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Growth and Preservation Parameters for Preparation of a Mixed Species Culture Concentrate for Cheese Manufacture[1]

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ABSTRACT

The production of a frozen concentrated cheese starter by batch culture fermentation of a mixed species cheese starter culture consisting of *Streptococcus cremoris* and *Streptococcus diacetilactis* was investigated. A response surface analysis to optimize growth conditions was made using 20% NaOH or 20% NH_4OH as neutralizers and pH and temperature as variables. The yield of cell mass was about two-fold higher using NH_4OH as the neutralizer. The optimal growth conditions for the latter were pH 6.1 to 6.5 and 24 to 27 C. Optimal balance of species of one *S. diacetilactis* per six *S. cremoris* cells was observed from a growth pH of 6.1 to 6.4 and temperatures of 23 to 26 C using NH_4OH. A pH of 6.2 to 6.5 and temperature of 25 to 27 C yielded cells of highest acid-producing ability when NH_4OH was the neutralizer. Cultures grown at 25 C using NH_4OH were also more resistant to freezing than NaOH neutralized cultures. Glycerol was the only cryoprotective agent examined which protected the culture during freezing and frozen storage. Lactose and malic acid appeared to have a destructive effect on the starter concentrate during frozen storage. Conditions of growth and type of cryoprotective agent affected each species individually. Gouda cheese prepared from a frozen concentrate was equivalent in quality to that prepared from a conventional bulk starter culture.

In spite of many scientific and technological advances, the daily production of bulk starter in the cheese factory remains a cumbersome and time consuming process which is never entirely free from the risk of failure due either to the activity of bacteriophage or the presence of inhibitors in the starter milk. The replacement of bulk starter by concentrated starter cultures produced at a central plant and used immediately or preserved for later use would be desirable and has been the objective of current dairy research and development. During the last several years one of the major considerations was whether to use continuous or batch culture techniques for producing cell biomass.

Batch culture methods for producing concentrated starter cultures have been extensively investigated by many workers and in some instances put into commercial production (*16*). The main parameters of batch culture that have the greatest influence on increasing the cell biomass and obtaining a highly biological active culture are: (*a*) composition of the growth medium (*2, 3, 12, 24*), (*b*) pH during growth and type of alkali used as the neutralizer (*4, 18, 21*), (*c*) temperature during growth (*11, 15, 27*), (*d*) time of fermentation (*16, 26*), and (*e*) method of harvesting the cells (*4, 16, 25*). However, it appears that only Gilliland (*11*) and Anderson & Leesment (*1*) have examined the growth relations on mixed strain starters concentrates as affected by the above parameters.

Several methods of preserving starter bacteria have been described and it has been established that freezing and storing the concentrates in liquid nitrogen (–196 C) is the best method for maintaining the viability and biological activity of the cells (*6, 10, 13, 16, 18, 20, 25*). The problems, however, that arise from the use of liquid nitrogen are many, i.e., the mass freezing of concentrates is not very effective due to small volumes that can be frozen at once and the high cost of the method, as well as the necessity of special equipment. It seems that the use of a method of freezing and storing the concentrated starters at temperatures between –10 to –30 C, which can be obtained easily by commercial freezers, should be a main research objective. In order to provide better viability and activity during freezing and frozen storage at subzero temperatures several compounds are used as cryoprotective agents (*10, 14, 16, 17, 25*). In all cases, neutralization of the lactic culture concentrates before freezing appears to be essential.

This paper describes the effects of growth conditions and type of alkali used as neutralizer on the total cell yield, balance of species, and activity of a mixed species starter culture used for Gouda cheese manufacture as well as the ability of the concentrated culture to withstand freezing and frozen storage at –10 and –30 C. The effect of several cryoprotective agents on the viability of the culture after frozen storage and on each component species, as well as on the activity of the starter upon freezing and frozen storage at –10 and –30 C was examined. Gouda cheese was made by using the frozen concentrates and was compared to cheese prepared from a normal bulk starter culture.

