AN ABSTRACT OF THE THESIS OF

JAMES WESLEY BLAINE (Name of student) for the Doctor of Philosophy (Degree)

in Microbiology (Major) presented on Jan. 27 1972 (Date)

Title: PREPARATION AND PRESERVATION OF LACTIC ACID STARTER CULTURE CONCENTRATES

Abstract approved: Redacted for Privacy

Dr. W. E. Sandine

Streptococcus lactis C2F and Streptococcus cremoris 459 grew to a maximum population of 1 to 2 x 10^9 colony forming units (cfu)/ml with a generation time of 96 min in 11% nonfat dry milk (NFDM) under static conditions at 22° C. Streptococcus diacetilactis 18-16 grew to a maximum population of 7 x 10^8 cfu/ml with a generation time of 95 min under the same conditions. Incubation of S. lactis C2F at 28° to 30° C resulted in a reduction of the generation time to 60 min but no significant increase in the maximum population. Maintaining the pH of S. lactis C2F cultures at 6.3 with sodium hydroxide, potassium hydroxide or calcium hydroxide increased the maximum population to 4.5 to 5.5 x 10^9 cfu/ml. When ammonium hydroxide, sodium carbonate or ammonium carbonate were used as neutralizing agents a maximum population of 8.4 to 8.9 x 10^9 cfu/ml resulted. No significant change in generation time was observed

except when ammonium hydroxide was used as the neutralizer when the generation time was reduced to 44 min. Sodium lactate formed during growth of S. lactis C2F cultures in which the pH was maintained with sodium carbonate was established as a contributing factor in limiting the maximum population. Increasing the percentage of NFDM above 11% (s/v) did not increase the maximum population level. However, there was an increase in the maximum population to 1.0×10^{10} cfu/ml when pH-controlled S. lactis C2F cultures were sparged with nitrogen gas at a low flow rate (controlled growth). S. cremoris 459 grown under these conditions (pH 6.3, 30° C) in NFDM had a generation time of 66 min and reached a maximum population of 8.7×10^{9} cfu/ml. Under the same conditions, S. diacetilactis 18-16 grew to a maximum population of 9.4×10^{9} cfu/ml in NFDM but only when the milk was supplemented with 1.5% yeast extract.

Concentrates of S. lactis C2F, S. cremoris 459 and S. diacetilactis 18-16 were prepared by adjusting NFDM cultures in the late logarithmic growth phase under controlled growth conditions, to pH 6.9 and adding sodium citrate to a final concentration of 4.5%. Cells were harvested from the partially cleared medium by centrifugation. The sedimented cells were reconstituted to one-tenth the original culture volume, lyophilized and stored at -196, -22, +7 and +22° C.

Lyophilization of S. lactis C2F cell concentrates (5.5×10^{10} cfu/ml) in 10% nonfat milk caused a 31% reduction in acid-producing activity of the cells measured immediately after freeze drying. When the cells were suspended in 5% monosodium glutamate, this effect was reduced to a 14% loss and use of 5% trehalose resulted in only a 6% loss in activity. Lyophilization of S. cremoris 459 and S. diacetilactis 18-16 cell concentrates in 5% monosodium glutamate, containing 4.2×10^{10} and 4.6×10^{10} cfu/ml respectively, caused a loss in activity of 31% for S. cremoris and 7% for S. diacetilactis 18-16.

Using activity of the lyophilized concentrate as 100%, the concentrates of S. lactis C2F cells lyophilized in 10% NFDM and stored at +22 and +7° C showed greater than 80% decrease in acid-producing activity in seven days, 64% at -22° in 21 days and no reduction in 21 days at -196° C. Lyophilization of S. lactis C2F cell concentrate in 5% monosodium glutamate showed a reduction in activity of 70% in 21 days when stored at +22 and +7° C. Lyophilization of S. lactis C2F cell concentrates in 5% monosodium glutamate supplemented with 0.16 M potassium iodide resulted in a 32% and 20% reduction in activity when stored at +22 and +7° C respectively for 21 days; there was no loss when stored at -22 and -196° C. The addition of 0.05% ascorbic acid or 0.05% butylated hydroxyanisole did not appreciably alter the results obtained with the use of glutamate alone.

There was no reduction in activity after 28 days of storage of lyophilized cell concentrates of S. lactis C2F, S. cremoris 459 or S. diacetilactis 18-16 when stored under vacuum in 5% monosodium glutamate at +22°, +7°, -22°, or -196° C. Lyophilized concentrates of S. lactis C2F stored in 10% NFDM under vacuum also showed no reduction in activity at +22°, +7°, -22° and -196° C.

Preparation and Preservation of Lactic Acid Starter Culture Concentrates

by

James Wesley Blaine

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1972

APPROVED:

Redacted for Privacy

Professor of Microbiology
in charge of major

Redacted for Privacy

Head of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented Jan. 27, 1972

Typed by Ilene Anderton for James Wesley Blaine

ACKNOWLEDGEMENTS

The author wishes to acknowledge the advice and direction offered by Dr. W. E. Sandine and Dr. P. R. Elliker during this study.

The author also wishes to acknowledge the financial assistance and research facilities provided by the Department of Microbiology of Oregon State University.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREPARATION AND PRESERVATION OF LACTIC ACID STARTER CULTURE CONCENTRATES

INTRODUCTION

Inherent in the making of fermented dairy products, such as cheese, buttermilk, cultured sour cream, etc., is the development of acidity and flavor in the milk. The first attempts to control acid and flavor development in these products consisted of keeping back clean-flavoured sour milk, buttermilk or whey. This portion was called the "starter" and it was used to inoculate or culture the new vat of milk from which the product was to be made. The recognition that bacteria were responsible for the development of acid and flavor and the separate propagation of the main bacterial species involved was the first step in the evolution of the starters presently used in the dairy industry. The preparation, selection and maintenance of these starters is an important and highly specialized part of the preparation of fermented or cultured dairy products.

Starter preparation and maintenance in the dairy plant is time consuming, expensive and frought with uncertainties; under the best conditions, which would include skilled personnel and most sophisticated of equipment, starter cultures can become contaminated with foreign bacteria, with culture-destroying viruses or lose acid producing activity for a variety of reasons.

A solution to the difficulties of handling starter cultures, currently in limited use in the United States, is the production of starter cultures in a central facility which supplies starters to the surrounding cheese making plants. Production of starter cultures in such a separate facility where no cheese making is conducted has the advantage of eliminating the potential hazards cited above. Under these conditions it is possible to maintain a high level quality control program which insures a uniform starter and thus a more uniform final product. The burden of the dairy plant of maintaining a highly skilled staff and providing expensive culture maintenance facilities is eliminated. Improvement of existing starter cultures or development of new types are additional activities to which the central facility is more suited than the dairy plant.

The present limitation on the production of starter cultures at a central facility is the difficulty and expense involved in preparing volumes of starter necessary to inoculate the cheese vat and the difficulty of maintaining the activity of starter cultures during transport and storage. A dairy plant using a one percent inoculum for a 10,000 lb. vat of milk (approximately 1163 gals.) would require 11.6 gals. or 100 lbs. of starter culture. Most dairy plants inoculate considerably more than 10,000 lbs. of milk per day, thus the difficulties of providing starter by this means becomes apparent. Dairy plants currently using starter cultures from central facilities are

limited to inoculation of intermediate or bulk cultures and direct vat inoculation is not possible. The problems associated with starter culture transfer and cultivation in the dairy plant are thus not entirely eliminated.

The solution proposed in this study is the production of starter culture concentrates capable of remaining viable under prolonged or adverse storage conditions. Concentration of starter cultures would result in small volumes of culture thus eliminating the difficulty and expense of transporting the large volumes of culture to the dairy plants. Concentrates sufficiently stable to remain active during transport and storage would insure the production of a uniformly high quality product.

LITERATURE REVIEW

Part I. Starter Cultures

Composition of Starter Cultures

Starter cultures used in the dairy fermentation industry are composed of a variety of microbial species. Starters consisting of the bacteria Streptococcus lactis, Streptococcus cremoris or Streptococcus diacetilactis are the most widely used in this country. Less commonly used starters contain strains of Propionibacterium shermannii, Lactobacillus bulgaricus, Streptococcus thermophilus or Leuconostoc citrovoroum. The microbial composition of the starter determines to a large extent the flavor and texture of the dairy product resulting from its use. Starters of Streptococcus lactis or Streptococcus cremoris are selected primarily for their souring ability and uniformity of performance. Strains of these species produce little or no aroma. Streptococcus diacetilactis typically produces lactic acid through a lactose fermentation and appreciable quantities of diacetyl and related aroma compounds through fermentation of citrate. All three of these organisms may be employed as single strain starters in which only one strain of a species makes up the culture or they may be used as mixed strain cultures. Mixed strain starters may consist of several strains of one species or of

combinations of several species. For cheeses like Cheddar, Monterey Jack and similar types where only a lactic acid fermentation is required, single or mixed strain starters of S. cremoris or S. lactis, or both, will be used. Products characterized by a diacetyl flavor, such as buttermilk and cultured sour cream require starters containing flavor producing microorganisms. These starters may consist of S. diacetilactis, alone, but more commonly they are mixed starters consisting of S. lactis, or S. cremoris and a flavor producing microorganism such as S. diacetilactis or Leuconostoc citrovorum.

Classification of Starter Culture Bacteria

Starter bacteria are classified in the sub-order Eubacteriineae. The Eubacteriineae include a physiologically diverse collection of bacteria but all appear morphologically as rods or spherical cells in various arrangements. Motility when it is present is by means of flagella and cell division is by simple fission. The family Lactobacteriiaceae, of the sub-order Eubacteriineae, include the gram-positive, nonmotile, microaerophilic or anaerobic rods or cocci. The spherical bacteria of the Lactobacteriiaceae are classified in the tribe Streptococcieae. The two genera of the tribe Streptococceae, Streptococcus and Leuconostoc, are both cocci arranged in chains. They can be separated by the relative inactivity in litmus milk

culture at 21° C and 30° C of the Leuconostoc as compared with the high activity of the Streptococcus. The streptococci are divided into four groups that can be separated on the basis of physiological characteristics and serological typing. The lactic group includes the S. lactis, S. cremoris and S. diacetilactis. Differentiation of these species can be achieved by consideration of relatively few tests. All three species give a positive precipitin reaction with group N antiserum and coagulate milk in 48 hours at 30° C. S. lactis and S. diacetilactis produce ammonia from the hydrolysis of arginine but S. cremoris does not. S. diacetilactis is the only one that produces diacetyl. Further simplification has recently been achieved by development of differential agar (Reddy, et al., 1969) and broth media (Reddy, et al., 1970). The agar distinguishes S. lactis from S. cremoris on the ability of the former to hydrolyze arginine but inability of S. cremoris to do so. The broth has the further advantage of differentiating all three species; S. diacetilactis is identified by the characteristic production of carbon dioxide, S, lactis and S. cremoris are differentiated, as in the agar medium, by arginine hydrolysis.

Cultivation of the Streptococci

Nutrition

The streptococci are a fastidious group of bacteria requiring, in addition to a nitrogen and carbon source, inorganic salts, vitamins and a minimum of six amino acids (Reiter and Oram, 1962).

The traditional medium for cultivation of starter bacteria is milk. Reiter and Moller-Madson (1963) reported that Orla-Jensen found that milk supplied the essential vitamins and nitrogenous substances for many of the lactic acid bacteria. It is possible, however, to stimulate the metabolic activity of streptococci in milk by adding various forms of nitrogen. Liver and yeast extracts (Smith, 1943), peptones (Garvie and Mabbitt, 1956, corn steep liquor (Kennedy and Speck, 1955), and pancreas extract and peptide fractions (Kizer, et al., 1955) have been found to stimulate lactic acid production. The addition of individual amino acids or of purines and pyrimidines fails to increase appreciably the activity of any starter but increases in the peptide content does increase starter activity (Williamson and Speck, 1962). Kihara and Snell (1960) pointed out that the activity of peptides resulted from their more efficient use as sources of limiting amino acids than the free amino acids themselves.

S. lactis, S. diacetilactis and S. cremoris require niacin,

pantothenate and biotin and are stimulated by pyridoxal (Reiter and Oram, 1962). Thiamine, folic acid and vitamin B_{12} are not required and riboflavin is required only by strains of S. cremoris. Milk contains all the vitamins required in sufficient concentrations but fluctuations in the content according to season, stage of lactation, etc., occur and may affect starters (Gregory, 1962).

Glutamic acid, valine, methionine, leucine, isoleucine and histidine are required amino acids by single strain starters. Aspartic acid, citrulline and ornithine are not required. Some strains of S. lactis and S. diacetilactis require arginine and phenylalanine in addition to the above required amino acids. Strains of S. cremoris require proline and phenylalanine and in most strains also tyrosine, alanine and lysine. A few strains require threonine, tryptophan, arginine and glycine. Milk contains low amounts of amino acids, thus it appears that the lactic acid streptococci must obtain most of the required nitrogen from proteins. The limited ability of these organisms to hydrolyze proteins to amino acids has prompted the suggestion that they are able to utilize peptides. Much of the support for this suggestion has come from the stimulatory effects of peptides mentioned above.

