a nonphosphated medium, Italian PHASE 4-NPC using calcium carbonate, to support the growth of the phosphate sensitive cultures. Both Italian PHASE 4 media were more effective than Thermostar in supporting the growth of the commercial cultures that were evaluated.

A measure of starter growth in the bulk tank is commonly made by pH and titratable acidity. With $CaCO_3$-buffered media it was possible to use pH but not titratable acidity as a criterion for "breaking" the starter. As lactic acid is produced the carbonate is transformed to bicarbonate which is further converted to carbonic acid which eventually decomposes spontaneously to CO_2 and water. As a bulk starter tank is an open system and aided by continuous agitation, the CO_2 is released into the atmosphere leaving free H_2O. Therefore, carbonate-buffered media prevent the accumulation of hydrogen ions and hence of free acids in the media. In a $Mg_3(PO_4)_2$-buffered medium, the developed acidity is absorbed by the conjugate base to form the conjugate acid. Upon titration with NaOH the conjugate acid releases the H^+ as it is converted back to the conjugate base. The amount of NaOH required gives a direct measure of the lactic acid that was produced.

A widespread fear of pH-controlled bulk starter media is the increased opportunity for growth by undesirable bacteria in such media. In reconstituted NDM, Gilliland and Speck (23) found the growth of both Staphylococcus aureus and Salmonella gallinarum to be markedly depressed with or without pH control (pH 6.6) when grown in combination with lactic starter cultures. The contamination level of S. gallinarum was considerably lower at 600-700 c.f.u./ml compared to 10^5 c.f.u./ml used in our research and may explain why we observed good growth of S. jacksonville in pH-controlled media in the presence of an active lactic culture.

Streptococcus diacetilactis also effectively inhibits a variety of food spoilage organisms and pathogens in milk. Involvement of one or more of the following factors has been suggested as possible mechanisms responsible for this inhibition: (a) production of antibiotic substances, (b) hydrogen peroxide production, (c) essential nutrient depletion, (d) reduction of oxidation-reduction potential and (e) production of organic acids in combination with low pH (15).

Barach (5) recently reported on the fate of S. aureus, B. subtilis and E. coli when grown in combination with lactic starter cultures in a conventional phage inhibitory medium (PIM) and in a whey-based medium externally neutralized at pH 6.0. Only the conventional PIM restricted the growth of all three contaminants. The neutralized medium prevented the growth of S. aureus and B. subtilis, but it could not contain the growth of E. coli.

It was reported by Thomas (82) that nearly 50% of the plants using external pH control have experienced some degree of contamination problems, coliforms being the most frequent contaminant isolated from the pH-controlled starter. He also presented data showing that coliforms increased dramatically in the neutralized starter from low contamination levels. Neutralized starter cooled to only 10 C continued to support coliform growth, and post fermentation contamination also resulted in coliform growth at 10 C. Coliforms were suppressed in a conventional medium and after cooling to 5 C in the neutralized medium.

Our results supported this increased risk of growth by gram negative bacteria in pH-controlled bulk starter media. Critical control of contamination is achieved with effective sanitation practices and not with a growth inhibitory bulk starter medium. Careful sanitation programs and a little common sense are necessary to prevent contamination of bulk starter culture with undesirable contaminants and preclude such contaminated bulk cultures from being used for cheese manufacture.

It is somewhat surprising that unbuffered plating media are used for the isolation and enumeration of lactic acid bacteria when the merits of plating on buffered media have been demonstrated as early as 1946 (33). The insoluble nature of $Mg_3(PO_4)_2$ or $CaCO_3$ makes it ideally suited for plating media in that acid production by individual colonies is localized and visually apparent in the form of a cleared halo surrounding the colonies. In addition,

whereas cells that are injured might be inhibited from recovery on a medium with a high soluble buffer content, optimum recovery should be allowed to take place on plating media such as PMP and FSDA-II.

FSDA was designed for the differentiation of fast acid-producing colonies of lactic streptococci from the slow acid-producers (30), and this medium has been successfully used in the industry for the improvement of commercial cultures during the past few years (A. R. Huggins-personal communication). FSDA-II offers no advantage over FSDA in the routine selection of 'fast' isolates for starter culture maintenance; however, FSDA-II allowed easier characterization of the genetic classes of slowness in lactic streptococci and it supported better growth of the thermophilic lactobacilli than did FSDA, where glycerophosphate is inhibitory to some strains (73).

The concept of internal pH control appears in general to be beneficial to the growth of lactic acid bacteria, not only for the propagation of mesophilic and thermophilic starter bacteria, but variations of PHASE 4 also appear very promising for growing the leuconostocs and pediococci.

Recent research (R. K. Thunell-personal communication) has shown PHASE 4 to be an excellent growth medium for the propagation and preparation of frozen bulk set cultures. These cultures retain excellent activity and viability during storage at -40 C.

Research done with L. acidophilus suggests the physiological state that is maintained or adapted through batch culture is an important factor in determining resistance to freezing and frozen

storage. Divalent cations are inductive of such morphological changes in some strains that are beneficial in the production of freeze-stable cell crops (92).

Thomas and Batt (83) observed prolonged survival of S. lactis grown in a lactose-limiting medium and resuspended in phosphate buffer with the addition of Mg^{+2}. Mg^{+2} decreased the toxicity of Cu^{+2} and provided a stabilizing effect by maintaining the integrity of cell ribonucleoprotein. Surviving organisms showed prolonged division lags in the absence of Mg^{+2}. Accelerated death, prompted by the addition of fermentable carbohydrate to the starved cells, was also reduced by the addition of Mg^{+2}.

Ough and Groat (53) observed increased fermentation rates by some strains of yeast in grape juice from the addition of insoluble solids coupled with continuous agitation.

Such research suggests that PHASE 4 may provide some additional physiological advantages beyond simply controlling the fermentation pH. Research is needed to study the physiological effects of PHASE 4 on the growth of various acid-producing bacteria. Greater knowledge is required in this area to more effectively apply the technology of internal pH control to other fermentation applications. Potential application to fermentations involving sausage production, silage, pickles, beer, wine, and fermented vegetables are just a few areas that require investigation. In addition, further research is needed in applying the use of insoluble buffers to various plating media for the isolation and

maintenance of acid-producing organisms, many of medical importance. Finally, applications are feasible in areas not directly involving fermentation. Recent research has shown eating Cheddar cheese aids in preventing tooth decay due to buffering effect of the cheese solids on the acid produced by S. mutans (54). Incorporation of $Mg_3(PO_4)_2$ into sugar-free chewing gum could effectively have the same results.