MATERIALS AND METHODS

Cultures

The mixed cheese starter culture was obtained from the collection maintaned in the Department of Food Science and Nutrition,

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University of Minnesota. The culture, designated N cheese starter culture, originated from the Netherlands and has been propagated in our laboratory for many years. The N culture was grown in 11% reconstituted nonfat dry milk and maintained by incubating at 21 C until coagulation (18 h) and stored at 4 C between transfers.

Media

In order to isolate and identify the acid and aroma producers in the N mixed cheese starter, the medium of Galesloot et al. (*9*) was used routinely. The differential agar medium of Reddy et al. (*19*) also was used. Total count was estimated by means of Elliker's agar (*8*).

Growth studies

A complete factorial experiment was performed using a response surface design and analysis (*7*). The levels of pH and temperature that would give the maximum population, and the best component species balance, as well as the highest activity were estimated. The total number of trials was 10 and the levels of the factors were: pH 5.5, 20 C; pH 7.0, 20 C; pH 5.5, 30 C; pH 7.0, 30 C; pH 5.5, 25 C; pH 7.0, 25 C; pH 6.25, 20 C; pH 6.25, 30 C; pH 6.25, 25 C; and pH 6.25, 25 C. The experiments were performed twice using 20% NaOH or 20% NH_4OH as neutralizer, in order to find which would provide the highest population, activity and species balance under similar growth conditions. The growth medium was 11% nonfat dry milk. The milk was steamed for 60 min and cooled immediately. Agitation control was used (150 rpm) during growth. Cells were harvested after 18 h which corresponded to the late log to early stationary phase as confirmed by growth curve. Growth studies were performed using a microfermentor (New Brunswick, N.B., N.J.) with automatic pH, temperature and stirring speed control. The jar of the microfermentor was sterilized separately from the growth medium. The volume of medium used for each trial was 4L and the inoculum was 1% (v/v). Samples were drawn after 18 h and viability (total count, species balance) as well as activity determined. Activity was measured by means of a Beckman pH meter and Beckman automatic titrimeter, using 0.111 N NaOH and an end point of pH 8.5. The samples for activity determinations were obtained from 100 ml of 11% nonfat dry milk. The milk was inoculated with 1% (v/v) of cultures to be tested and incubated for 6 h at 32 C. Ten ml samples were used and the activity was measured by subtracting the titratable acidity of the milk from that of the test culture. Nutrient agar was used for the estimation of contaminants developed during the batch fermentation. All tests were performed in triplicate. At the end of the growth period, the temperature of the medium was dropped to 0 C and sodium citrate was added to a final concentration of 2%. The pH was then adjusted when necessary to 6.9 and the medium was allowed to stand for 10 min. The cells were harvested and concentrated by using a Sorval centrifuge (refrigerated) at 8,900 × g for 30 min. The cell mass was washed three successive times with 0.05 M phosphate buffer, pH 6.8, to clean the cells from residual milk which might contain undesirable fermentation end products. The cell mass was then resuspended in 11% nonfat dry milk at a rate of 3% of the original volume. Uniform resuspension was accomplished by using a Waring blender and blending the mixture (cells + milk + cryoprotective agent, if added) for 1.5 min. At this point count, species' balance as well as activity were determined by diluting the concentrates to their original volume (1/33). The balance of strains was the same since viability and activity showed a 4 to 5% decline compared to the culture before concentration. The concentrates were frozen and stored at −10 or −30 C in screw cap tubes (10 ml) or screw cap plastic bottles (125 ml). Cultures were periodically examined for their viability, strain balance and activity. The cultures were thawed quickly in a water bath (45 C), diluted to their original volume (1/33) and were plated. Dilution blanks contained 0.11% nonfat dry milk. The effects of several compounds as cryoprotective agents were examined, namely; 5% yeast extract, 7.5% lactose, 10% sucrose, 2% L-malic acid, 1% L-glutamic acid 0.06 M, pH 7.0, 10% glycerol and 5% N-Z amine NAK (percentages refer to the final concentration of the compound in the cell suspensions). The cultures were examined as above and controls (without cryoprotective agents) were run at the same time.