Snell and Mitchell (1941) prompted by reports that hydrolytic products of nucleic acids might become factors limiting growth of various organisms under certain conditions, investigated the effect

of purine and pyrimidine bases on the growth of lactic streptococci. Adenine and thymine were found to be essential for growth of S. lactis. Niven (1944), however, found that xanthine, adenine, quanine and uracil served as growth stimulates for S. lactis, but could be eliminated from the medium without seriously affecting growth. In an investigation into the stimulatory effects of peptides, Anderson and Elliker (1953a) found that all strains of S. lactis and S. cremoris tested required adenine and most required quanine or uracil or were stimulated by them. The compounds, which were essential for growth in a synthetic medium, were found to be stimulatory in nonfat milk (Anderson and Elliker, 1953b). Koburger, et al., (1963), in an investigation of the stimulatory properties of pancreas extract, also found nucleic acid derivatives to be stimulatory to S. lactis when grown in milk. In this investigation, the active compounds were inosine, hypoxanthine and adenine.

Lactose is the principal source of carbon and energy for the lactic streptococci when they grow in milk. Other sugars may be fermented, including sucrose, mannose, fructose, and maltose (Sandine, et al., 1962). Citrate can be utilized by Streptococcus diacetilactis (Fryer, et al., 1970) and Kizer and Speck (1955) have observed a significant affect of citrate on growth of S. lactis. Kizer and Speck (1955) have also confirmed an acetate requirement of S. lactis and S. cremoris strains reported by Collins,

et al., (1950). Citrate has an inhibitory affect on S. cremoris.

Potassium and magnesium are required in large amounts by starter culture organisms (Reiter and Moller-Madson, 1963). In iron-deficient milk, Pulay, et al. (1959) reported a decrease in acid production by S. lactis and S. cremoris. Reiter and Oram (1968) confirmed the iron requirement of S. cremoris and S. lactis strains but reported that vanadium at high concentrations would replace iron. Molybdenum, cobalt, zinc and copper have no effect on growth. Manganese stimulates growth of starter betacocci (Leuconostoc), but has no effect on the lactic streptococci including Streptococcus diacetilactis (DeMan and Galesloot, 1962). The absence of any effect on single strain starters when grown in a calcium free phage resistant medium suggests that calcium is not a requirement for growth (Reiter, 1956).

Growth Factors

In addition to the nutritional demands of the organism, temperature, pH and the gaseous environment are factors which affect growth of the lactic streptococci.

Pont and Holloway (1968) reported an optimum growth rate for S. lactis in whey at a temperature of 30° C. Bergere (1968) reported a maximum growth rate at 35° C in a tryptone-yeast extract-lactose medium for the same strain but the cell yield was

greater at 25° C.

Davis and Thiel (1939) studied the effect of pH on growth of a large selection of streptococci and lactobacilli. The pH range for S. cremoris was 5.4 to 7.7 with an optimum of 7.0. S. lactis grew in a range of 4.8 to 9.0 with the optimum between 6.0 and 8.5. S. diacetilactis grew in a pH range similar to S. lactis, but the optimum pH was restricted to 6.5. Harvey (1965) reported a pH optimum of 6.3 for S. lactis and found that below pH 5 there was a reduction in the specific activity of the cellular enzymes. The optimum pH reported by Bergere (1968) was 6.5. Pont and Holloway (1968) have probably taken the most realistic approach in stating that the generation time of S. lactis did not differ significantly between pH 6.0 and 6.5.

The presences of oxygen in milk can result in the formation of hydrogen peroxide by the starter organisms and subsequent inhibition of their growth and metabolism. This has been brought out by Hogg and Jago (1970) and proof has been provided by Pont and Holloway (1968), Gilliland and Speck (1969) and Rahn, et al. 1938).

Production of Lactic Starter Cultures

The hazards inherent in conventional starter culture preparation have prompted an extensive search for better methods of preparation. The majority of studies in this area have been on the

application of batch methods of cultivation presumably because of the simpler techniques and equipment required. Attempts to cultivate the lactic streptococci by dialysis, a technique covered extensively in a recent review by Schultz and Gerhardt (1969), has proven of limited value. Cultivation of streptococci by continuous method is a recent consideration resulting from the development and continuing interest in mechanized cheese making.

Production of starter cultures by batch methods has essentially taken the approach of manipulating the growth variables to produce high yields of cells, then concentrating the cells into a smaller volume.

Lamprech and Foster (1963) grew S. lactis and S. diacetilactis in a tryptone-yeast extract-glucose broth at 25° C. The medium was buffered with $Mg_3(PO_4)_2$ and aerated to provide agitation. Yields of 1.0 to 1.2 x 10^{10} for S. lactis and 4 to 5 x 10^9 for S. diacetilactis were obtained. The cells yields were essentially identical whether nitrogen gas or air was used to agitate the culture and a dried autolyzed brewers yeast could be substituted in the medium for Trypone and yeast extract with only slight reductions in cell yields. The insoluable magnesium phosphate presented a problem, however, when attempts were made to concentrate the cells from the medium.

Pont and Holloway (1968) studied the growth of S. lactis C10 in a variety of whey media under different conditions. The highest

yields were obtained with a whey broth containing 1% tryptone and 5.8% yeast extract. The optimum temperature was found to be 30° C and the optimum pH, which was maintained by automatic addition of 5N sodium hydroxide, was 6.3.

Peebles, et al. (1969) have also used an automated pH control system. They used single strains of S. cremoris which they grew in a tryptone-yeast extract-glucose-lactose broth. The optimum pH was found to be 6.0 and the use of ammonium hydroxide as a neutralizer resulted in higher yields than when sodium hydroxide was used.

Stadhouders, et al. (1969) compared cultivation of single and mixed strain starter bacteria in skim milk and in a tryptone-yeast extract-lactose medium (T_1P). Higher yields and activity were reported for single strains of S. lactis and S. cremoris in the T_1P medium but skim milk cultures proved to be more satisfactory for the cultivation of mixed strain starters.

In addition to the growth studies, Stadhouders, et al. (1969) reported on a technique for removing bacterial cells from a skim milk medium. The technique involved solubilization of the calcium-casein-phosphate complex in milk by adjustment of the pH to 6.9 and the addition of sodium citrate. The cells could then be removed from the milk by centrifugation. Continuous culture methods have essentially been based on the use of pH control to maintain the flow rate (Linklater and Griffin, 1971). In this technique, additional medium,

which is skim milk in most cases, is input when the continuously monitored pH drops below a predetermined setting. The input of medium into the reaction vessel results in the flow of culture fluid containing actively growing cells through the output orifice. Precise adjustment of the flow rate can result in a continuous operation with a theoretical life span of infinity. The pH setting is a critical factor and must be determined for each organism. Contamination, obstruction of output and input openings due to wall growth and difficulty in adjusting the apparatus for optimum flow rate are some of the difficulties that have been encountered. The production of log phase cells is also a disadvantage since these cells are more susceptible to storage conditions. There is presently no known commercial operation using a continuous method of starter preparation although workers in both Australia and Russia have apparently used the process on a pilot scale (Lloyd, 1971). The use of continuous starter production integrated with a mechanized cheese making process holds the greatest promise for this procedure.

Part II. Lyophilization

Definition and Historical Development

Lyophilization, or freeze drying, is the dehydration of a frozen aqueous material through the sublimatation of ice. Meryman

(1966) has credited Altman with the first reported use of freeze drying in 1890. There was an apparent lack of interest in the procedure however until the report of Shachell (1909). His report was followed by reports of the application of freeze drying to the preservation of a variety of biological materials. One such report was by Hammer (1911) who demonstrated that lyophilized bacterial cultures survived longer than cultures dried from the liquid state. This was the first report of the lyophilization of bacteria. Several excellent reviews have since reported the progress made on preservation of bacteria by lyophilization (Fry, 1964; Heckly, 1961; Meryman, 1966).

Factors Affecting Survival of Microogranisms During Lyophilization and Storage

There are five stages in the lyophilization process: (1) the preparation of the suspension for drying; (2) freezing; (3) drying; (4) storage; and (5) rehydration. A reduction in the survival of the microorganisms may occur at any point due to unfavorable conditions (Meryman, 1966).

Preparation of the Suspension

At this initial stage, the type of organism and the nature of the suspending fluid are the most critical factors. The recommended procedure is to make the time that the organisms are in the

suspending fluid before freezing as short as feasible. Any injury occurring to the cells during this stage will be compounded in subsequent processes.

Freezing

The freezing of bacteria has been intensively studied, but more as a means of preservation than as a step in the lyophilization process. Maqur (1960) has stated that survival during freezing depends on the temperature to which cells are cooled, the rate at which they are cooled, the rate at which they are thawed, and the nature and physical state of the medium in which they are suspended during the process. The formation of intracellular ice during freezing has been suggested as the most important factor contributing to injury and death of the cell.

Drying

The susceptibility of microorganisms to the drying process depends upon a number of factors. An examination of the literature reveals a wide variance in the sensitivity of organisms to drying. The genera of bacteria most resistant are Staphylococcus, Sarcina and Micrococcus. The genera Vibrio and Neisseria are the least resistant (Rhodes, 1950). The streptococci are only slightly less resistant than the staphylococci, Sarcina or micrococci.

The significance of nutrition as a factor in survival has not been ascertained and there are conflicting reports on the effect that culture age has on survival. The bulk of the data appears to support the conclusions of Naylor and Smith (1946) that maximum survival is attained in cultures that are harvested at the maximum stationary phase of growth.

Record and Taylor (1953) have shown an increase in survival of Escherichia coli in phosphate buffer with increases in cell concentration. This cell concentration effect may be the result of protective materials extruded from injured or dead cells. Support for this hypothesis was given by Bergmann, et al. (1957), who showed that survival of Brucella abortus during freeze-drying was enhanced when extracts from the supernatent of aged cultures were added.

The cell suspending medium has received more attention than any other single factor in the lyophilization process. Many substances have been tried and recipes proposed. A list of these recipes have been cataloged by Heckly (1961). The substances most often reported to have a protective effect have been carbohydrates, (Heller, 1941; Fry and Greaves, 1951) serum, (Elser, et al., 1935) sodium glutamate, (Cho and Obayaski, 1956) and skim milk (Hammer, 1911). Despite the numerous studies on the cell suspending medium, there has been little effort to uncover the nature of the protecting effect offered by various compounds. The first such study

of this kind has been on sodium glutamate by Morichi, et al. (1963). The protective effect of glutamate, according to Morichi, is associated with the hydrogen-bond generating group ($-NH_2$) and the two acid groups (α-COOH and γ-COOH), which have a high affinity for water. This quality insures the retention of adequate moisture in the dried preparation.

Storage

The factors which are important in the drying process are also important during storage. In addition, three other factors are involved during storage. These are: (1) atmosphere in which the cultures are stored; (2) temperature of storage; and (3) residual moisture.

Dried cultures have been stored in air, in a vacuum or in nitrogen gas, but there is substantial evidence that storage under vacuum is essential for maintaining a high viability of dried cultures. Air or oxygen has been reported to have a lethal effect (Meryman, 1966). The effect of oxygen on freeze dried cultures has been investigated rather extensively by Heckly, et al. (1963). who showed a correlation between death of dried organisms and spontaneous free radical formation; data of Swartz (1970) support this idea. Free radical formation was more extensive when lyophilized S. lactis cultures were stored in air than in the absence of air. Storage under

nitrogen insured better survival than air or oxygen, but not as good as storage under vacuum (Naylor and Smith, 1946). The effect of temperature on viability during storage seems to be the most clearly defined. The higher the storage temperature, the lower the survival rate. This relationship may be affected to some degree by the nature of the suspending fluid and the storage atmosphere.

The assumption that the dryer the culture, the greater the survival, made by early workers, was questioned by Fisher (1950), and Fry and Greaves (1951) provided experimental evidence that a prolonged drying time lowered the survival. From the work of Obayashi and Cho (1957) and Scott (1958) there does appear to be an optimum moisture content. This optimum level is affected to some degree by the suspending substance and may vary with the type of organism.

Rehydration

The solution used for rehydration, the rate of addition, the temperature of rehydration, and the recovery medium have been implicated as factors in the optimum recovery of lyophilized bacteria (Leach and Scott, 1959; Speck and Meyers, 1946; Postagate and Hunter, 1963).

Lyophilization of Starter Cultures

Parallel with the studies on lyophilization have been attempts to apply the discoveries to the preservation of dairy starter cultures.

The first reported attempt to produce lyophilized starter cultures was by Rogers (1914). His objective in using lyophilized culture for cheese starters is still applicable.

> The ideal culture for distribution is in a dry form, sufficiently active to produce rapid growth when it is added to milk and yet so dormant that it can be held a long time without losing its activity.

Rogers used the Shachell Method (Shachell, 1909) which involves exposing cultures in a frozen condition over sulfuric acid in a vacuum. He used pure cultures of Lactobacillus bulgaricus and "cultures occurring in Swiss cheese". The variability in survival to lyophilization of the starter organisms led him to confess some doubts as to the value of this procedure.

Nichols and Hoyle (1949) reported on the activity of commercial starters and strains of S. lactis and S. cremoris stored for up to three and one-half years in the lyophilized state. The cultures were stored in milk or horse serum under vacuum. All cultures could be rejuvinated but activity was measured only after daily transfers for three to seven days giving rise to a question of the original activity of the dried preparation.

Czulack and Hammond (1953) reported on their experience with freeze drying of single strain starter cultures as a means for maintaining them in an active state. They suspended cells grown in tryptone-yeast extract broth for 48 hours at 30° C in a diluted horse serum-lactose suspension. The cells were lyophilized in 0.5 ml amounts sealed under vacuum and stored at room temperature. The activity, measured over a period of six to 16 months did not show a significant decrease. Unfortunately, as in the work of Nichols and Hoyle, the activity was measured only after transfer of the original lyophilized culture, thus leaving the activity of the original dried preparation in question.