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APPENDIX I

EVALUATION OF CONVENTIONAL AND pH-CONTROLLED BULK STARTER MEDIA[1]

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ABSTRACT

Conventional phosphated bulk starter media were compared to pH-controlled media, both external and internal neutralization, for their abilities to promote the growth of lactic starter cultures and to suppress bacteriophage proliferation. The pH-controlled media produced bulk cultures with greater activity per unit volume than the conventional media. Only a conventional medium with a boosted buffering capacity generated bulk cultures that had comparable activities to the pH-controlled media.

However, the conventional media were very effective in preventing phage proliferation in the bulk starter while the externally pH-controlled media were not phage inhibitory. Only the internal pH control medium and the highly buffered conventioanl medium were capable of producing superior activity while still providing effective phage inhibition.

INTRODUCTION

It is well established that calcium is necessary for maximum bacteriophage proliferation in the lactic streptococcal host (5,6,23,24,26,31). Reiter (27) observed in 1956 that lactic phage replication was inhibited in milk in which the calcium ions had been removed. Hargrove (11,12,13) demonstrated that by adding phosphates (1-3% final conc) to milk most lactic phage proliferation was suppressed, yet host growth was as good as in milk without phosphates. Zottola (34) later formulated various combinations of orthophosphates (2% reconstituted conc) that could be dry-blended with nonfat milk powder-electrodialyzed whey combinations. Upon reconstitution, pasteurization and inoculation, these formulations successfully inhibited most phage replication. Subsequently, several commercial products emerged that were phosphated with a nonfat dry milk or dried sweet whey base and are still widely used by the dairy industry for the production of bulk starter cultures.

Gulstrom et al. (10) recently evaluated the growth-promoting and phage-inhibitory properties of several commercial bulk starter media and found only 2 out of 7 media to effectively suppress phage replication. Since then, externally neutralized starter media (4,18,25,28) are being used by more cheese plants for the production of bulk starters. The pH of these media is generally controlled by the external addition of gaseous NH_3 or concentrated liquid alkali such as NH_4OH or NaOH. Another pH-controlled bulk starter medium in which the pH is internally controlled (34) by a patented

buffering complex (30) is also successfully being used throughout the dairy industry with increasing acceptance.

The purpose of this investigation was to compare the performance of the more traditional, milk-based media containing conventional phosphate buffering to the more recent pH-controlled bulk starter media.

MATERIALS AND METHODS

Commercial bulk starter media and laboratory scale bulk fermentations

Seven commercial bulk starter media were prepared according to the manufacturer's instructions. Two of the products were conventional milk-based media (CB). Three of the media used external pH control (EN). The fermentation pH for these media was maintained at 5.8 ± 0.05 by the external addition of 20% (w/w) NH_4OH. An internally neutralized medium (IN) and a conventionally phosphated medium with increased buffering (HCB) were also evaluated. Reconstitution of the seven products is summarized in Table 45.

Pasteurization of the reconstituted media was at 85-90 C for 40-45 min on a Corning magnetic stirrer-hot plate with high agitation. An outer stainless steel bath filled with water was used to evenly distribute the heat around the 1000-ml fermentor vessel during heating. Following pasteurization the vessels were cooled to 24 C in ice water.

Laboratory bulk fermentations were run with a bench-scale fermentor (Fermentation Design Inc., Allentown, PA) equipped with automatic pH and temperature control. Modification was made to provide top-stirring agitation with a stainless steel paddle powered by a variable-speed electric motor and controller (GT-21, GK Heller Corp., Floral Park, NY). Incubation was at 24 C for 16 h. The pH-controlled media were agitated continuously throughout the

Table 45. Reconstitution of the seven commercial bulk starter media.

Medium	% Total Solids (w/w)	Medium Supplements[a]
EN-1	7.5	None
EN-2	7.0	25% whey + 50% NDM
EN-3	8.0	25% whey + 50% NDM
CB-1	11.5	50% NDM
CB-2	11.5	50% NDM
HCB	8.1	None
IN	7.8 (8.7)	None

[a]The additional ingredients, dried sweet whey (Tillamook County Creamery, Tillamook, OR) and NDM (Galloway West Co., Fond du Lac, WI), were used to make the complete media as instructed by the product manufacturers.

fermentation period while the three conventionally buffered media were only agitated initially for 5 min to mix in the inoculum and for 5 min prior to sampling.

Lactic cultures and phages

Three different lactic culture systems were used to evaluate the bulk starter media: (a) commercially-prepared frozen bulk set cultures used according to the manufacturers' instructions (b) a multiple strain starter consisting of six phage insensitive strains (7) grown separately in reconstituted NDM (11% w/w) for 16 h at 21 C and then combined and inoculated into the various media at a 1% total inoculation rate (S. lactis BA-1, BA-2, S. cremoris 108, 290-P, 224 and $SK_{11}G$) (c) single strains of S. cremoris (205, 134 and $SK_{11}G$).

Lab propagated strains were maintained as described by Daniell and Sandine (7) and phages were maintained, propagated and assayed on M17 medium as previously described (32). Frozen commercial bulk set cultures were stored at -80 C.

Phage-host combinations hp-S. cremoris HP and ml_8-S. lactis ML_8 along with two apparently low calcium-requiring combinations, 407-S. lactis 407 and T189-S. cremoris 205, were used to determine phage inhibition of the various media. Phage-host 407-407 was found by Gulstrom et al. (10) to increase in titer in several commercial media, and phage-host T189-205 is not completely inhibited by the best medium (F) of that study (16,33).

Buffering capacity

CB-2, HCB and IN were reconstituted at their respective solids levels in 100-ml volumes. The buffering capacity of each medium was determined by measuring the ml of 10% (w/w) lactic acid necessary to lower the pH to 5.0.

Culture growth

Duplicate tubes of 10 ml - reconstituted NDM (11% w/w) were pasteurized (62.8 C for 30 min) and inoculated with the mature bulk starters and subjected to a controlled temperature profile (CTP) activity test as described by Pearce (19) and Heap and Lawrence (15). Culture activity was measured as the total change in pH (ΔpH) from 0.5, 1.0 and 2.0% inocula.

Total cell counts were determined by a micro-drop plating technique (submitted to Appl. Environ. Microbiol.).