Cheese making trials

Gouda cheese was made from the same lot of milk by using the frozen concentrates and conventional starter. Quantities of 125 ml of frozen concentrated starter cultures were thawed quickly in a 45 C water bath and 4 liters of 11% nonfat dry milk were inoculated and allowed to stand at room temperature for 1 h to give the organisms the opportunity to partially recover from frozen storage. The conventional starter before inoculation had a titratable acidity of 1.05 to 1.10% expressed as % lactic acid, and the frozen concentrate had an acidity of 0.25 to 0.45. The entire amount of frozen concentrate (125 ml) was used for vat inoculation (450 lbs of milk) and a 1% inoculum (v/v) from the conventional starter. Organoleptic evaluation of the cheese was made after one month and at one month intervals for up to four months.

RESULTS AND DISCUSSION

By using the differential agar media of Galesloot et al (*9*) and Reddy et al. (*19*), it was found that *S. cremoris* and *S. diacetilactis* were the component species of the N starter cultures. The total population was about 5.0×10^8 CFU/ml, and the ratio of component species (aroma/acid producers) was about 1/7 during the exponential to early stationary phase of growth.

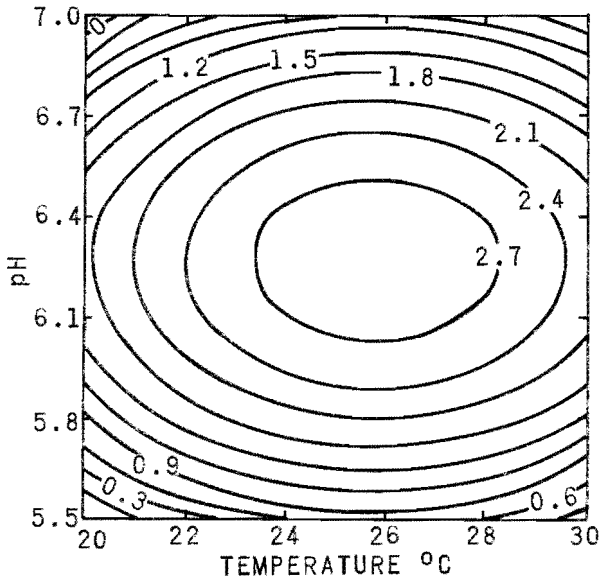

Figure 1. *Response surface indicating yields of cell mass of N starter culture as influenced by the pH and temperature of growth. 20% NaOH was used as the neutralizer. Numbers identifying contour lines are yields of cell mass, e.g. a pH of 6.0 to 6.5 and temperature from 23.5 to 28.0 C is predicted to give a yield of at least 2.7 × 10⁸ CFU/ml.*

Figures 1 and 2 show that the highest viable count was obtained by using a pH between 6.10 and 6.45 and a temperature between 24.0 and 28.0 C, as well as pH from 6.0 to 6.50 and temperature from 23.5 to 28.0 C when 20% NH_4OH or 20% NaOH was used as neutralizer, respectively. From Fig. 4 it appears that the best strain balance (1/7) was obtained by using a variety of pH and temperature conditions, i.e., pH range 5.80 to 6.80 and 26.0 to 29.0 C as well as pH 5.80 to 6.80 and 20.0 to 23.0 C or pH 6.4 to 6.8 and 20.0 to 29.0 C as well as pH 6.1 to 5.8 and 20.0 to 29.0 C. The highest cell yield was also obtained at this range of pH and temperature. The best strain balance obtained using 20% NaOH as neutralizer was 1/8 which was obtained at pH 6.0 to 6.6 and 23.5 to 26.5 C (Fig. 3). The conditions necessary to