MATERIALS AND METHODS

Source of Microorganisms

Frozen milk cultures labeled Streptococcus lactis C2F, Streptococcus cremoris 459 and Streptococcus diacetilactis 18-16 from the stock culture collection of the Department of Microbiology at Oregon State University were used in this study.

Preparation and Maintenance of Stock Cultures

The frozen stock cultures were thawed at 30° C and 0.1 ml was used to inoculate 10 mls of 11% nonfat dry milk (Matrix, Galloway West Co., Fond du Lac, Wis.). The inoculated milk was incubated at 22° C for 18 to 22 hrs. At the end of the incubation period, the cultures were streaked on lactic agar, incubated at 32° C for 42 hrs. and individual colonies transferred to 10 ml of 11% nonfat dry milk (NFDM). The S. lactis and S. cremoris cultures were incubated at 22° C, the S. diacetilactis at 30° C, until coagulation was noted. One-tenth ml from those cultures showing coagulation was added to each of 20 glass screw cap tubes containing 5.0 ml of 11% NFDM. The inoculated cultures were quick frozen in a dry ice-acetone bath and placed in the freezer at -22° C. A streak plate of the culture on lactic agar and a methylene blue stained smear was also made to

check for purity of the culture. At monthly intervals, one frozen culture of each species was removed to prepare a new lot of stock cultures according to the procedure above.

Preparation of Working Cultures

Working cultures were prepared by the following procedure. One frozen culture of the organism to be used was thawed at 30° C and 0.1 ml inoculated into 10 mls of NFDM. The culture was incubated at 22° C for 16 to 18 hours and 0.1 ml transferred to 10 mls of NFDM and incubated as before. The procedure was repeated once more before using the culture in the scheduled experiment.

Characterization of Stock Cultures

Stock cultures of each of the three lactic streptococci were characterized by serological and physiological tests to establish the validity of the stated genus and species.

Serological Testing. The serological identification of cultures as members of the Group N streptococci was performed according to directions of Difco (Detroit, Mich.) for use of their Bacto-Streptococcus Group N antigen and antiserum in the group precipitin test. The extract was prepared using Todd Hewitt Broth grown cells and the autoclave method. The capillary tube method was used for the precipitin test.

Coagulation of Milk in 48 Hrs. Eleven percent NFDM was inoculated with 1% of the stock culture and incubated at 30° C until coagulation was noted.

Diacetyl Production. The method of King (1948) was used to measure diacetyl production in eighteen hour milk cultures.

Arginine Hydrolysis. The method of Niven, et al. (1942) was used to determine ammonia production from arginine. Niven's broth was inoculated with 18 hour NFDM cultures of the test organisms.

Differential Broth. One-tenth ml of 18 hour NFDM cultures were used to inoculate the lactic streptococci differential broth of Reddy, et al. (1970). The broth was incubated at 30° C and observations made at 24 hr. intervals for 72 hrs.

Carbon Dioxide from Citrate. 18 hour NFDM cultures of the stock organisms were used to inoculate the citrate broth of Sandine, et al. (1962). Visual observations for carbon dioxide production were made at the end of 24 hrs. of incubation at 30° C.

Culture Media

Nonfat dry milk was used at a concentration of 11% (w/v), unless otherwise stated, for propagation of stock cultures and production of cells for lyophilization studies. S. lactis C2F was also grown in the T_1P medium of Stadhouders, et al. (1969) consisting of 2% Tryptone, 0.7% yeast extract and 5% lactose and the medium of

Pont and Holloway (1968) consisting of 11% whey, 1% Tryptone and 0.5% yeast extract to compare with growth in NFDM. Lactic agar was used for plate counts; it consisted of Tryptone, 2%; yeast extract, 0.5%; gelatin, 0.25%; dextrose, lactose, sucrose, 0.5%; sodium chloride, 0.4%; sodium acetate, 0.15%; ascorbic acid 0.05%; agar, 2%.

Growth Apparatus

A 14 liter Fermentation Design fermentor or a 1 liter Scientific Products Micro-fermentor was used in growth studies and for the production of cells for the lyophilization studies. The pH was monitored and maintained by a Fermentation Design pH control module with an Ingold pH electrode. The base was prepared in concentrations of 30-35% (w/v) and sterilized by autoclaving. Non-sterilized solutions of ammonium hydroxide and ammonium carbonate were used in concentrations of 25% (w/v). Bases were added automatically to the culture medium by peristaltic pumps controlled by the pH module.

Growth Curves

Growth curves were determined by removal of a sample of culture at specified intervals. One ml of the sample was diluted by serial dilution and pour plates in lactic agar made of the appropriate

dilution. The plates were incubated at 32° C for 48 hrs. and the colonies counted. Cell concentrations were expressed as colony forming units per ml (cfu/ml). Nine ml dilution tubes containing 0.1% NFDM were used for serial dilution of the sample. The first tube of each dilution series contained 6 gm of 3 mm glass beads, 1.0 ml of 20% sodium citrate and 8.0 mls of 0.1% NFDM.

The generation time was determined by the formula g=t/n where "t" is the time of logarithmic growth and "n" is the number of generations in time "t"; $n=(\log N_1 - \log N_0)/\log 2$. The specific growth rate (sgr) was calculated according to the following formula: sgr=ln 2/g.

Harvesting of Cells and Preparation of Concentrate

Cells were harvested from the NFDM cultures at the end of the logarithmic phase of growth. The culture was first adjusted to pH 6.9 with 35% sodium carbonate. Sodium citrate was then added to a final concentration of 4.5% while stirring the culture. The culture was stirred for an additional ten minutes then centrifuged at 7000 rpm (7970 x g) for one hour in a Sorvall refrigerated centrifuge. The sedimented cells were reconstituted in the specified suspension fluid to approximately 1/10 the volume of the original culture. Unless otherwise specified, the cell suspension was adjusted to pH of 7.0 with sterile 2N sodium hydroxide. The cell suspension was referred

to as the "cell concentrate".

Harvesting and Activity of Cells From T_1P and Whey Media

Cells were harvested from T_1P and whey medium without addition of sodium citrate by centrifugation for one hour at 7970 x g. The cells were reconstituted in 10% NFDM to 1/10 the original volume of the culture. Activity tests were determined on the concentrates using 0.1 to 0.6 ml of concentrate to inoculate 50 ml of steamed milk. The inoculated milk was incubated four hours at 30° C and the acid formed was titrated to pH 8.3 using 0.1N NaOH. The plots of activity versus size of inoculum for concentrates prepared from T_1P, whey and NFDM media were used to determine the activity of the same concentrate of cells prepared in the different media (Figure 1).

Studies on Factors Affecting Lyophilization and Storage Survival

Effect of Cell Suspending Fluid

Cell concentrates were prepared in 10% NFDM, 5% monosodium glutamate or 5% trehalose (Sigma Chemical Co., St. Louis, Mo.) to a cell concentration of 3 to 5 x 10^{10} cfu/ml, the pH adjusted to 7.0, and 3.0 ml distributed into each of approximately 18 screw cap scillination vials. The contents of each vial was quick frozen

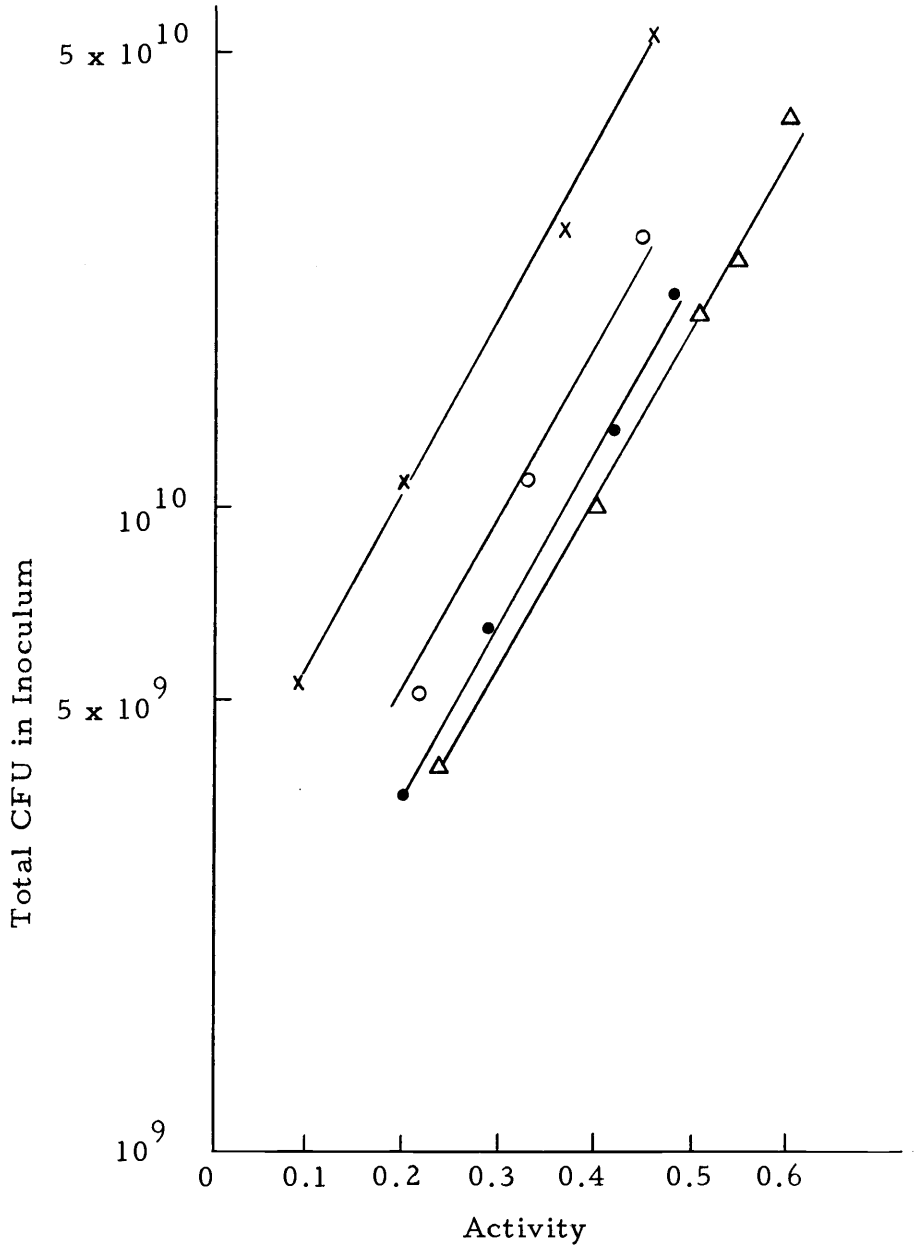

Figure 1. Plots of activity versus size of inoculum for cell concentrates harvested from T_1P; 0-0, whey; x-x, NFDM (controlled conditions); ●-●, NFDM (static conditions) Δ- Δ . See text for details of procedure.

at -76° C in a dry ice-acetone bath and placed in the well of a Virtis Freeze-mobile with the caps removed. The frozen material was lyophilized for 24 hours then the vial removed and the cap replaced. One vial was reconstituted to the original volume with 0.1% NFDM immediately after lyophilization and the activity determined; this activity was considered 100% for comparison of concentrates receiving different storage treatments. Three vials were used for moisture determinations. The remaining vials were distributed between four storage temperatures: +22°, +7°, -22° and -196°; and examined at weekly intervals for activity.

Effect of pH of Cell Suspending Fluid

The procedure was as above with the exception that only 5% glutamate was used as the suspending fluid and in one of the concentrates the pH was not adjusted to 7.0.

Effect of Ascorbic Acid, Potassium Iodide and Butylated Hydroxyanisole

All concentrates were prepared in 5% monosodium glutamate which contained either 0.16M potassium iodide, 0.05% ascorbic acid or 0.05% butylated hydroxanisole (BHA; Sigma). To effect suspension of BHA it was necessary to add 5% Tween 80. Concentrates were lyophilized in vials.

Effect of Atmosphere

Cell concentrates prepared in 5% sodium glutamate was distributed in 1.5 ml amounts to 10 ml lyophilization ampules and lyophilized. Ampules containing concentrate to be stored in vaco were sealed by gas torch. Ampules containing concentrate to be stored in air were removed from the lyophilizer and stoppered after filtered air had been admitted. For determination of activity the concentrates were reconstituted to the original volume of 1.5 mls with 0.1% NFDM.

Determination of Activity

Concentrate activity was determined by adding 0.5 ml of sample to 50 mls of 11% NFDM which had been steamed for 30 minutes. The inoculated milk was incubated in a 30° C water bath for four hours then placed in ice. The milk was titrated with approximately 0.1 N Sodium hydroxide to a pH of 8.3 with a Fisher Automatic Titrimeter. The activity was expressed as the percent lactic acid calculated according to the following formula: percent lactic acid = (ml of base x normality of base x 9)/ml of sample.

Determination of Moisture

The moisture content of lyophilized concentrates was

determinated by comparing the initial weight of the lyophilized vial with the weight of the vial after decication over phosphorous pentoxide at 45° C for one week. The loss in weight was divided by the initial weight of the water in the sample and multiplied by 100 to give the percentage of moisture.