Phage inhibition

Each medium was inoculated with 1% host bacteria and infected with sufficient phage to provide approximately 10^5 pfu/ml. The final phage titer was determined by the double layer plaque assay of Adams (1) on M17 agar. One ml of the phage-infected starter was transferred to 9 ml of chilled sterile saline (0.85%) and centrifuged at 5000 x g for 10 min. The supernatant was serially diluted in saline and titered.

Commercial cheesemaking trial

The bulk starter media HCB and IN were used to prepare 100 and 200 gallons, respectively, of bulk cultures using the same frozen commercial culture as the inoculum. Both media were ripened according to the manufacturers' instructions. Cheddar cheese (640 lb block) was made in 45,000 lb capacity vats with each of the bulk cultures.

RESULTS

Buffering capacity

CB-2, HCB and IN (100 ml of each) required 15, 26 and 40 ml, respectively, of 10% lactic acid to lower the medium pH to 5.0. HCB had 73% more buffering capacity than the conventional medium CB-2, but the buffering capacity of HCB was still 35% less than the internal pH control medium, IN.

Culture growth

Figures 19, 20 and 21 present the activity and cell count data for the tested media. Culture activity appears to closely correspond to total cell numbers. With all three culture systems, the pH-controlled media (EN-1, 2, 3 and IN) had higher activity and greater cell populations than the two conventional buffered bulk starter media, CB-1 and 2. A notable exception resulted with the EN-1 medium's failure to support the growth of S. cremoris 205.

Phage inhibition

Figures 22 and 23 illustrate the phage inhibitory dimensions for 6 of the 7 commercial media as tested with four different phage-host combinations. Reconstituted NDM (11% w/w) served as the non phage inhibitory control. The two conventionally buffered media, CB-1 and 2, along with the internally neutralized medium IN, effectively prevented any increase in phage titer with the hp phage.

Figure 19. Growth of a commercial frozen bulk set lactic culture in various bulk starter media.

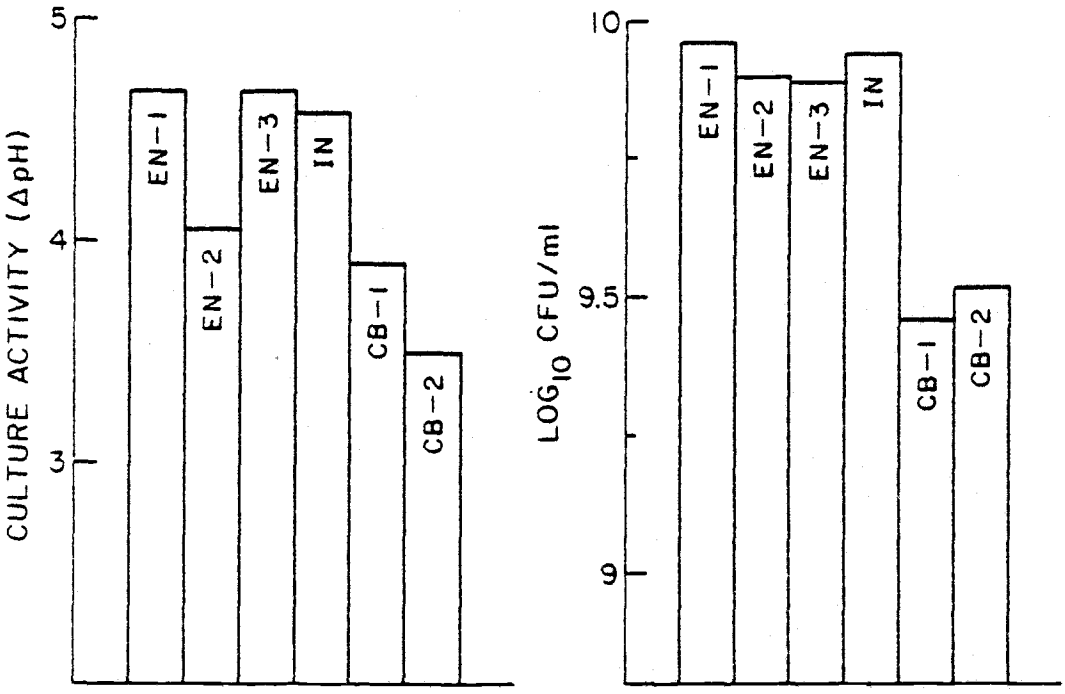
CULTURE ACTIVITY (ΔpH)
5
4
3
EN-1
EN-2
EN-3
IN
CB-1
CB-2
LOG10 CFU/ml
10
9.5
9
EN-1
EN-2
EN-3
IN
CB-1
CB-2

Figure 19.

Figure 20. Growth of a multiple strain starter culture composed of six phage insensitive Streptococcus lactis and Streptococcus cremoris strains in various bulk starter media.

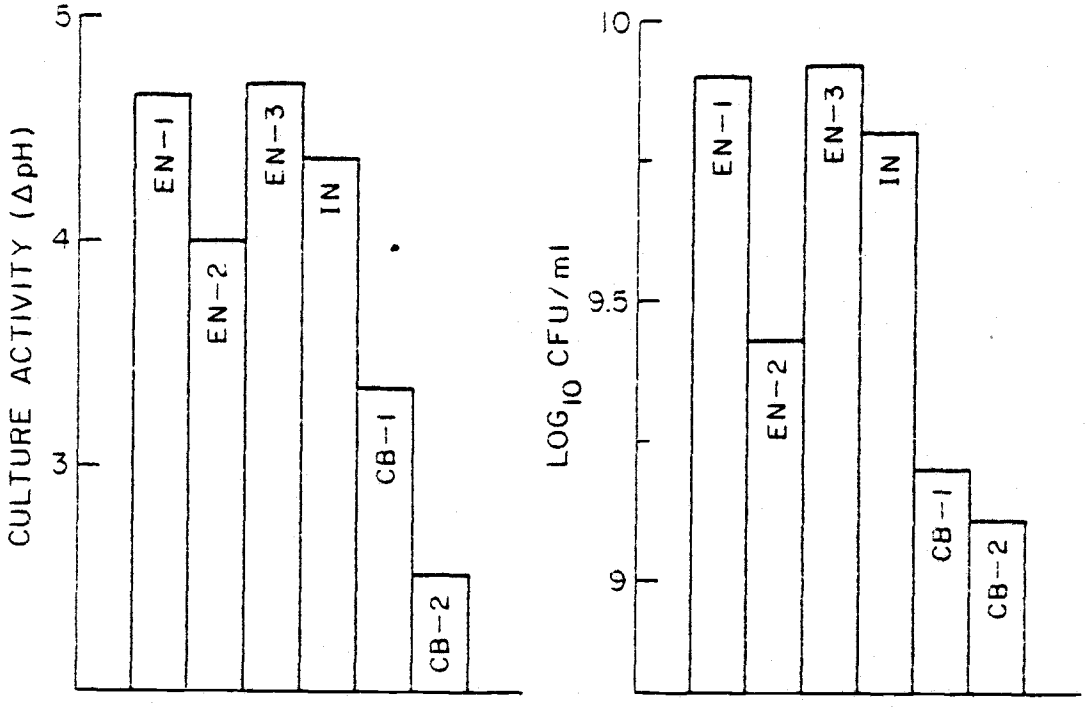
CULTURE ACTIVITY (ΔpH)
5
4
3
EN-1
EN-2
EN-3
IN
CB-1
CB-2
LOG10 CFU/ml
10
9.5
9
EN-1
EN-2
EN-3
IN
CB-1
CB-2