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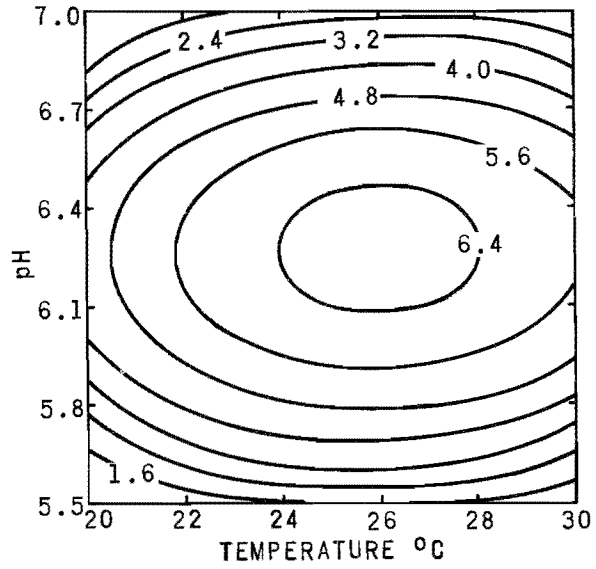

Figure 2. *Response surface indicating yields of cell mass of N starter culture as influenced by the pH and temperature of growth. 20% NH_4OH was used as the neutralizer. Numbers identifying contour lines are yields of cell mass, e.g., pH of 6.10 to 6.45 and temperature from 24.0 to 28.0 C is predicted to yield at least 6.4 × 10^9 CFU/ml.*

obtain the highest cell mass were also in this range. By conducting similar response surface analysis for the observed activities of the cultures, by using inocula having equal viable counts we found that when 20% NaOH was used as the neutralizer highest activity was obtained at pH 5.90 to 6.60 and 22.5 to 28.0 C, or at pH 6.25 to 6.45 and 25.0 to 27.0 C when 20% NH_4OH was used as the neutralizer (Fig. 5, 6). The optimal conditions necessary for growth using 20% NaOH as the neutralizer were a pH between 6.0 and 6.50 and a temperature between 23.5 to 26.5 C, or pH from 6.1 to 6.45 and a

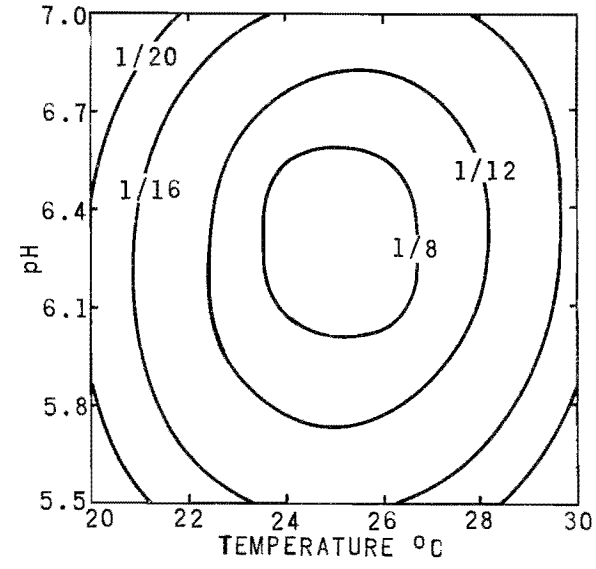

Figure 3. *Response surface indicating balance of species in the N starter culture* (S. diacetilactis/S. cremoris) *as influenced by the pH and temperature of growth, 20% NaOH was used as the neutralizer. Numbers indentifying contour lines are the species balance rates, e.g., pH of 5.75 to 6.80 and temperature from 23.5 to 26.5 C is predicted to give a strain balance of at least 1/12.*