RESULTS

Characterization of Stock Cultures

The cultures used in this study were characterized to confirm the stated genus and species. The results are given in Table 1. All cultures were gram positive cocci in pairs or in chains. The positive precipitin reaction with group N antiserum established the cultures as members of the lactic group of streptococci. S. diacetilactis was confirmed on the ability to produce carbon dioxide from citrate and diacetyl in milk. S. cremoris was characteristically unable to produce ammonia from arginine. S. lactis was established on the basis of no diacetyl production in milk and the ability to produce ammonia from arginine. Further support for the designated genus and species was supplied by the typical reactions of each organism in the differential broth as defined by Reddy, et al. (1970).

Conditions for Measurement of Growth

Growth curves were determined on the three lactic streptococci in milk under a variety of growth conditions in an effort to establish the conditions necessary for optimum cell production. In the initial experiments of this study erratic growth curves were obtained especially as the culture approached the stationary phase of

Table 1. Characterization of Cultures Used in the Present Study.

Test	Streptococcus lactis C2F	Streptococcus cremoris 459	Streptococcus diacetilactis 18-16
Reaction to Group N antiserum	+	+	+
Coagulation of milk in 48 hrs.	+	+	+
Diacetyl Produced in milk	-	-	+
NH_3 from arginine	+	-	+
CO_2 from citrate	-	-	+
Differential	acid, NH_3	acid	acid, CO_2, NH_3

growth. Since this phenomena coincided with the period when coagulation of the milk was first observed, it was concluded that particles of coagulated milk were protecting aggregated groups of bacteria from complete dispersal. Methylene blue stained smears on the culture seemed to confirm this. In an effort to eliminate this problem, four growth curves were determined simultaneously on a culture of S. lactis grown in milk at 22° C. Each growth curve was determined by using different dilution conditions for the plate count. The growth curves are shown in Figure 2. An interpretation of the results is limited because a sufficient number of experiments was not done to evaluate the results statistically. It appeared, however, that there was an advantage in having citrate in the first dilution tube. More dramatic evidence of this is shown in Figure 3, which gives the results of an experiment similar to the one described above. The advantages of using glass beads or of using 0.1% NFDM diluent instead of water was not as obvious. In subsequent experiments, the standard procedure was to use a dilution series containing 0.1% NFDM diluent with glass beads and sodium citrate in the first tube.

Conditions for Optimum Growth

Optimum Temperature for Growth

The optimum temperature for growth of S. lactis in NFDM is

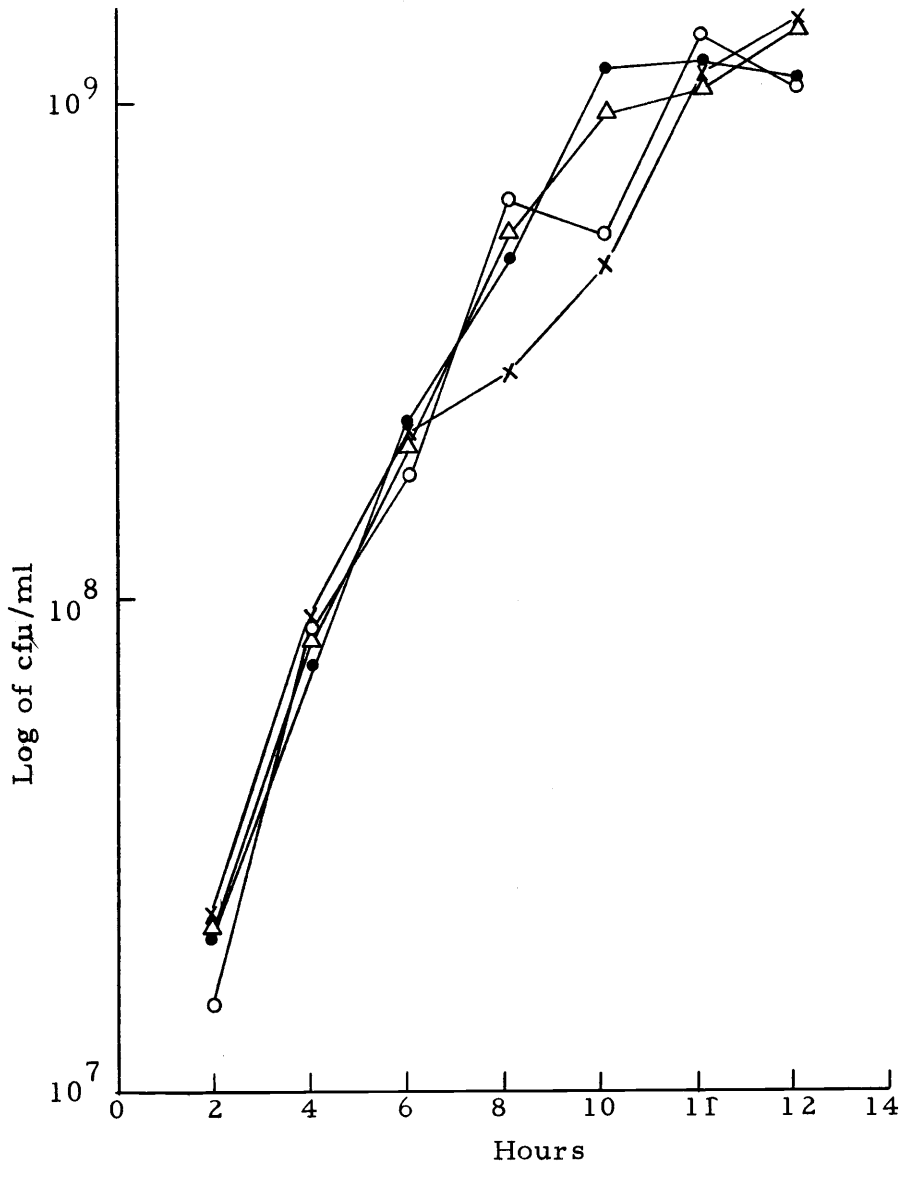

Figure 2. Growth curves of S. lactis in NFDM at 22 C. using four methods of sample dilution. 0-0, 0.1% NFDM in all dilution tubes; x-x, 0.1% NFDM in all tubes, glass beads in first tube; Δ-Δ, 0.1% NFDM in all tubes, 2% sodium citrate and glass beads in first tube; ●-●, distilled water in all tubes, 2% sodium citrate and glass beads in first tube.

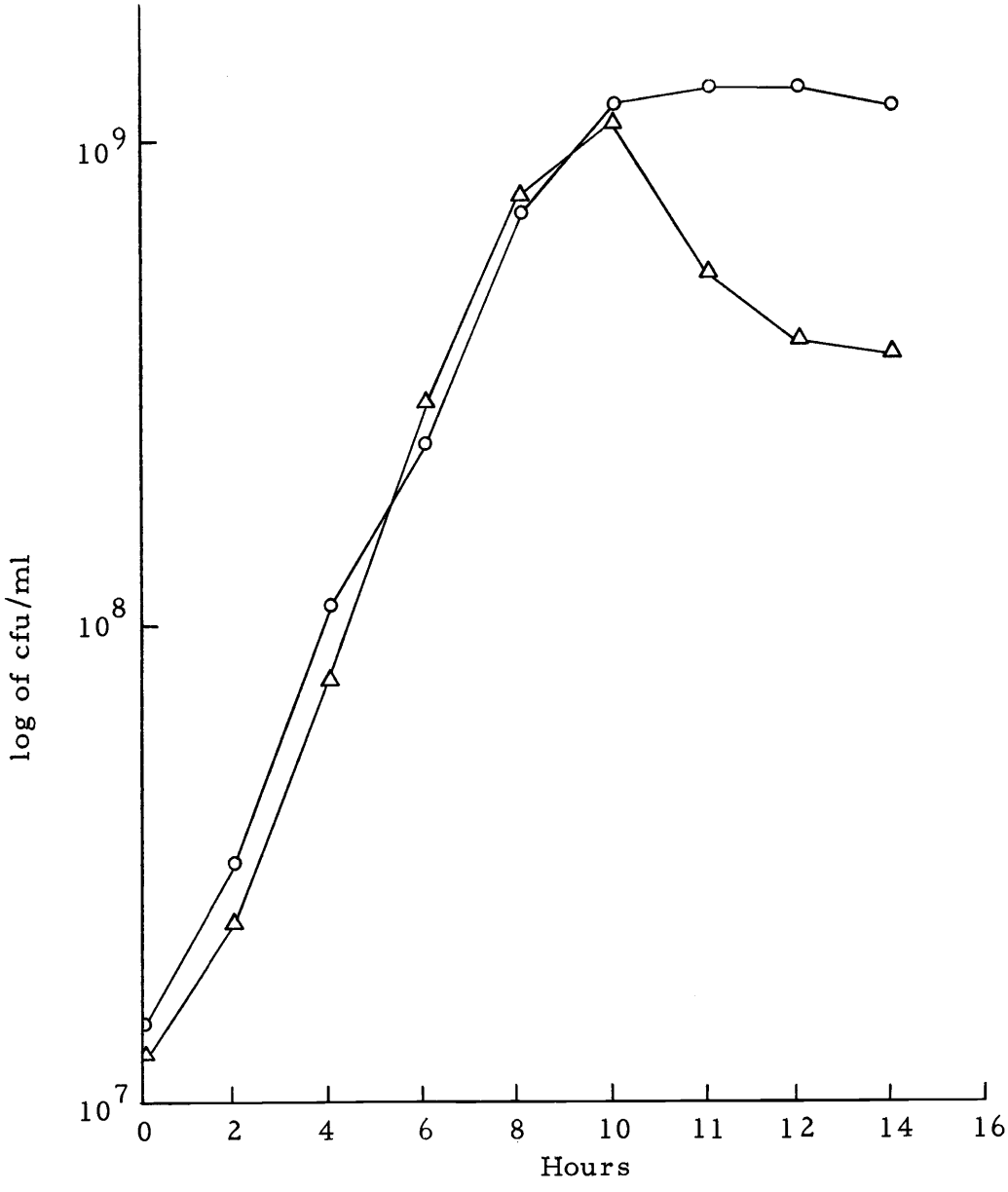

Figure 3. Growth curves of S. lactis in NFDM at 32 C using sample dilutions with, 0-0, and without Δ- Δ, 2% sodium citrate in the first tube; 0.1% NFDM in all tubes and glass beads in first tube.

from 28 to 30° C as seen from the results given in Table 2. Growth in this temperature range gives a maximum cell yield and minimum generation time. A temperature of 30° C was, therefore, used in subsequent experiments for the growth of all three species of test organisms.

Selection of Neutralizer for pH Maintenance

Based on the reports of other workers (Harvey, 1965), that the optimum pH for the lactic streptococci was between 6.0 and 6.5, a pH of 6.3 was selected as the optimum for growth. Effective maintenance of the pH of a growing culture of S. lactis at 6.3 could be attained using a variety of neutralizers as shown in Table 3. Only two of the neutralizers tested were ineffective; potassium carbonate and calcium carbonate. The use of hydroxides of sodium, potassium or calcium as a means of controlling the pH resulted in a two-fold increase in the maximum cell yield. Maintaining the pH with ammonium hydroxide, ammonium carbonate or sodium carbonate resulted in a four-fold increase in the maximum population above the uncontrolled culture. The generation time was not affected by the neutralizer used except where ammonium hydroxide was used, in which case the generation time was reduced, also when ammonium carbonate was used, the generation time was increased. Sodium carbonate was used for maintaining the pH in subsequent

Table 2. Effect of Growth Temperature on Generation Time and Maximum Cell Yield of S. lactis Grown in 11% NFDM.

Temperature	Generation Time (min)	Population (cfu/ml)
22 C	95	1.8×10^9
25 C	86	1.6×10^9
28 C	61	2.0×10^9
30 C	58	2.2×10^9
32 C	83	1.3×10^9
34 C	162	4.1×10^8

Table 3. Effect of Neutralizer on Generation Time and Maximum Cell Yield of S. lactis Grown in 11% NFDM at 30° C with pH Maintained at 6.3.

Neutralizer	Generation Time (min)	Maximum Population (cfu/ml)
None	59	2.2×10^9
NaOH	58	4.4×10^9
KOH	58	4.9×10^9
$Ca(OH)_2$	58	5.5×10^9
NH_4OH	44	8.9×10^9
Na_2CO_3	60	8.4×10^9
K_2CO_3	--	-
$CaCO_3$	--	-
$(NH_4)_2CO_3$	67	8.6×10^9

"controlled" growth because of the high population which could be attained with its use and because of the ease in handling.

Effect of Agitation on Growth

A minimum agitation rate of 100 rpm was necessary under controlled growth conditions to insure proper mixing of neutralizer. Increasing the agitation rate to 200 rpm did not have a significant effect on the generation time or the maximum population attained (Table 4).

Effect of Atmosphere on Growth

When S. lactis C2F was grown at 30° C in 11% NFDM and the pH controlled with sodium carbonate at 6.3, the cell yield could be slightly increased if nitrogen was added continuously at a low flow rate or was used to flush the culture for five minutes at "0" growth time (Table 5). The addition of nitrogen gas had no significant effect on the generation time.

In a similar experiment, carbon dioxide gas was added continuously at a slow flow rate to a S. lactis culture. The culture was grown at 30° C in 11% NFDM with the pH maintained at 6.3 with calcium hydroxide. The generation time was not significantly different from that of cultures grown without CO_2, but the maximum population was increased from 5.5 to 8.2 x 10^9 cfu/ml (Table 6).