Figure 20.

Figure 21. Growth of Streptococcus cremoris 205 in various bulk starter media.

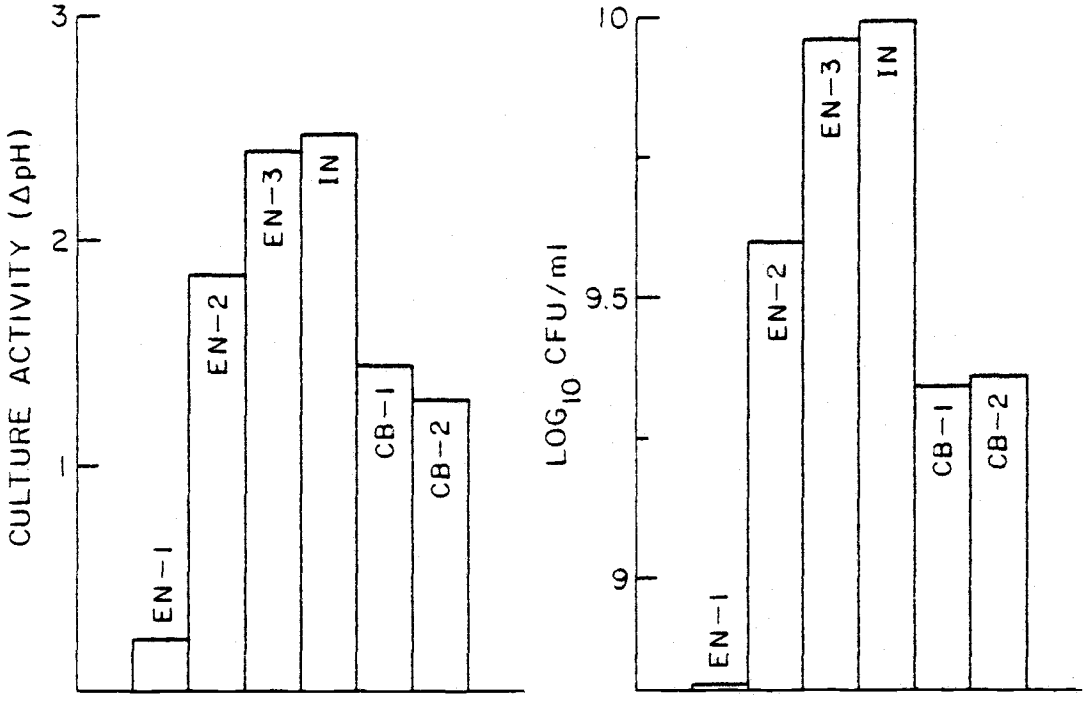
CULTURE ACTIVITY (ΔpH)
3
2
1
EN-1
EN-2
EN-3
IN
CB-1
CB-2
LOG10 CFU/ml
10
9.5
9
EN-1
EN-2
EN-3
IN
CB-1
CB-2

Figure 21.

Figure 22. Proliferation of phages hp and T189 in various bulk starter media following infection with 10^5 pfu/ml.

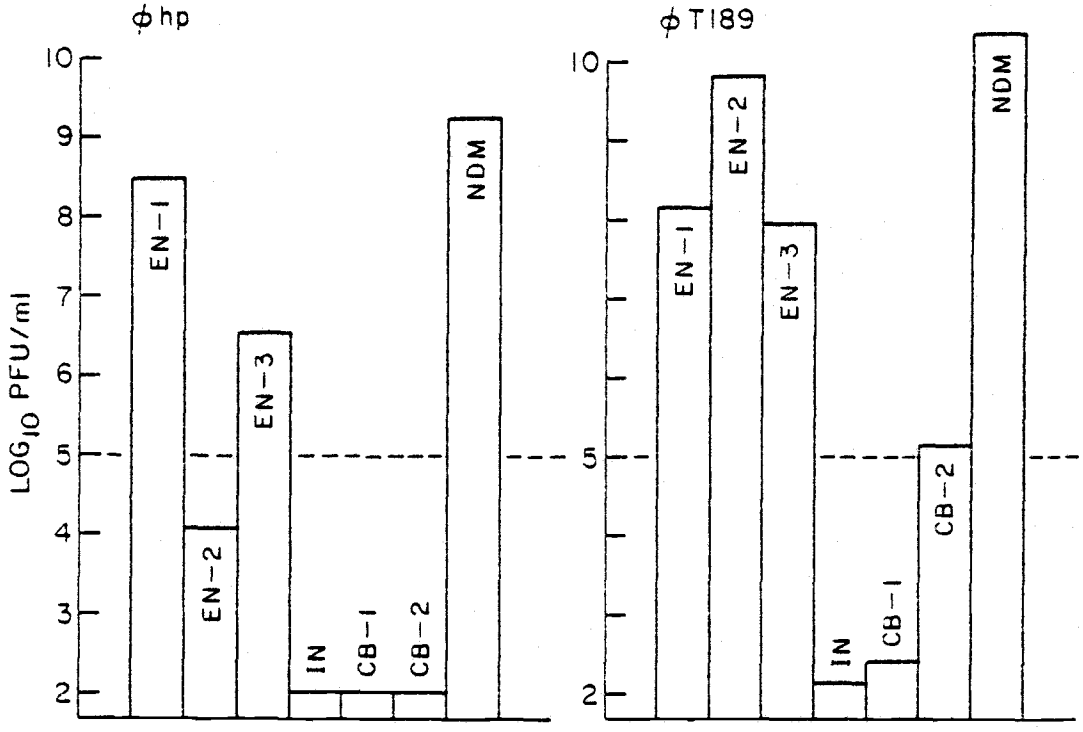

Figure 22.