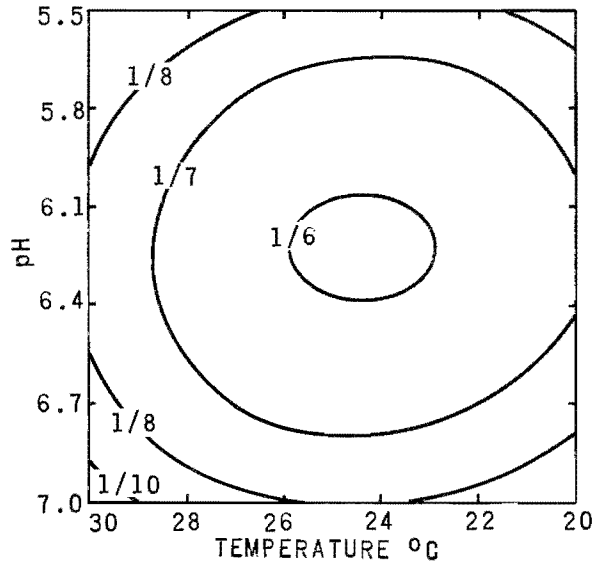

Figure 4. *Response surface indicating balance of species of the N starter culture* (S. diacetilactis/S. cremoris) *as influenced by the pH and temperature of growth, 20% NH_4OH was used as the neutralizer. Numbers identifying contour lines are the species balance rates, e.g., a pH of 6.1 to 6.4 and temperatures from 23.0 to 26.0 C is predicted to give a strain balance of at least 1/6.*

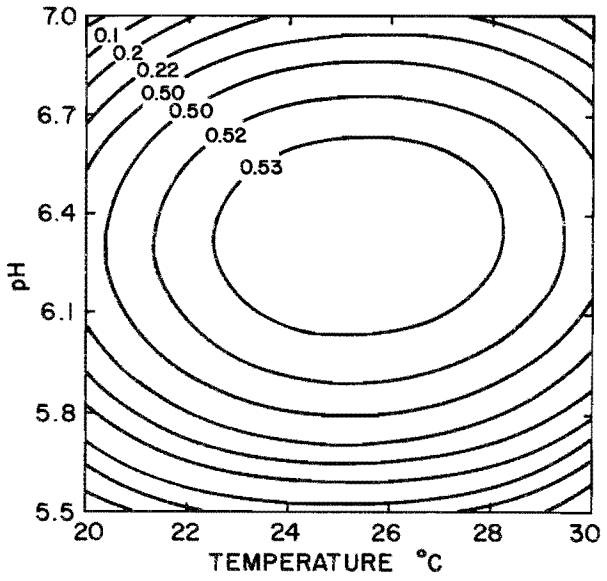

Figure 5. *Response surface indicating activities of the N starter culture as influenced by the pH and temperature of growth, 20% NaOH was used as the neutralizer. Numbers identifying contour lines are the obtained culture activities, e.g., a pH of 5.90 and 6.60 and temperature from 22.5 to 28.0 C is predicted to give a culture having an activity of at least 0.53.*

temperature from 24.5 to 27.0 C when 20% NH_4OH was used as the neutralizer. The use of NH_4OH gave a two-fold increase in cell yield, which agrees with results of other investigators (*4, 11, 18, 22, 25*). Concentrates prepared using NH_4OH were also found to have higher acid producing ability than those prepared with NaOH.

Several workers (*3, 5, 18*) reported that growth of lactic streptococci at pH 6.0 to 6.5 favors production of maximum cell numbers. However, these investigators worked with single strain starter cultures and did not

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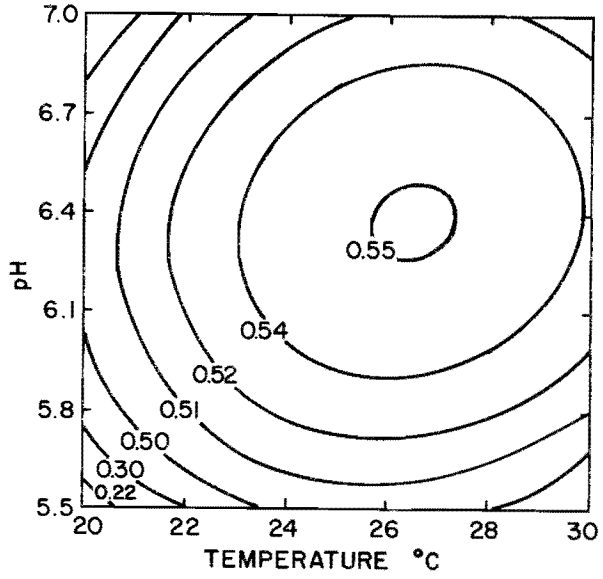