Table 4. Effect of Agitation Rate on Generation Time and Cell Yield of S. lactis Grown at 30°C in 11% NFDM with the pH Controlled at 6.3 with Na_2CO_3.

Agitation Rate (rpm)	Generation Time (min)	Maximum Population (cfu/ml)
100	60	8.4×10^9
200	63	8.8×10^9

Table 5. Effect of Nitrogen Gas on Generation Time and Cell Yield of S. lactis C2F Grown in 11% NFDM at 30° C; pH was Maintained at 6.3 with Na_2CO_3.

Atmosphere	Generation Time (min)	Maximum Population (cfu/ml)
Air	60	8.4×10^9
Continuous N_2	63	1.2×10^{10}
Flushed with N_2	62	1.0×10^{10}

Table 6. Effect of Carbon Dioxide Gas on Generation Time and Cell Yield of S. lactis C2F Grown in 11% NFDM at 30° C. The pH was Maintained at 6.3 with Calcium Hydroxide.

Atmosphere	Generation Time (min)	Population (cfu/ml)
AIR	58	5.5×10^9
CO_2	60	8.2×10^9

Comparison of Controlled and Non-Controlled Growth

The generation times and maximum population levels were determined for S. lactis C2F, S. cremoris 459 and S. diacetilactis 18-16 grown in the conventional manner (22° C, static, 11% NFDM). The results obtained by this method of cultivation, referred to as non-controlled growth, were compared with generation time and maximum population data of controlled growth experiments. Controlled growth refers to cultivation in 11% NFDM at 30° C, 100 rpm agitation and maintenance of pH at 6.3 with 35% Na_2CO_3 during growth. The comparisons are shown in Table 7 and Table 15. In all organisms there was a reduction in the generation time when grown under controlled conditions. In S. lactis C2F and S. cremoris 459 there was a greater than four-fold increase in the maximum population level under controlled conditions. In S. diacetilactis 18-16, however, there was no significant difference in the maximum population level between the two growth conditions.

Effect of Yeast Extract on Growth of S. diacetilactis

In an effort to obtain higher yields of S. diacetilactis 18-16 under controlled growth conditions, the 11% NFDM medium was supplemented with yeast extract. The addition of yeast extract up to 1.5% resulted in a higher maximum population level but above 0.5%

Table 7. Comparison of Controlled and Non-Controlled Growth of Lactic Streptococci.

Organism	Growth	Generation Time (min)	Maximum Population (cfu/ml)
S. lactis C2F	non-controlled[a/]	95	1.8×10^9
	controlled[b/]	60	8.4×10^9
S. cremoris	non-controlled	97	1.6×10^9
	controlled	66	8.7×10^9
S. diacetilactis 18-16	non-controlled	96	7.4×10^8
	controlled	55	7.0×10^8

a/ Static culture incubated at 22° C for 18 hr. in 11% NFDM.

b/ NFDM culture incubated at 30° C for 18 hr. with agitation at 100 rpm and pH maintenance at 6.3 with 35% Na_2CO_3.

the cell yield per gram percent yeast extract decreases (Table 8).

Growth of S. lactis in Other Media

Growth of S. lactis C2F in 11% NFDM under controlled growth conditions was compared with controlled growth in media used by other workers for cultivation of starter culture streptococci (Table 9). The whey medium of Pont and Holloway (1969) gave a slightly higher cell yield than NFDM but the T_1P medium of Stadhoulders, et al (1969) gave only half the cell yield that could be attained with NFDM.

Limitations to Growth

Effect of NFDM Concentration

Cultivation of Streptococcus lactis C2F in 5% NFDM under controlled conditions gave a generation time of 70 min and a maximum population of 1.5×10^9 as shown in Table 10. This yield is of the same magnitude as that obtained with 11% NFDM grown under non-controlled growth conditions. Increasing the concentration of NFDM to 11% decreased the generation time to 60 min and increased the maximum population to 8.4×10^9. The generation time and maximum population obtained using higher than 11% NFDM were not significantly different from those at the 11% level. The efficiency of cell production, measured as the maximum cfu/ml/gram of NFDM

Table 8. Generation Time and Maximum Population of S. diacetilactis 18-16 Grown in Yeast Extract Supplemented NFDM Under Controlled Conditions.

Concentration of Yeast Extract (%)	Generation Time (min)	Max. Pop. (cfu/ml)	Max. Pop. / gm. % Y. E.
0	55	6.5×10^8	-
0.5	37	4.1×10^9	8.2×10^{11}
1.0	35	5.9×10^9	5.9×10^{11}
1.5	44	9.4×10^9	6.3×10^{11}

Table 9. Comparison of S. lactis C2F Growth in 11% NFDM, Whey-Tryptone-Yeast Extract, and T_1P Under Controlled Growth Conditions.

Medium	Generation Time (min)	Maximum Population (cfu/ml)
11% NFDM	60	8.4×10^9
Fortified Whey[a/]	56	9.3×10^9
T_1P[b/]	100	3.9×10^9

a/ 11% whey, 1% tryptone, 0.5% yeast extract.

b/ 2% tryptone, 0.7% yeast extract, 0.5% lactose.

Table 10. Effect of NFDM Concentration on Generation Time and Cell Yield of Streptococcus lactis C2F Grown Under Controlled Conditions.

Concentration of NFDM (%)	Generation Time (min)	Maximum Population (cfu/ml)	cfu/gm. NFDM
5	70	1.5×10^9	3.0×10^{10}
11	60	8.4×10^9	7.6×10^{10}
15	57	8.2×10^9	5.5×10^{10}

was greatest at the 11% concentration.

Effect of Sodium Lactate

Growth of S. lactis C2F in 11% NFDM is logarithmic up to eight hours when the pH was maintained at 6.3 with sodium carbonate (Figure 4). At this point, the calculated amount of sodium lactate was 0.23%. The calculated amount at the maximum population level was 0.72%. As shown in Table 11, sodium lactate was inhibitory to S. lactis C2F at concentrations as low as 0.5%.

Harvesting of Cells

The procedure for harvesting bacteria grown in NFDM was similar to that of Stadhouders, et al. (1969). Adjustment of the pH of the culture with 35% sodium carbonate above 6.9 did not result in further clarification of the culture. Raising the level of sodium citrate to 4.5%, final concentration, increased solublization. Rapid stirring of the culture for ten minutes after addition of sodium citrate was necessary for complete solublization. No advantage was gained in extending the time beyond ten minutes.

The Sorvall refrigerated centrifuge was efficient in the removal of cells from the partially cleared culture fluid when centrifugation was for one hour at 7000 rpm (Tables 12 and 15). A centrifugation time of less than one hour or speeds less than 7000 rpm resulted in

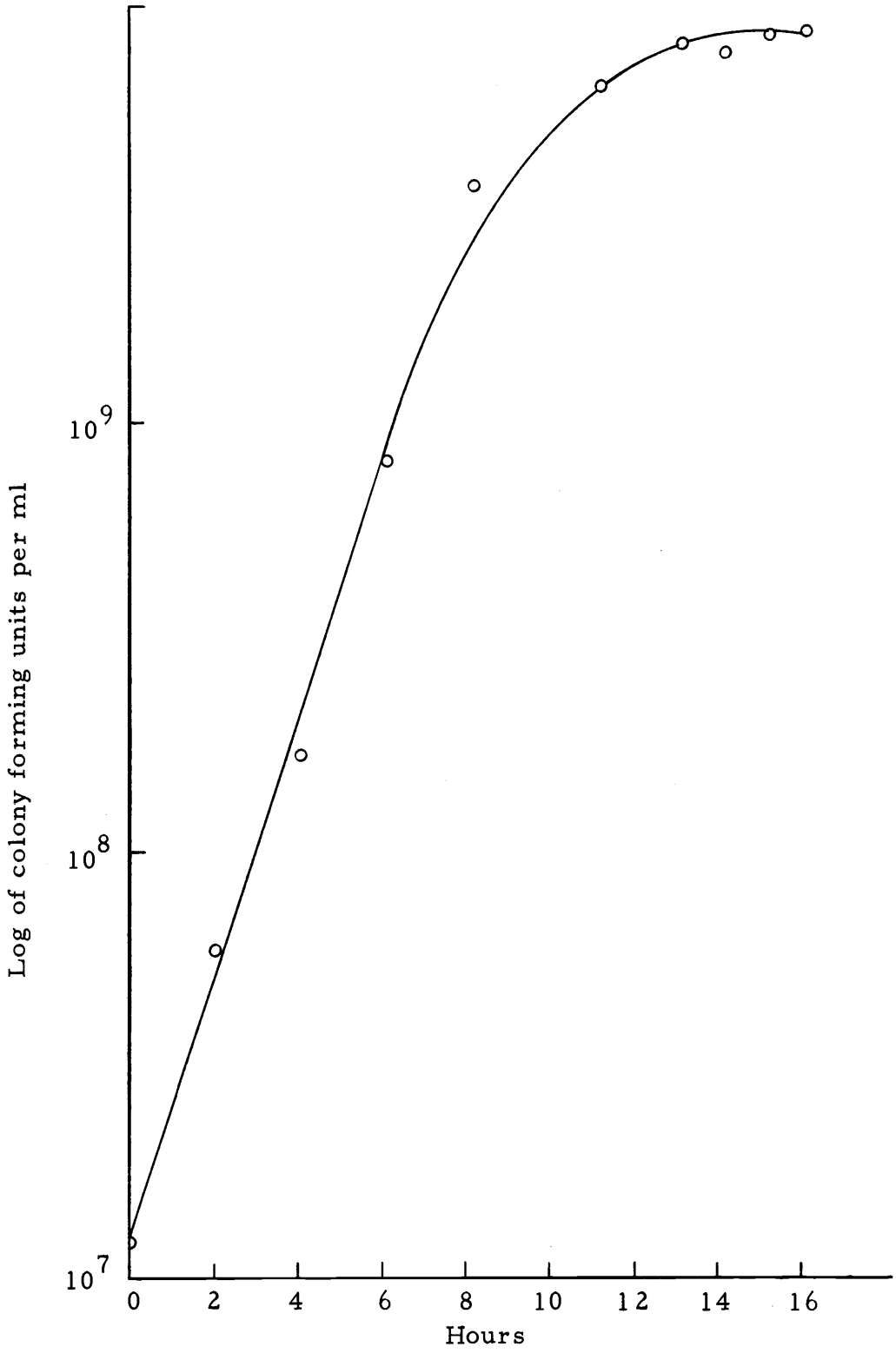

Figure 4. Growth curve of S. lactis C2F in 11% NFDM at 30 C with the pH maintained at 6.3 with Na_2CO_3.

Table 11. Effect of Sodium Lactate on Growth of Streptococcus lactis C2F.

Concentration of Sodium Lactate (%)	Specific Growth Rate[a]
0	0.759
0.5	0.667
0.75	0.414
1.0	0.368
2.0	0.000

[a]The specific growth rate is an expression of the number of generations per unit of time and was calculated according to the following formula: specific growth rate = $(\log_n 2)/g$ where g is the generation time.

Table 12. Evaluation of Procedure for Harvesting of S. lactis C2F Cells From NFDM Cultures Using the Sorvall RC-2 Centrifuge at 7000 rpm for One Hour.

Sample	cfu/ml	Total Colony Forming Units	Percent of Initial Total
Before Harvest[a/]	5.5×10^9	4.2×10^{12}	100
Cell Concentrate[b/]	6.8×10^9	4.5×10^{12}	107
Supernatant[c/]	1.2×10^7	1.2×10^{10}	0.3

a/ Colony count determined on 15 hr culture with total volume of 764 ml. The culture was grown in a 14L Fermentation Design fermenter in 11% NFDM at 30° C. The pH was controlled at 6.3 with 35% Na_2CO_3. A colony count was determined at 15 hrs of growth.

b/ Fifteen hr culture was centrifuged at 7000 rpm for one hour in Sorvall RC-2. The cell paste was reconstituted with 11% NFDM to give a total volume of 66 ml.

c/ Total volume of supernatant liquid was 1024 ml.

less efficient recovery of cells. The Sharples centrifuge was used for removal of cells from larger culture volumes (7 to 10 liters). The efficiency, as measured by the number of cells recovered in the effluent, was equal to that obtained with the Sorvall. Removal of the cell paste from the bowl under asceptic conditions, however, was extremely difficult. An inner bowl lining of aluminum foil aided in removal of most of the cell paste but removal of cells from the lining was as difficult as removing them from the inner bowl wall. To circumvent the difficulties encountered in removal of the cell paste, the cells were lyophilized in the bowl then removed in the dried state. This facilitated removal of the cells but presented additional problems. The dried cells cell paste was extremely difficult to disperse in solution; complete solubilization in milk could not be achieved. This solubility problem prevented an accurate evaluation of the effect of this procedure on the viability of the cells. In addition, difficulty was encountered in maintenance of an adequate flow rate of culture through the centrifuge. When the flow was too slow, medium constituents were deposited in the bowl in addition to the cells. When the flow rate was too rapid, cells were carried out in the effluent.

Activity of Concentrate

There was no significant decrease in the activity of S. lactis cells during the harvesting procedure, but considerable variation

occurred in activity of the concentrate depending upon the medium in which the cells were grown. This is shown in Table 13. All cells were harvested from cultures in the late logarithmic phase of growth and suspended in 10% NFDM. The highest activity was exhibited by cultures grown in NFDM under static conditions at 22° C. Concentrates prepared from controlled growth NFDM cultures were slightly less active than those prepared from non-controlled cultures. Concentrates from T_1P or whey medium were 25% and 53% less active respectively than cells from non-controlled NFDM cultures.