Figure 23. Proliferation of phages 407 and ml_8 in various bulk starter media following infection with 10^5 pfu/ml.

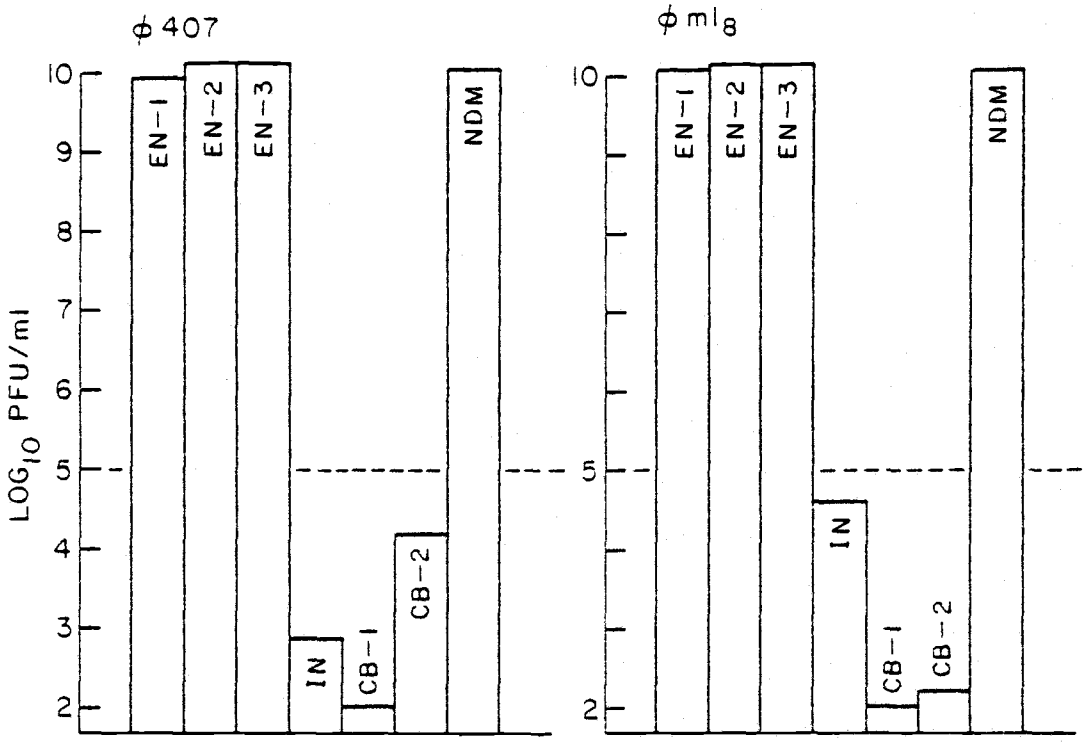

Figure 23.

Only one of the three externally neutralized products, EN-2, was able to completely suppress the proliferation of the hp phage.

With the low calcium-requiring phage T189, only CB-1 and IN were completely inhibitory. CB-2 possessed borderline phage inhibition with the T189 phage; but if commercially used for cheese manufacture the CB-2 bulk culture would have probably produced normal activity in the vat.

Generally, the same pattern resulted with phages 407 and ml_8. The three externally neutralized media and the NDM control provided very poor phage inhibition, whereas the conventionally buffered media and IN did not allow the phage titers to increase.

IN vs HCB

The highly buffered medium (HCB) using conventional phosphates was compared to the IN medium for bulk starter performance in the laboratory and in the cheese vat (Table 46). Even though culture activity as measured in the laboratory was slightly less than with the IN medium and holdover activity decreased more rapidly with HCB, the performances in the cheese vat were very similar for the two products. The HCB medium also prevented phage proliferation when challenged with the hp and T189 phages.

Table 46. Bulk starter comparison between the internally neutralized medium and a highly buffered conventional medium.

	HCB	IN[a]
BULK STARTER FERMENTATION[b] (26 C)		
Initial pH	6.85	6.80
16 h pH	5.23	5.35
Developed T. A. (%)	1.93	2.37
Activity[c] (pH)	1.6	1.7
BULK STARTER[d] HOLDOVER[e] ACTIVITY[f] (ΔpH)		
Fresh	1.75	1.75
3 day	1.45	1.60
7 day	0.70	1.10
PHAGE INHIBITION[g]		
hp/HP	1.0×10^2	1.0×10^2
T189/205	5.5×10^4	2.0×10^2
CHEDDAR CHEESE MANUFACTURE[h]		
Inoc rate[1]	400	400
T. A./pH at draw	.16/5.91	.16/5.89
T. A./pH at milling	.59/5.09	.59/5.11
pH of cheese (10 day)	5.08	5.10

[a] 8.7% (w/w) solids.

[b] Inoc with S. cremoris 134 (1%).

[c] CTP (1% inoc rate).

[d] Inoculated with S. cremoris $SK_{11}G$ (1%).

[e] Held at 40 F.

[f] Activity measured as change in pH following incubation at 32 C for 4 h (2.5% inoc rate).

[g] Phage/ml recovered after 16 h-24 C fermentation with homologous hosts (initial infection of 10^5 pfu/ml).

[h] Manufactured at Galloway West Co., Fond du Lac, WI.

[i] Pounds of bulk starter added per 45,000 lb vat.

DISCUSSION

Much higher cell populations are attained when the pH is maintained above 5.0 (20). In bulk starter media buffered with conventional orthophosphates (Na^+, K^+ and NH_4^+), growth continues until the pH drops to a point where it is slowed and eventually checked by the hydrogen ion concentration (29). With pH-controlled media the pH does not drop low enough to suppress the growth, the cells continue to multiply and the limiting factor becomes nutrient exhaustion or the production of auto-inhibitory metabolites such as hydrogen peroxide (9), D-leucine (8) and/or high levels of lactate (3,21,22).

Superior activity and higher cell numbers were achieved with the pH-controlled media in this study. Whether neutralized externally with NH_4OH (EN-1, 2, 3) or internally (IN), neutralization of the lactic acid end product enabled the cells to grow to greater populations. The high conventional buffered medium (HCB) was also effective in extending the growth period and increasing the overall activity.