Figure 6. *Response surface indicating activities of the N starter culture as influenced by the pH and temperature of growth. 20% NH_4OH was used as the neutralizer. Numbers identifying contour lines are the obtained culture activities, e.g., a pH of 6.25 to 6.45 and temperature from 25.5 to 27.0 C is predicted to give a culture having an activity of at least 0.55.*

examine the effect of pH on species balance. In this study, pH and temperature appeared to affect the balance of strains but temperature appeared to be the predominant factor. It is known that every organism has its own requirements of pH and temperature for optimal growth. In the case of lactic streptococci, the optimum pH range appears to be more narrow than that for temperature. According to our results, a temperature of about 25 C was favorable not only for obtaining maximum population but also for good species balance. These results are in agreement with other investigators (*2, 3, 15, 18*). From our data it is obvious that the combined effects of pH, temperature and type of neutralizer during growth should be taken into consideration and not each variable individually. In order to confirm the computer obtained plots (Fig. 1-6), pH and temperature growth levels were selected randomly and the mixed starter culture was grown as described before. The total viable counts, the ratio of the component species and the biological activity were determined. The range of the obtained data was ± 5% of the levels corresponding to the plots.

In preparing the starter concentrates the method of harvesting the cells was important. When Na-citrate was used and the pH of the medium was adjusted to 6.9, recoveries of cell yield up to 96% were obtained, compared to 75% without Na-citrate treatment. The adjustment of pH was necessary only when cultures were grown at pH 6.24 or less. These results agree with others (*4, 11, 25*).

This study showed that the most significant single factor influencing the survival of starter culture organisms was the temperature of freezing and frozen storage. In general, the highest recovery was obtained when cultures were frozen and stored at −30 C in 11% nonfat dry milk with or without additives.

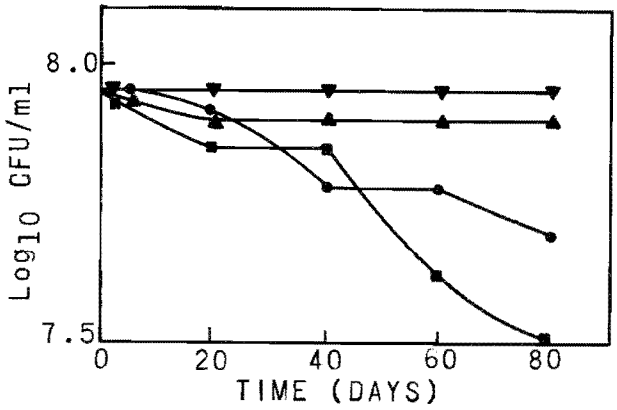

Figure 7. *Effect of glycerol used as a cryoprotective agent on the ability of the N starter to withstand freezing and frozen storage at −10 and −30 C respectively. Cultures were grown at pH 6.25 and 25 C with 20% NaOH used as the neutralizer. Control (●—●) and with 10% glycerol added (▲—▲) cultures were frozen and stored at −10 C. Control (■—■) and with 10% glucerol added (▼—▼) cultures were frozen and stored at −30 C.*