Freezing of Concentrate

There was no significant reduction in the colony counts of S. lactis concentrates during the freezing of the concentrate in preparation for lyophilization. This was true regardless of whether the concentrate was quick forzen at -76° C for one minute in a dry-ice acetone bath or whether it was slow frozen at 22° C for two hours in the freezer. The composition of the cell suspending fluid used had no influence in survival to freezing; 10% NFDM and 5% monosodium glutamate solutions were used.

Table 13. Activity of S. lactis Cells Concentrate Prepared From Whey Medium, T_1P or NFDM.

Growth Medium	Growth Conditions	Activity of Concentrate[a] (inoculum = 10^{10} cfu)
11% NFDM	non-controlled[b]	0.40
11% NFDM	controlled[c]	0.37
T_1P[d]	controlled	0.31
Whey-tryptone-yeast extract	controlled	0.19

[a] Activity expressed as mls of 0.1N NaOH required to titrate to pH 8.3 the acidity developed in 4 hr at 30° C when 50 ml of steamed (30 min) NFDM was inoculated with 10^{10} cfu.

[b] Static culture incubated at 22° C for 15 hr in 11% NFDM.

[c] NFDM culture incubated at 30° C for 15 hr with agitation at 100 rpm and pH maintenance at 6.3 with 35% Na_2CO_3.

[d] 2% tryptone, 0.7% yeast extract, 5.0% lactose.

Lyophilization

Effect of Suspending Fluid on Survival During Lyophilization and Storage

When S. lactis C2F cells were suspended in 10% NFDM and lyophilized, there was a 31% reduction in activity. When the cells were suspended in 5% monosodium glutamate, the loss in activity due to lyophilization was only 14% and when 5% trehalose was used, 6% (Table 14). Loss of activity in stored lyophilized concentrates was greatest when 10% NFDM was the suspending fluid (Table 14). This was especially true at storage temperatures of +22° and +7° C. where greater than 80% reduction in activity occurred in seven days of storage. Five percent monosodium glutamate was the most effective suspending fluid in maintaining the activity of lyophilized concentrates. Five percent trehalose was slightly less protective than glutamate except at the storage temperature of -196° C where both were equally protective. A summary of cell populations achieved for each species in the lyophilized concentrate and other culture types is shown in Table 15.

Effect of pH of Suspending Glutamate-Cell Suspension

The importance of the pH of the concentrate before lyophilization is shown in Table 16. There was a greater reduction in activity

Table 14. Effect of Cell Suspending Fluid on Storage Survival of Lyophilized Streptococcus lactis C2F Cell Concentrates[a/]

Suspending Fluid[b/]	0 days[c/]	Percent Reduction in Activity[d/] in 21 days at			
		+22	+7	-22	-196
10% NFDM	31	80	80	54	11
5% glutamate	14	63	64	0	0
5% trehalose	6	69	67	18	0

a/ The cell concentrate before lyophilization contained 9.8×10^{10} cfu/ml.

b/ Cells were grown under controlled conditions (see b of Table 7), harvested by centrifugation and resuspended in 3.0 ml aliquots of the indicated suspending fluid and lyophilized for 24 hours.

c/ Activity of the concentrated cell suspension before lyophilization was called 100% to determine the loss in activity at 0 days due only to the lyophilization. Activity was determined as indicated in footnote a to Table 13.

d/ Activity of the freshly lyophilized concentrate readjusted to 3.0 ml volume in 0.1% NFDM for comparative purposes. Activity was determined as indicated in footnote a to Table 13.

Table 15. Cell Populations Achieved in Various Types of Cultures of Lactic Streptococci.

	Colony Forming Units/ml		
	S. lactis C2F	S. cremoris 459	S. diacetilactis 18-16
(1) Regular[a/]	1.8×10^9	1.6×10^9	7.4×10^8
(2) Controlled[b/]	7.2×10^9	8.7×10^9	4.1×10^9
(3) Concentrated[c/]	9.8×10^{10}	8.1×10^{10}	1.0×10^{11}
(4) Lyophilized[d/]	5.9×10^{10}	4.2×10^{10}	6.1×10^{10}

a/ Nonfat milk (11%) culture (milk steamed 30 min) incubated at 22° C for 18 hrs.

b/ Nonfat milk (11%) culture grown in fermentor at 30° C, pH controlled at 6.3 with Na_2CO_3, incubated to late log phase. S. diacetilactis 18-16 grown in 11% NFDM supplimented with 0.5% yeast extract.

c/ Cells centrifuged from culture medium and reconstituted in 50 to 70 mls of suspending fluid.

d/ Three ml of concentrate lyophilized for 24 hours in 5% monosodium glutamate.

Table 16. Effect of pH on Cell Suspending Fluid on Storage Survival of Lyophilized S. lactis C2F Concentrates.

Suspending Fluid pH[a]	Percent Reduction in Activity[b] after Storage at 21 Days at			
	+22	+7	-22	-196
pH 7.0	63	64	0	0
pH 5.8	76	73	27	10

[a] Suspending fluid, 5% monosodium glutamate.

[b] See footnotes b and d of Table 14 for experimental details.

during storage of the dried concentrate when left at the original pH of 5.8 than when adjusted to pH 7.0. The reduction in activity as a result of lyophilization was not significantly different between the two preparations.

Effect of KI, BHA and Ascorbic Acid

When 0.16M potassium iodide (KI) was added to S. lactis cells suspended in 5% glutamate, the storage stability of the lyophilized concentrate was increased by greater than 50% (Table 17). Butylated hydroxyanisole (BHA) and ascorbic acid, added at concentrations of 0.05% had a deleterious effect on storage stability. There was no significant reduction in activity of the concentrates containing KI or ascorbic acid as a result of the lyophilization process, but there was an 18% reduction in activity in concentrates containing BHA.

Effect of Storage Atmosphere

No reduction in activity after 28 days of storage was observed in lyophilized concentrates at any of the storage temperatures when the dried concentrates were stored under a vacuum (Table 18). The storage atmosphere appears to be a more significant factor than the suspending medium as illustrated in Table 19. Nonfat dry milk suspended concentrate lost more than 80% activity in seven days when stored in an atmosphere of air but no reduction occurred when the

Table 17. Effect of Additives to Glutamate Cell Suspension on Storage Survival of Lyophilized S. lactis C2F Concentrates.

Suspension Medium	Percent Reduction in Activity in 21 days at			
	+22	+7	- 22	- 196
5% glutamate	63	64	0	0
+Potassium iodide (0.16M)	32	20	0	0
+Butylated hydroxyanisole (0.05%)	75	59	27	0
+Ascorbic acid (0.05%)	65	60	15	8

Table 18. Effect of Air on Storage Survival of Lyophilized *S. lactis* C2F Concentrates.

Storage Atmosphere[a]	Percent Reduction in Activity in 28 Days at			
	+22	+7	-22	-196
Air	57	47	10	0
Vacuum	0	0	0	0

[a] Cells suspended in 5% monosodium glutamate, pH 7.0.

Table 19. Comparison of Storage Survival of S. lactis C2F Concentrates Lyophilized in 5% Monosodium Glutamate or 10% NFDM and Stored in vacuo.

Suspension Fluid	Percent Reductions in Activity in 21 Days at			
	+22	+7	-22	-196
5% Glutamate	0	0	0	0
10% NFDM	0	0	0	0

dried concentrate was stored in a vacuum. The suspending fluid does have some protective effect during the lyophilization process because there is a greater reduction in activity of milk suspended concentrates than glutamate suspended concentrates when measured immediately after freeze-drying (Table 14).

Lyophilization and Storage of Lactic Streptococci Concentrates

There was no significant reduction in activity of S. lactis or S. diacetilactis concentrates during the lyophilization process or during subsequent storage, as shown in Table 20. S. cremoris was more susceptible to lyophilization and storage. There was a 31% reduction in activity of S. cremoris concentrate due to lyophilization and a significant reduction in activity of the stored concentrates at all temperatures.

Residual Moisture of Lyophilized Concentrates

The average residual moisture was 6.9% in all lyophilized concentrates not prepared for storage under vacuum. There was no correlation between residual moisture and effects of lyophilization and storage on activity of the resulting cells.

Table 20. Effect of Storage Temperature and Time on Activity of S. lactis C2F, S. cremoris 459 and S. diacetilactis 18-16 Concentrates Lyophilized in 5% Monosodium Glutamate and Stored Under Vacuum.[a/]

Organism	0 Days[b/]	Percent Reduction in Activity[c/] in 28 Days Storage at			
		+22	+7	-22	-196
S. lactis	7	0	0	0	0
S. cremoris	31	23	13	13	16
S. diacetilactis	0	0	0	0	0

a/ Cells were grown under controlled conditions, harvested by centrifugation and resuspended in 5% monosodium glutamate. Aliquols of 1.5 ml were lyophilized in 10 ml amplues.

b/ Values at 0 days reflect loss in activity due to lyophilization of the concentrate.

c/ See footnotes to Table 14 for experimental details and explanation of table values.

DISCUSSION

The objective of this study was the production of starter culture concentrates of small volume capable of remaining viable under prolonged or adverse storage conditions. The study was conducted in three phases. The first phase was an investigation into the optimum conditions for cultivation of high cell yields of the more commonly used starter culture bacteria. The second phase was the development of the methodology for harvesting cells and preparation of concentrates. The final phase was an investigation of the conditions under which cell concentrates could be lyophilized and stored with a minimum loss in activity.

The three bacteria, S. lactis C2F, S. cremoris 459, and S. diacetilactis 18-16 were selected as the study organisms because they are representative of the starter bacteria most widely used in the dairy industry. Variable responses to growth conditions, lyophilization and to storage conditions among different strains may occur, but these should be minor enough to require only slight modification in the developed methodology.

Growth

Growth of most microorganisms is influenced by several factors. These are (1) temperature; (2) pH; (3) nutrient

requirements; (4) relationship to other organisms in the same environment; (5) gaseous environment, especially atmospheric conditions, important for obligate aerobes or anaerobes; (6) oxidation-reduction potential; and (7) presence or formation of growth inhibitory substances to which the organisms are sensitive. Batch cultivation of microorganisms in the laboratory is convenient for studying these variables since control can be exerted on the influence of each factor. Oxidation-reduction potential was not examined *per se* in the present study, but the gaseous environment was controlled. The fourth factor was not considered because only single strain starters in the defined environment were used.

The relationship between growth temperature and cellular metabolic function has been explained in terms of the optimum temperature range for maximum enzymatic activity (Ingraham, 1962). In the present study, the optimum range was found to be from 28° to 30° C, based on generation time and maximum population level data. In view of the work of Dorn and Rahn (1939), this range should be accepted with some qualification. These authors found that the optimum temperature for maximum growth rate, maximum population, greatest rate of fermentation and highest amount of acid production for *S. lactis* was not the same for all functions. The activity of cultures grown at the various temperatures was not examined in the present study, but the temperature range of 28° to

30° C was consistent with previously recommended temperatures for activity determinations (Pont and Holloway, 1968). Yield of cells obtained was the primary criterion for selection of this temperature range for subsequent experiments. The same temperature optimum used for growth of S. lactis was used for growth of S. cremoris and S. diacetilactis. The physiological similarity of these bacteria made it unlikely that widely varying optimum temperatures for maximum cell yield would exist between species. Subsequent experimental data did not weaken this assumption.

Reports in the literature on the optimum pH for growth of streptococci are contradictory, and, incomplete. In view of recent work on production of concentrates, however, it was felt that a pH of 6.3 was within the optimum range for maximum cell production (Pont and Holloway, 1968; Stadhouders, et al., 1969). As in the case of temperature, the pH is believed to directly affect the activity of the enzymes of the cell. Also, as in the case of temperature, the optimum pH for one metabolic activity may not be the same for another. The work of Gilliland, et al. (1970) provides an illustration. They found that L. citrovorum cell concentrates produced large quantities of diacetyl if they had been obtained from cultures grown at pH 5.5, although higher cell yields were obtained if the cultures were grown at pH 6.5.

The limiting effect of pH on the growth of S. lactis is

illustrated by the increased cell yields obtained when the pH was neutralized with sodium, potassium or calcium hydroxide (Table 3). The differences in the maximum population observed among the three neutralizers may reflect the differences in toxicity of the three lactates or different effects of the cations supplied. The toxicity of sodium lactate was demonstrable (Table 11) in confirmation of the work of Pont and Holloway (1968). The higher population levels attained with calcium hydroxide as compared with sodium hydroxide also confirmed the data of Stadhouders, et al. (1969) that the hydroxide of calcium is less toxic to S. lactis than the hydroxide of sodium.

Neutralization with ammonium carbonate or ammonium hydroxide resulted in higher cell populations than with either calcium or sodium hydroxide neutralizers. Peebles, et al. (1969) reported similar results in a paper comparing sodium hydroxide and ammonium hydroxide neutralization of S. cremoris cultures. Snell (1952) stated that ammonia can serve as a source of nitrogen for the synthesis of nonessential amino acids and other nitrogenous compounds that are essential components of the cell but are not supplied preformed in the medium. It has also been found by Zarlengo and Abrams (1963) that ammonia penetrates cells of S. faecalis more easily than K^+ or Na^+ and is effective in restoring glycolytic activity of cells. They hypothesized that free NH_3 rapidly equilibrated across the cell membrane by simple diffusion and once inside the cell neutralized some

of the intracellular acid, thus raising the pH within the cell. If the same mechanisms proposed by Snell (1952) and Zarbengo and Abrams (1963) are involved it would account for the results observed for NH_4OH neutralized cultures and possibly ammonium carbonate neutralized cultures. Stadhouders, et al. (1969) stated that ammonium and sodium lactate are each more toxic than calcium lactate, thus less toxicity of ammonium lactate does not account for the increased cell yield. The decreased generation time in NH_4OH neutralized cultures lends support to the possibility that ammonium is utilized as a source of nitrogen.