Since higher cell numbers are produced per unit volume, the pH-controlled bulk starters are essentially biologically concentrated, which translates to reduced starter usage for the cheesemaker in the cheese vat. This means considerable economic savings in the form of less starter media used, less media inventory and less freight costs.

PH-control also protects the cells from acid injury at pHs below 5.0 (14) which enables excellent holdover activity of the mature starter for several days (33).

In general, the orthophosphate concentration in conventional media is approximately 2% of the reconstituted medium. Increase in clotting time of milk results from inoculation with cultures grown in phosphate buffered phage inhibitory media (2). Ledford and Speck observed metabolic injury and considerable loss of proteinase activity for cultures grown in three commercial phage resistant media containing high phosphates (17). Since most of the buffering capacity in external pH-control media is provided by the added alkali, lower phosphate levels are practical which should reduce the adverse effects of high phosphates. Damage from high phosphate in the internally neutralized medium (IN) should also be lower than with conventional media (including HCB) since much of the IN phosphate exists in an insoluble form during a major portion of the bulk fermentation.

Use of more starter strains that are sensitive to the cook temperature in Cheddar cheesemaking is made possible by neutralization of the bulk culture (18). Good activity in the cheese vat is possible because of the higher cell density. In addition, use of a wider spectrum of phage insensitive strains is more practical when their cell numbers are boosted with pH-control.

Effective phage inhibition was notably lacking with the three external pH-controlled media (EN-1, 2, 3). On the other hand, the

two conventional media (CB-1 and 2) along with the internal pH-controlled IN and the highly buffered HCB were very effective in suppressing phage proliferation in the bulk starter. Examination of the phage inhibition results shows a general decrease in phage titer from the 10^5 pfu/ml infection level to a final titer 2-3 logs lower in those media found phage inhibitory. Huggins (16) found this phenomenon only when phage and host bacteria were present together in the medium. He proposed that in the absence of free Ca^{+2} an abortive - type of infection occurs in which the phage DNA is incompletely injected into the host and is subsequently degraded in the medium.

In summary, pH-controlled bulk starter media offer several advantages over the conventionally buffered media. Economics is a major factor, but more importantly pH-control optimizes the growth and holdover conditions for the propagation of lactic starter cultures. This results in a more reliable bulk culture for the manufacture of fermented dairy products. The results of this study showed that only the internally neutralized medium (IN) and the highly buffered medium (HCB) generated superior activity while still maintaining effective phage inhibition. The pH-control media are relatively new and reformulation is needed to improve the phage inhibition of the external pH-control media. However, pH-controlled bulk starter media without phage inhibition may also have become more accepted when coupled with starter strains that are carefully selected for their phage insensitivity (33).

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APPENDIX II

MICRO-DROP TECHNIQUE FOR THE ENUMERATION OF LACTIC STREPTOCOCCI AND THEIR PHAGES[1]

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Submitted to: Applied and Environmental Microbiology

Surface-drop plating procedures were developed for the enumeration of lactic streptococci and their phages. These procedures were most useful in field research situations with inadequate laboratory facilities.

Enumeration of lactic streptococci by standard pour plate (2) or surface spread plate techniques are time-consuming procedures and require basic laboratory equipment. Such restrictions often make on-site total cell counts impractical in field research situations. This is also the case for quantitative phage plaguing by the standard double layer technique of Gratia as cited by Adams (1).

A simplified plating technique where micro-drops are dispensed onto an agar surface (6,7) was developed as a method for enumerating lactic streptococci and their phages. This method required less equipment and supplies, was relatively quick to perform and provided accurate results which make it more suitable for field research situations.

For the enumeration of lactic streptococci, 0.1 ml of sample was blended with a Waring blender in chilled 0.1% (w/v) peptone (Difco) diluent (100 ml) at high speed for 60 sec to break up the chains of cocci (5). In situations where blending was not possible, suitable shaking procedures were followed to reduce the chain length to a uniform length (3). The blended sample was then serially diluted in sterile 0.1% peptone and dispensed in four separate 0.025 ml micro-drops with an Oxford Micro-Doser repetitive pipette onto pre-dried (48 h-ambient temperature) plates of PMP agar.

Each petri dish was gently rotated with gradual tilting to allow the drops to cover the maximum surface area without running into each other. The plates were allowed to stand a short period to allow the medium to absorb the drops before inverting the plates and incubating at 30 C for 24-48 h or at ambient temperature (20-25 C) for 48 h.

PMP agar was a modified formulation of M17 (9) using the insoluble buffer trimagnesium phosphate instead of disodium-B-glycerophosphate. PMP was prepared as two separate components. One component consisted of polypeptone (BBL, Cockeysville, MD) 20.0 g; Phytone peptone (BBL), 5.0 g; yeast extract (Ardamine YEP, Yeast Products, Inc., Clifton, NJ), 5.0 g; lactose (Sigma Chemical Co., St. Louis, MO), 5.0 g; dipotasium phosphate trihydrate (Baker, reagent grade), 2.5 g; 0.25% (w/v) bromcresol purple (Sigma), 10.0 ml; Davis agar (Davis Gelatine Ltd, Christchurch, New Zealand), 10.0 g and 900 ml of distilled water. The other component contained trimagnesium phosphate (Stauffer Chemical Co., Westport, CT) 2.5 g and 100 ml of distilled water. The two components were autoclaved for 15 minutes at 121 C. After cooling to approximately 55 C the $Mg_3(PO_4)_2$ suspension was added to the other component. The complete medium (pH 6.9-7.1) was further cooled to approximately 45 C and then aseptically poured into petri dishes. Occasional swirling of the flask during pouring was necessary to keep the insoluble $Mg_3(PO_4)_2$ in suspension.

Lactic streptococci produced bright yellow colonies surrounded by halos of clearing contrasted against a purple medium. The clear halos resulted from the acid-solubilization of the suspended trimagnesium phosphate in the medium. The insoluble $Mg_3(PO_4)_2$ served to localize the acid produced by the individual colonies. Halos were usually observed surrounding individual colonies after 24 h incubation (Figure 24), whereas with extended incubation or in regions with several closely associated colonies halos would overlap and form large areas of clearing (Figure 25). Cell counts were determined by averaging the colony counts of the four drops and multiplying by the appropriate dilution factor.

Insoluble $CaCO_3$ has also been used in agar media to help preserve neutral conditions for the isolation and cultivation of acid-producing bacteria. Calcium carbonate is used in a plating medium for the differential enumeration of S. lactis, S. cremoris and S. diacetilactis (8), and it is also used in a medium to differentiate S. thermophilus and L. bulgaricus (3).