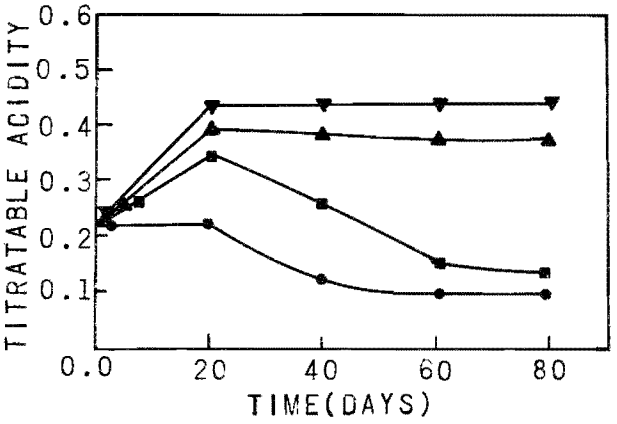

Figure 8. *Effect of glycerol used as a cryoprotective agent on the ability of the N starter to maintain its biological activity upon freezing and frozen storage at −10 and −30 C respectively. Cultures were grown at pH 6.25 and 25 C with 20% NaOH used as the neutralizer. Control (●—●) and with 10% glycerol added (▲—▲) cultures were frozen and stored at −10 C. Control (■—■) and with 10% glycerol added (▼—▼) cultures were frozen and stored at −30 C.*

Glycerol was the most advantageous in preserving the viability and biological activity of the N starter (Fig. 7, 8). This effect may have existed because of the -OH groups which stabilized the conformation of the cellular constituents (*17*). Glycerol enhanced rather than just protected the biological activity of the frozen culture (Fig. 8). Gibson et al. (*10*) found that glycerol did have a protective effect on *S. lactis* and *S. cremoris* when these cultures were stored at −23.3 C, but retarded acid development during incubation after thawing.

Data not presented here show that only glycerol provided protection for *S. diacetilactis* and lactose, sucrose and L-malic acid did not provide protection to this aroma producer. L-glutamic acid and N-Z Amine NAK appeared to be equally protective for both species. Yeast extract appeared to affect equally both strains

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when cultures were grown at pH 5.5 and 30 C with 20% NaOH as the neutralizer, but provided better protection to *S. cremoris* when 20% NH_4OH was used as the neutralizer. Both component organisms appeared to be more resistant to freezing and frozen storage when grown at 25 C with NH_4OH used as the neutralizer. When NaOH was used as the neutralizer the resistance of *S. diacetilactis* seemed to be influenced more (depending on the growth conditions) than that of *S. cremoris*. The results indicate that careful selection of the proper cryoprotective agent as well as strain selection (*11, 23*) should be the main consideration in preparing frozen starter concentrates.

Cheese prepared by using the concentrated frozen N culture stored at −10 to −30 C for as long as 150 days was evaluated after one month and at one-month intervals for up to four months for taste and flavor. During the cheese making process the frozen starter culture exhibited a delay in acid production, but the final pH of the cheese was the same as that of the control. The cheese prepared by using the frozen starter concentrates was equal or better in quality than that prepared by using conventional N starter culture. The cheese also did not develop any unusual organoleptic characteristics during storage (up to 3 months). However, the most striking property of the cheese was that it did not develop bitterness which was present in the control cheese.

The results suggest that appropriate growth conditions must be chosen to obtain a high cell yield with high biological activity and optimum species balance able to withstand freezing and frozen storage. Between the two sub-zero temperatures employed, −30 C was the better storage temperature. Of the cryoprotective agents examined, only glycerol was found to be protective to both cell viability and biological activity. However, some of these compounds appeared to individually affect the viability of the species or their acid producing ability. Further investigations on how freezing and frozen storage influence the membrane system and biological activity of each species are required.