The same mechanisms used to explain an increased population level when NH_4OH was used as neutralizer may also explain the effect of ammonium carbonate. However, an additional factor must be considered: the effect of carbon dioxide on growth. When sodium or ammonium carbonate are used as neutralizers, the carbonate ion picks up a hydrogen ion yielding water and carbon dioxide. Whitehead, et al. (1958) showed that S. lactis and S. cremoris require carbon dioxide in the medium although there was a variation in the level required among different strains. A comparison between maximum population levels of S. lactis cells neutralized with calcium hydroxide, and grown in air and in CO_2 (Table 6) demonstrated the effect of carbon dioxide on growth. This supported the previous findings of Whitehead, et al. (1958) and Reiter and Oram (1961) that carbon

dioxide is a growth requirement of S. lactis, and it helps to explain the observed increase in cell yield when the carbonates were used as neutralizers.

There is no evidence that S. diacetilactis has an exogenous requirement for CO_2 and since it produces CO_2, not only from citrate but from glucose (or lactose), its demand may be satisfied in this manner. If an exogenous requirement exists, this might account for the lower population maximum obtained by S. diacetilactis even when the milk was supplmented with yeast extract (Table 8).

The increased maximum population achieved when nitrogen gas was added continuously or at the initial stage of growth is difficult to explain. As pointed out above, there is no evidence that the streptococci utilized nitrogen gas. Some of the increase could be an artifact caused by the breakup of cell chains as a result of the increased agitation. This seems unlikely because of the similar maximum population levels achieved by both cultures to which nitrogen gas was added. Peroxides which would be formed under aerobic conditions but not when nitrogen was added might account for the observed differences in maximum population levels. Peroxides have been found to be produced by lactic streptococci in the early stages of growth resulting in extended lag times (Gilliland and Speck, 1969). This may account for the equal effect of adding nitrogen for only a short time at the beginning of growth.

There are three possible causes for the observed limitation in growth. These are: (1) decrease in space below minimum requirements; (2) lack of essential nutrients; or (3) accumulation of growth inhibitors.

Limitations of space would be difficult to ascertain but it would seem unlikely that it is worthy of serious consideration in the present study. Agar growth yields more dense populations than that which would occur in broth. Pont and Holloway (1968) have calculated that at a population level of 5×10^{10} cells/ml, each cell would occupy an average of 17.5 cubic microns. This is from 35 to 70 times its own volume assuming a cell size of 0.5 to 1.0 microns in diameter. At any one loci in an interval of time, space limitations might become a factor. This would be especially true as the viscosity of the fluid increased due to growth. While this might become a problem in a static culture, it would probably be minimal in agitated cultures. The absence of any significant effect in the maximum population when agitation was increased (Table 4) is support for assuming that space limitations were not a primary factor in growth limitation.

The fastidious nature of the lactic streptococci suggests a second possibility as an explanation for limitations to growth. Milk supplied the required vitamins and nitrogenous substances necessary for growth, but the essential nature of these substances gives rise to a potential growth limiting condition. There are two sets of data

that argue against the nutrient condition of the culture as a primary factor in limiting growth. When the NFDM concentration was increased from 11% to 15% there was no increase in the maximum population of the culture. These data are recorded in Table 10. If nutrient limitation were causing a cessation of growth, cultures grown in higher concentrations of NFDM would be expected to reach higher cell populations. The level of nutrients is not a neutral entity, however, as evidenced by the low maximum population attained in cultures grown in 5% NFDM. At this low concentration, the nutrient concentration is unquestionably a primary limiting factor but at higher concentrations of NFDM other causal explanations are necessary.

When nitrogen gas was added to cultures continuously or as an initial flush, the maximum cell level was increased approximately ten-fold (Table 5). Since the lactic streptococci have no way of fixing atmospheric nitrogen, it would appear that an increase in the maximum population was achieved independent of metabolism of added nitrogen. The possible explanation for this increase was given above.

The bulk of the data supplied by this research favors the third possibility of growth limitation; the accumulation of growth inhibitors. The influence of pH on growth is illustrated by the increased cell populations attained when the culture pH was maintained at the optimum (Table 3). The magnitude of the differences in maximum

populations between non-controlled and controlled growth cultures is evidence that increased acidity of the culture is a primary growth limiting factor. This phenomona is presumably a result of the lowered enzyme activity at an acid pH as described by Harvey (1965).

The elimination of one cause of growth limitation seemingly produced another. The data given in Table 11 shows that a build-up of sodium lactate during maintenance of the pH can affect the growth adversely. These findings substantiate the findings of Pont and Holloway (1968) that sodium lactate affects growth. These workers showed that a higher level of sodium lactate was required for complete cessation of growth than was found in this study. The explanation for this is unclear. Species differences should not be that substantial.

In addition to sodium lactate, inhibition of growth by other compounds may be involved. Gilliland and Speck (1968) reported on a compound isolated from spent broth of a culture of lactic streptococci which inhibited growth of the organism. This inhibitory compound was identified as D-leucine. Other instances of D-amino acids causing growth inhibition have been reported for the lactobacilli (Terri, 1954).

Peroxides formed by the lactic streptococci have been shown to be inhibitory, but these are formed in the early stages of growth (Gilliland and Speck, 1969). They decrease rapidly to nontoxic

levels as acid production increases, and, thus, may not be a significant factor, except only during growth inhibition.

The nutrient limitations of NFDM for growth of S. diacetilactis are obvious from the data in Tables 7 and 8. The nature of this deficiency is not known, but it can be eliminated by the addition of yeast extract. Addition of greater than 0.5% yeast extract results in a higher maximum population but the yield of cells per gram percent of yeast extract drops. This reflects the effect of additional growth limiting factors which become prominent at the higher population levels.

Nonfat dry milk competes favorably with other media used in the production of starter concentrates as was illustrated in Table 9. Growth of S. lactis in the whey medium of Pont and Holloway (1968) resulted in slightly higher maximum population level of S. lactis but concentrates harvested from milk were substantially more active. The high generation time and low maximum population level of S. lactis in the T_1P medium of Stadhouders, et al. (1969) does not agree with the results they have reported. The reason for this discrepancy is unclear. Differences in the species, in growth techniques and in source of ingredients may partially account for this aberration.

From a commercial view point, the use of NFDM may or may not compete favorably, cost wise, with the media used in this study or other media presently in use by suppliers of starters. This

would depend on the feasibility of using inexpensive substitutes for tryptone and yeast extract and the bulk rate cost of the various ingredients. In any evaluation cell yield per unit of medium should not be the only consideration. The ease at which cells can be harvested and the activity of the concentrate that is produced are also major considerations. Considerable difficulty was encountered in harvesting cells and resuspending the sediment from the whey medium. The use of ammonium hydroxide as a neutralizer resulted in a high cell yield and a low generation time but it was more difficult to handle, is more expensive than sodium carbonate, and according to Peebles, et al. (1969), cells prepared from cultures neutralized with ammonium hydroxide are less active than those neutralized with NaOH.

Harvesting and Preparation of Concentrate

The use of milk as a medium for production of starter culture concentrates has not been given serious consideration in the past because of cost and the difficulty in removing the bacterial cells from the medium. Previous attempts to sediment the cells by centrifugation resulted in precipitation of the milk proteins as well. In this study, the bacteria were efficiently removed from the milk by a procedure recently developed by Stadhouders, et al. (1969). A possible alternative would be to digest milk proteins with an

appropriate proteolytic enzyme before growing and harvesting cells.

The milk proteins are present in the milk in a colloidal dispersion. Casein, the major milk protein, exists as complex particles or micelles containing calcium, inorganic phosphate, magnesium and citrate (Jenness and Patton, 1959). This calcium caseinate-phosphate particle, which varies from 30 to 300 mμ in diameter, precipitates from milk when the pH is adjusted to the isolectric point of the casein, 4.6. Phosphate and citrates form undissociated complexes with calcium and magnesium which results in dispersion of the particles to smaller sizes. Sodium and potassium have an identical effect. The Stadhouders procedure utilizes these properties to bring about solubilization of the calcium caseinate-phosphate particles. The pH of the culture is adjusted to the point of minimum insolubility of the caseinate particle and sodium citrate added to aid in further dispersal of the particle.

The Sorvall centrifuge used in this work was convenient and adequate for the small volumes involved (1000-1200 ml). The efficiency of cell recovery using this apparatus was in agreement with the results reported by Stadhouders, et al. (1969). The Sharples centrifuge proved adequate for handling the large volumes (7 to 10 L) that could not be handled conveniently by the Sorvall. However, the problems involved with removal of the cell paste, the limitation on the volume of fluid that can be efficiently centrifuged and the

difficulties in adjusting and maintaining the optimum flow rate for cell removal are serious arguments against use of this apparatus. The drying of cells in the bowl permits convenient removal of the cells but in the absence of a suspending matrix the cells form insoluble aggregates.

For large scale mass production of cell concentrates, both the Sorvall and Sharples would be inadequate, though industrial models of the latter are available. The bactofuge described by Simonart (1959) would be ideal in industry application since the efficiency is only slightly less than the Sorvall and the volume capacity is considerably greater (3000-6000 L/hr).

The lactic streptococci require an organic source of nitrogen for growth which can be attributed to a requirement for certain amino acids. When these organisms are propagated in milk, the milk proteins provide the required amino acids (Cowman, et al., 1970). S. lactis has been shown to contain a membrane and an intracellular proteinase which can function to provide the cell with amino acids by breakdown of milk proteins (Cowman and Speck, 1967). When grown in milk, S. lactis would be expected to have an active proteinase system, but when grown in T_1P medium or whey supplemented with yeast extract and tryptone, there would be a reduced need for a proteinase system because required free amino acids would be supplied in the tryptone and yeast extract. This difference in

proteinase levels may account for the observed differences in activity of the concentrates given in Table 13. Agitation may be another factor to account for the differences in activity of concentrates prepared from controlled and non-controlled milk cultures. In this regard, Pont and Holloway (1968) observed a decrease in acid production when S. lactis cultures were stirred as compared to unstirred cultures. Gilliland and Speck (1969) made similar observations in lactic cultures to which air was added. Both groups attributed the reduced acid to production of peroxides which were formed under aerobic conditions by the lactic streptococci.

The production of concentrates on a commercial scale in NFDM under non-controlled growth conditions would not be economically feasible because of the low cell yield per gram of medium used. The high cell yields of lactic streptococci obtained under controlled growth in NFDM make this a more practical means for production of starter concentrates, especially if more active cells with short lag periods in milk are consistently produced. The T_1P and whey media appear less practical because of the lower activity of the resulting cell concentrates and the greater cost of the media constituents.

Lyophilization

Five stages in the lyophilization process have been listed as potential points where cell injury or loss in viability can occur.

During the preparation, freezing, drying and rehydration stages, without storage, there was a 14% decrease in activity when S. lactis C2F cells were suspended in 5% glutamate. This reduction amounted to 31% of the activity when suspended in NFDM; there was no significant reduction when the cells were lyophilized in trehalose. The absence of any significant loss in activity or cell count during the harvesting procedures rules out the preparation stage as a problem step. Failure to detect a decrease in viable cells during the freezing stage does not exclude this as a potential problem step, because cell activity and lag due to injury must still be considered. This injury which could have taken the form of loss or impairment of the proteinase system, would not have been detected by colony counts since the cells were plated on a rich medium (lactic agar). Lactic agar supplies the cell with the required amino acids thus eliminating the need for an active proteinase system. Westhoff, et al. (1970) found that S. lactis and S. cremoris showed a reduced activity in milk when stored at low temperatures, but no reduction in cell numbers. This loss in activity was attributed to injury of the proteinase system when it was found that proteinase enzyme activity was also diminished during low temperature storage.

A study of losses in activity occurring during the rehydration step was not conducted, thus it is difficult to separate losses occurring during rehydration from losses occurring during drying. It was

assumed that rehydration losses were insignificant because there was no loss in dried cultures stored in vacuo. If a loss in activity was occurring because of rehydration, this loss should have appeared despite the conditions of storage.

Loss in viability during the drying step has been attributed to two causes. One is the denaturation of proteins by carbonyl groups which react with amino groups of the cell protein (Scott, 1958). The second is loss in bound water which results in essentially the same effect. Sucrose has been cited as an effective suspending substance for maintaining viability of lyophilized bacteria (Meryman, 1966). The hydrophilic (water-loving) characteristic of sucrose has been hypothesized to prevent loss of bound water, thus preventing denaturation of cellular enzymes. Trehalose closely resembles sucrose in structure, hydrophilic nature and absence of reducing ability and these facts may help explain the protective effect of this compound. The presence of reducing sugars have been implicated in decreased viability of dried preparations and may explain the differences observed between NFDM and trehalose. The results obtained with glutamate are consistent with those obtained by Cho and Obayashi (1956) and Lion and Bergmann (1961). The work of Morichi, et al. (1963), indicating that glutamate reacts with the carbonyl groups of the cell, may explain the protective effect of this compound. While milk has often been used in the past as a suspension fluid, it is

obviously inferior to glutamate and trehalose. Suspension in trehalose results in greater lyophilization stability than glutamate, but glutamate is the more acceptable compound for two reasons. First, subsequent storage stability of concentrates in trehalose was slightly less than that of concentrates in glutamate. Second, the cost of trehalose makes its use on a large scale prohibitive.