For the enumeration of lactic streptococcal phages, 1.0 ml of phage-infected sample was transferred to 9.0 ml of chilled sterile saline (0.85% NaCl), centrifuged at 5000 x g for 10 min and then serially diluted in sterile saline. One drop (0.05 ml) of 1.0 M $CaCl_2 \cdot 2H_2O$ was added to each tube containing 0.8 ml HS (host + saline), prepared by mixing 1.0 ml M17-grown host (16 h-30 C) with 9.0 ml saline. Diluted phage (0.2 ml) was then added to the CHS (HS + Ca^{+2}) tubes and vortexed. With the Oxford Micro-Doser,

Figure 24. Colonies of S. cremoris 205 on PMP agar after 24 h incubation at 30 C. Note the halos around the colonies resulting from acid-solubilization of the $Mg_3(PO_4)_2$ suspended in the medium.

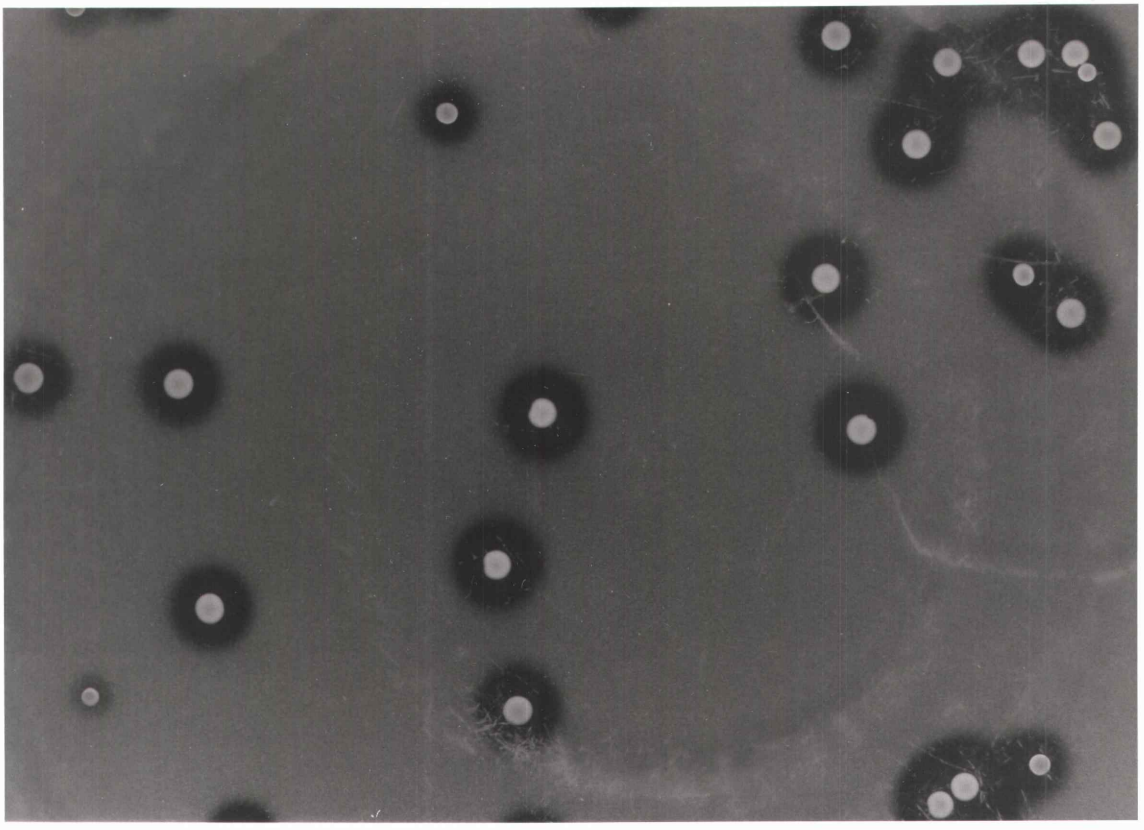

Figure 24.

Figure 25. Growth of S. cremoris 205 colonies in the four 0.025-ml drop spots after 48 h incubation at 30 C on PMP agar.