REFERENCES

1. Anderson, I., and H. Leesment. 1962. Growth relation in mixed starters with *S. diacetilactis* or *L. citrovorum*. XVI Int. Dairy Congr. B., 217-224.
2. Bergere, J. L. 1968. Production massive de cellules de Streptocoque lactiques. I. Methods generales d'etude et facterus de la crossance de "*Streptococcus lactis*" souche C10. Lait 48:1-11.
3. Bergere, J. L. 1968. Production massive de cellules de streptocoques lactiques. III. Production de differentes souches en culture a pH constant. Lait 48:131-139.
4. Blaine, J. W., W. E. Sandine, and P. R. Elliker. 1970. Preparation of concentrates of lactic streptococci from milk cultures. J. Dairy Sci. 54:637.
5. Cogan, T. M., D. J. Buckley, and S. Condon. 1971. Optimum growth parameters of lactic streptococci used for the production of concentrated cheese starter cultures. J. Appl. Bacteriol. 34:403-409.
6. Cowman, R. A., and M. L. Speck. 1963. Activity of lactic streptococci following ultra-low temperature storage. J. Dairy Sci. 46: 609.
7. Davies, O. L. 1971. Design and Analysis of Industrial Experiment (2nd ed). Hafner Publishing Co., New York.
8. Elliker, P. R., A. W. Anderson, and C. Hanneson. 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. 39:1611.
9. Galesloot, T. E., F. Hassing, and J. Stadhouders. 1971. Agar media for the isolation and enumeration of aroma bacteria in starters. Neth. Milk Dairy J. 15:145-150.
10. Gibson, C. A., G. R. Landerkin, and P. M. Morse. 1966. Effects of additives on the survival of lactic streptococci in frozen storage. Appl. Microbiol. 14:665-669.
11. Gilliland, S. E. 1971. Strain balance of multiple lactic streptococcus concentrated cultures. J. Dairy Sci. 54:1129-1133.
12. Gilliland, S. E., E. D. Anna, and M. L. Speck. 1970. Concentrated cultures of *L. citrovorum*. Appl. Microbiol. 19:890-893.
13. Gilliland, S. E., and M. L. Speck. 1974. Frozen concentrated cultures of lactic starter bacteria: A review. J. Milk Food Technol. 37: 107-111.
14. Kawashima, T., and M. Maeno. 1964. Studies on preservation of lactic acid bacteria by freezing. Japan J. Zootech. Sci. 35:48-54.
15. Lamprech, E. D., and E. M. Foster. 1973. The survival of starter organisms in concentrated suspensions. J. Appl Bacteriol. 26:359-369.
16. Lloyd, G. T., and E. G. Pont. 1973. The production of concentrated starters by batch culture. Aust. J. Dairy Technol. 28:104-107.
17. Morichi, T. 1972. Mechanism and prevention of cellular injury in lactic acid bacteria subjected to freezing and drying. 56th Annual Session of I.D.F. Subject 1.
18. Peebles, M. M., S. E. Gilliland, and M. L. Speck. 1969. Preparation of concentrated lactic streptococcus starters. Appl. Microbiol. 17:805-810.
19. Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1972. Agar medium for differential enumeration of lactic streptococci. Appl. Microbiol. 24:947-952.
20. Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1974. Component balance in mixed lactic starters as influenced by preservative procedures. J. Dairy Sci. 57:124-127.
21. Richardson, G. H. 1973. Improved lactic bulk culture system utilizing a whey-based medium under pH control. J. Dairy Sci. 56:637.
22. Snell, E. E. 1952. The nutrition of the lactic acid bacteria. Bacteriol. Rev. 16:235-241.
23. Speckman, C. A., W. E. Sandine and P. R. Elliker. 1974. Lyophilized lactic acid starter culture concentrates: preparation and use in inoculation of vat milk for cheddar and cottage cheese. J. Dairy Sci. 57:165-173.
24. Stadhouders, J., L. A. Jansen, and G. Hup. 1969. Preservation of starters and mass production of starter bacteria. Neth. Milk Dairy J. 23:182-199.
25. Stadhouders, J., G. Hup, and L. A. Jansen. 1971. A study of the optimum conditions of freezing and storing concentrated mesophylic starters. Neth. Milk Dairy J. 25:229-239.
26. Swartling, P., and B. Lindgren. 1960. Must starters be subcultured every day? Svenske Mejrritidy 52:631. Dairy Sci. Abstr. 23:230, 1961.
27. Wolk, J., and R. T. Tittsler. 1958. Effect of temperature on acid production by *Streptococcus lactis* and *Streptococcus cremoris* J. Dairy Sci. 41:705.

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