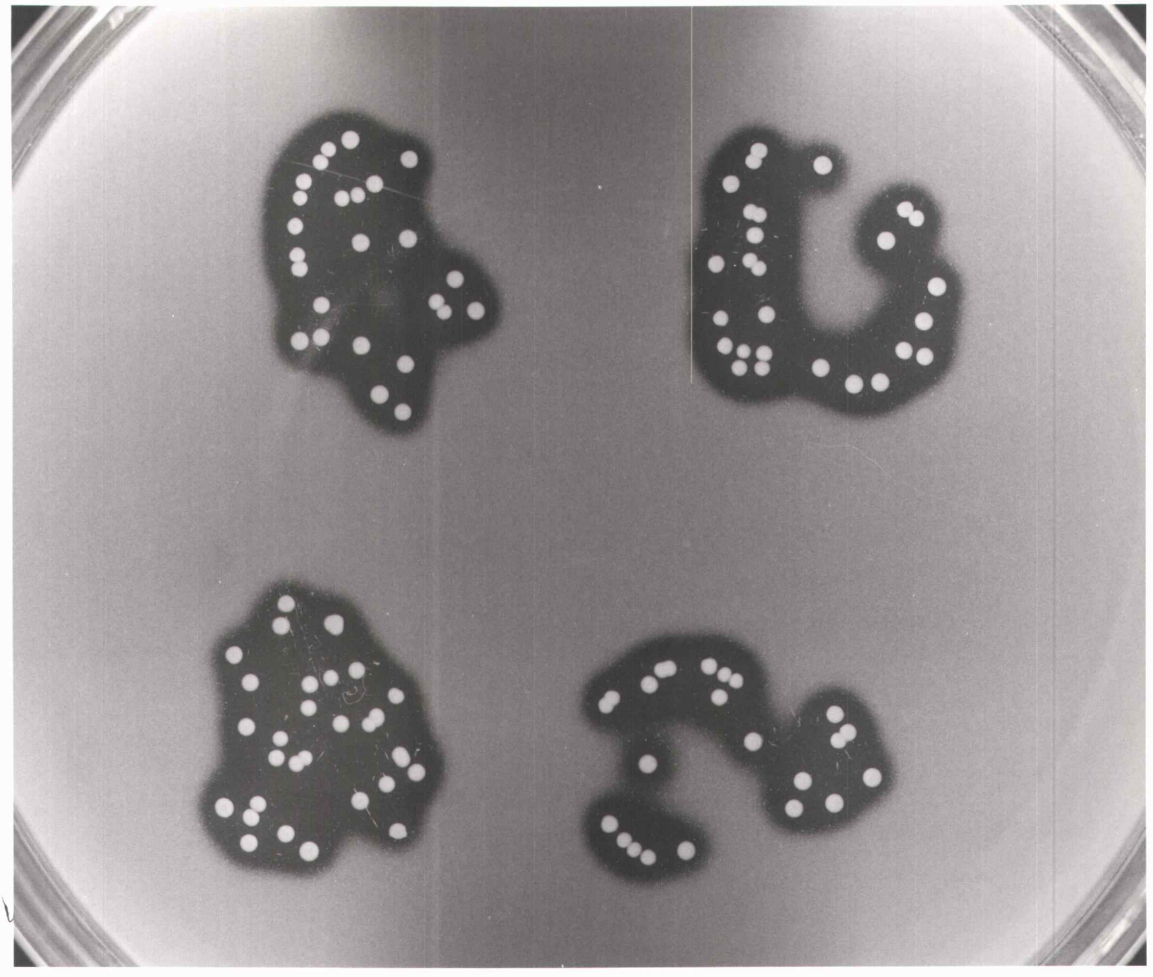

Figure 25.

three 0.050 ml (2 x 0.025) drops of phage + CHS were applied to the surface of a prepoured plate of M17 agar with 0.0025% bromcresol purple (air dried plates ambient temp for 48 h). The plates were carefully rotated by hand to allow the drops to spread out on the surface (maintaining three separate drops). After the medium had absorbed the drops, the plates were inverted and incubated at 30 C for 12-24 h.

Three large circles of host lawn containing individual plaques (Figure 26) appeared after the incubation period. Final phage titer was determined by averaging the three plaque counts and multiplying by the appropriate dilution factor.

The micro-drop procedure has been applied to the enumeration of other bacteria in our laboratory. A similar technique was used to determine survival of E. coli in membrane filter diffusion chambers (6). Differentiation of nonacid-producing bacteria from lactic streptococci was also possible on PMP agar based on halo formation and colony color.

Both techniques have been widely used in our laboratory and in field research situations at various cheese plants. Raw counts of 10-100 colonies and 10-50 plaques per drop were countable and resulted in the most reproducible counts.

However, the cell count technique is best suited for total counts; differential counts are more difficult because of the closely associated colonies. Colony enumeration with the micro-drop

Figure 26. Plaquing of T189 phage (S. cremoris 205 host) on M17 agar (with 0.0025% bromcresol purple) after 18 h incubation at 30 C. The arrow points out an individual plaque within a circular lawn of host growth.

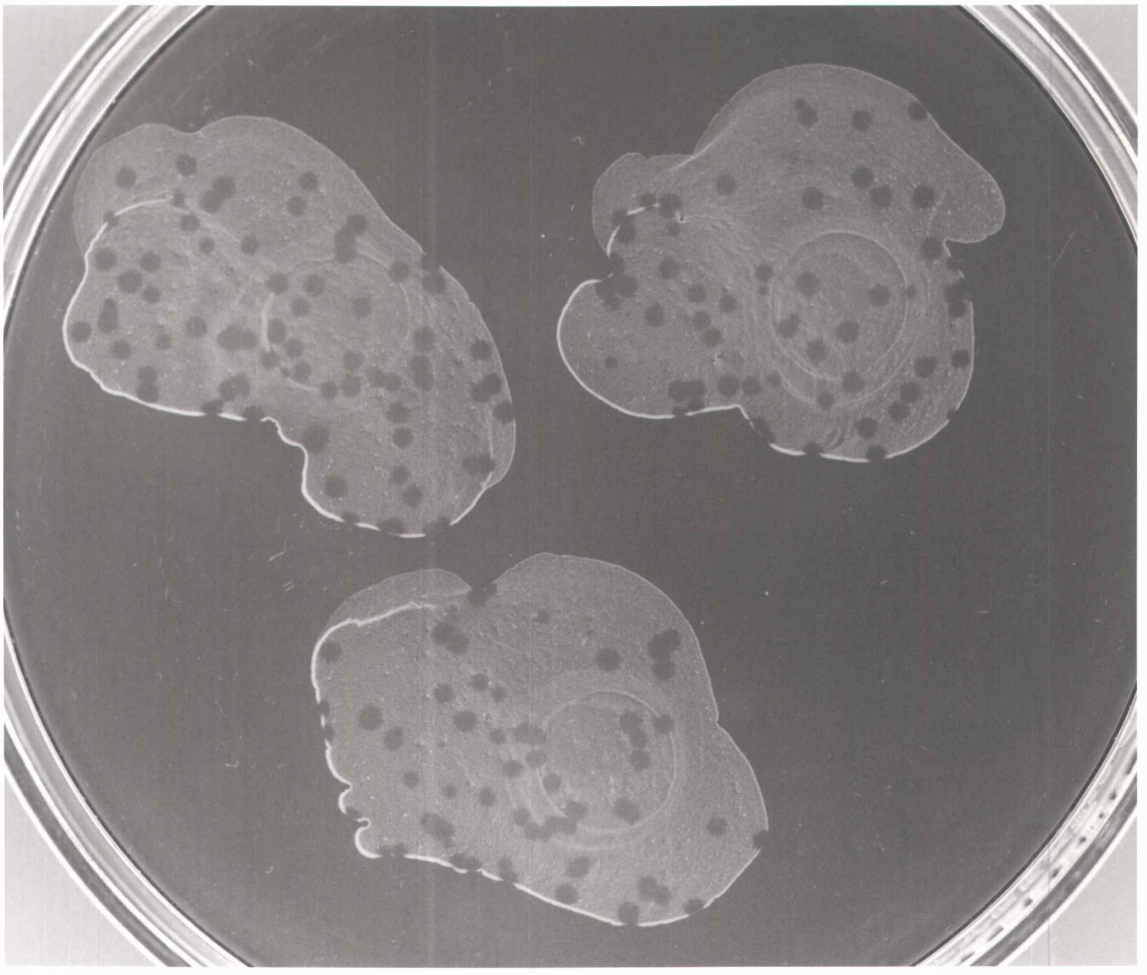

Figure 26.