There are several explanations for the protective effect of glutamate at the neutral pH. The additional stress of the lower pH on the cell would prevent recovery of injured cells. This stress may cause a reduced efficiency of repair of key enzymes. The pH may also effect binding of glutamate to carbonyl groups resulting in less protective effect by the glutamate molecules.

Rogers (1914) was the first to observe the marked increase in stability of lyophilized cultures when stored in vacuo; this was confirmed in the present study (Table 18). The same phenomenon has since been reported by Naylor and Smith (1946), Czulak and Hammond (1953) and Maister, et al. (1958). The nature of the suspending fluid is apparently of little significance when cultures are stored in vacuo as shown in Table 19. When cultures are stored in air, however, the suspending fluid is an important factor in culture stability as evidenced by the protective effect of glutamate, trehalose and, especially glutamate plus KI. The cause for the deleterious effect of air, or oxygen, is not clear. Webb (1969) has suggested that

oxygen inactivates a membrane-bound repair system necessary to repair injury occurring during lyophilization. Lion, et al. (1961) and Dimmick and Heckly (1961) have shown a correlation between free radical formation in lyophilized bacteria, measured by electron paramagnetic resonance, and viability.

If oxygen has a deleterious effect on storage stability, then the antioxidants, ascorbic acid and butylated hydroxyanisole, would be expected to exert a protective effect on dried cells during storage. This was not the case and may suggest that the reaction between oxygen and dried bacteria is not preventable by action of the antioxidants. Potassium iodide is an effective additive, however, in support of, the findings of Lion and Bergmann (1961).

The effect of moisture on storage survival of dried bacteria must also be considered. Concentrates not stored under vacuum contained an average 6.9% residual moisture. This is higher than the 1% value recommended by Meryman (1966) for optimum stability. Concentrates stored under vacuum would have lower residual moisture since they were not exposed to the atmosphere. Whether the lower residual moisture or the lack of oxygen is the more significant, cannot be deducted from the data available. Scott (1958) has hypothesized that removal of the most firmly held water molecules may result in loss of stability of the cellular constituents, especially in the presence of air. During lyophilization, drying could occur beyond

the acceptance level so that more firmly held water molecules would be removed.

The greater susceptability of S. cremoris to lyophilization and storage as compared with that of the S. lactis and S. diacetilactis strains used in this study is difficult to explain. Attempts to explain the phenomenon in terms of species dissimilarities would be hazardous without consideration of additional strains of each organism. However, Gibson, et al. (1965) have shown in studies of frozen storage of the lactic streptococci that considerable variation among strains of the same species may occur. Attempts to understand differences in susceptibility among strains, species or genera of bacteria are further complicated by an inadequate understanding of the mechanisms involved in viability loss during lyophilization and storage in the dried state. Differences in the susceptibility of the cell wall to stress or of key enzymes to denaturation may account for the differences observed among different organisms, but this is only speculative at the present time.

The differences in susceptibility among strains and species becomes of great importance from a practical standpoint. Mixed starters containing strains or species with different susceptibilities to lyophilization and storage would not retain the original ratios during these processes. This alteration in the ratio of one strain to another could result in undesirable effects in the final product such

as poor flavor. Thus, in addition to the physiological considerations in the selection of starter cultures, the susceptibility to lyophilization and storage stress may well be an additional criterion demanding more attention.

To place the technique of lyophilization of cell concentrates in the perspective of the commercial operation, it is necessary to return to some figures given in the introduction. It was stated that a cheesemaker using the standard one percent inoculum for a vat containing 10,000 lbs of milk would require 100 lbs of starter culture. Starter cultures prepared in the dairy plant by conventional methods contain approximately 2×10^9 cells/ml. The approximate total cell population of the 100 lb inoculum would be 2×10^9 cells times the number of mls in 100 lbs (44,000 mls), or an approximate total cell population of 8.8×10^{13} cells. During this study, units of lyophilized cell concentrates were prepared with an average of 0.506 gms and containing an average total cell population of 1.0×10^{11}. Dividing the total cell population required to inoculate 10,000 lbs of milk (8.8×10^{13}) by the total population of a unit of lyophilized concentrate (1.0×10^{11}) gives 880 units of lyophilized concentrate required. This number of units weighs a total of 445 gms or, approximately, 1 lb of lyophilized concentrate; a considerable reduction from the 100 lbs of starter culture required by conventional procedures.

Lyophilization is not an inexpensive process and preparation of

concentrates under vacuum conditions on a large scale could involve complications not considered in this study. The minimum weight and volume and the storage stability, however, are arguments in favor of lyophilizated concentrates for use as starters and would seem worthy of consideration by the dairy industry.

BIBLIOGRAPHY

Anderson, A. W. and P. R. Elliker. 1953a. The nutritional requirements of lactic streptococci isolated from starter cultures. I. Growth in a synthetic medium. Journal of Dairy Science 36:161-167.

Anderson, A. W. and P. R. Elliker. 1953b. The nutritional requirements of lactic streptococci isolated from starter cultures. II. A stimulatory factor required for rapid growth of some strains in reconstituted nonfat milk solids. Journal of Dairy Science 36:608-613.

Bergere, J. L. and J. Hermier. 1968. Mass production of lactic streptococci. II. Growth of Streptococcus lactis in media at constant pH. Le Lait 48:13-30.

Bergmann, Donald E., Frank E. Halleck, B. J. Byron, J. Mechales and Robert I. Tenney. 1957. Characteristics of a factor protecting the viability of lyophilized Brucella abortus cells. Journal of Bacteriology 74:101-105.

Cho, Chujo and Yoji Obayaski. 1956. Effect of adjuvant on preservability of dried BCG vaccine at 37 C. Bulletin of the World Health Organization 14:657-669.

Collins, E. B., F. E. Nelson and C. E. Parmelee. 1950. Acetate and oleate requirements of the lactic group of streptococci. Journal of Bacteriology 59:69-74.

Cowman, R. A., and M. L. Speck. 1967. Proteinase enzyme system of lactic streptococci. I. Isolation and partial characterization. Applied Microbiology 15:851-856.

Cowman, R. A., D. C. Westhoff, H. E. Swaisgood and M. L. Speck. 1970. Proteinase system of lactic streptococci. IV. Relationship between proteinase activity and growth at 32 C. Journal of Dairy Science 53:126-131.

Czulak, J. and L. A. Hammond. 1953. Freeze dried starter cultures. Australian Journal of Dairy Technology 8:89-91.

Davis, J. G. and C. C. Thiel. 1939. The effect of pH on growth and gas production by streptococci and lactobacilli. Journal of Dairy Research 10:455-463.

DeMan, J. C. and Th. E. Galesloot. 1962. The effect of the addition of manganese to milk upon the growth of starter bacteria. Netherlands Milk and Dairy Journal 16:1-23.

Dimmich, R. L. and R. J. Heckly. 1961. Free radical formation during storage of freeze-dried Serratia marcescens. Nature 192:776-777.

Dorn, F. Leslie and Otto Rahn. 1939. Definition versus measurement of optimal temperature. Archiv fur Mikrobiologie 10:6-12.

Elser, W. J., R. A. Thomas and G. I. Steffen. 1935. The desiccation of sera and other biological products (including microorganisms) in the frozen state with preservation of the original qualities of products so treated. Journal of immunology 28:433-473.

Fisher, P. J. 1950. A note on the immediate death rate. Journal of General Microbiology 4:455.

Fry, R. M., M. R. C. S. and R. N. Greaves. 1951. The survival of bacteria during and after drying. Journal of Hygiene 49:220-246.

Fry, R. M. 1954. The preservation of bacteria. In Biological Applications of Freezing and Drying. R. J. C. Harris (ed). Academic Press, Inc., New York pp. 215-252.

Fryer, T. F., M. Elisabeth Sharpe and B. Reiter. 1970. Utilization of milk citrate by lactic acid bacteria and "blowing" of film-wrapped cheese. Journal of Dairy Research 37:17-28.

Garvie, E. I. and L. A. Mabbitt. 1956. Acid production in milk by starter cultures--The effect of peptones and other stimulatory substances. Journal of Dairy Research 23:305-314.

Gibson, C. A., G. B. Landerkin and Pamela M. Morse. 1965. Survival of strains of lactic streptococci during frozen storage. Journal of Dairy Research 32:151-156.

Gilliland, S. E. and M. L. Speck. 1968. D-leucine as an auto-inhibitor of lactic streptococci. Journal of Dairy Science 51:1573-1578.

Gilliland, S. E. and M. L. Speck. 1969. Biological response of lactic streptococci and lactobacilli to catalase. Applied Microbiology 17:797-800.

Gilliland, S. E., E. D. Anna and M. L. Speck. 1970. Concentrated cultures of Leuconostoc citrovorum. Applied Microbiology 19:890-893.

Gregory, M. E. 1962. Reviews of the progress of dairy science. Section D. Nutritive value of milk and milk products. Part 2. Water-soluble vitamins in milk and milk products. Journal of Dairy Research 29:220-232.

Hammer, B. W. 1911. A note on the vacuum desiccation of bacteria. Journal of Medical Research 24:527.

Harvey, R. J. 1965. Damage to Streptococcus lactis resulting from growth of low pH. Journal of Bacteriology 90:1330-1336.

Heckly, R. J. 1961. Preservation of bacteria by lyophilization. Advances in Applied Microbiology 3:1-76.

Heckly, Robert J., R. L. Dimmick and J. J. Windle. 1963. Free radical formation and survival of lyophilized microorganisms. Journal of Bacteriology 85:961-966.

Heller, George. 1941. A quantitative study of environmental factors involved in survival and eath of bacteria in the desiccated state. Journal of Bacteriology 41:109-126.

Hogg, D. McG. and P. R. Jago. 1970. The influence of aerobic conditions on some aspects of the growth and metabolism of group N streptococci. The Australian Journal of Dairy Technology 25:17-18.

Ingraham, John L. 1962. Temperature relationship. In the Bacteia eds. I. C. Gunsalus and R. Y. Stanier. Academic Press. New York. 4:265-296.

Jenness, R. and S. Patton. 1959. Principles of Dairy Chemistry. John Wiley and Sons, Inc., New York, 446 pp.

Kihara, Hayato and E. E. Snell. 1960. Peptides and bacterial growth. VIII. The nature of strepogenin. Journal of Biochemistry 235:1409-1414.

King, N. 1948. Modification of the Voges-Proskauer test for rapid colorimitric determination of acetylmethylcarbinol plus diacetyl in the butter cultures. Dairy Industries 13:860-861.

Kizer, D. E., L. Hankin, M. L. Speck and L. W. Aurand. 1955. Stimulation of lactic acid bacteria by substances concomitent to contain proteolytic enzymes. Journal of Dairy Science 38:303-309.

Koburger, J. A., M. L. Speck and L. W. Aurand. 1963. Identification of growth stimulants for Streptococcus lactis. Journal of Bacteriology 85:1051-1055.

Lamprech, R. and E. M. Foster. 1963. The survival of starter organisms in concentrated suspensions. Journal of Applied Bacteriology 26:359-369.

Leach, R. H. and W. J. Scott. 1959. The influence of rehydration on the viability of dried microorganisms. Journal of General Microbiology 21:295-307.

Linklater, P. M. and C. J. Griffin. 1971. The design and operation of a continuous milk fermentation system. Aust. J. Dairy Tech. 26:22-26.

Lion, M. B. and E. D. Bergmann. 1961. Substances which protect lyophilized Escherichia coli against the lethal effect of oxygen. Journal of General Microbiology 25:291-296.

Lion, M. B., J. S. Kirby-Smith and M. L. Randolph. 1961. Electron spin resonance signals from lyophilized bacterial cells exposed to oxygen. Nature 192:34-36.

Lloyd, G. T. 1971. New developments in starter technology. Dairy Science Abstracts 33:411-416.

Maister, H. G., V. F, Pfeiffer, W. M. Bogart and E. N. Heger. 1958. Survival during storage of Serratia marcescens dried by continuous vacuum sublimation. Applied Microbiology 6:413-419.

Maqur, Peter. 1960. Physical factors implicated in the death of microorganisms at subzero temperatures. Annuals New York Academy of Sciences 85:610-629.

Meryman, Harold, T. 1966. Freeze-Drying. In Cyobiology. Harold T. Meryman ed. Academic Press. New York. pp. 609-663.

Morichi, Toshiki, Ryozaburo Irie, Nobuhiro Yano and H. Kembo. 1963. Protective effect of glutamic acid and related compounds on bacterial cells subjected to freeze-drying. Journal of General and Applied Microbiology 9:149-161.

Naylor, H. B. and P. A. Smith. 1946. Factors affecting the viability of Serratia marcescens during dehydration and storage. Journal of Bacteriology 52:565-573.

Nichols, Agnes A. and Margery Hoyle. 1949. Bacteriophage in typing lactic streptococci. Journal of Dairy Research 16:167-208.

Niven, C. F., Jr,, K. L. Smiley and J. M. Sherman. 1942. The hydrolysis of arginine by streptococci. Journal of Bacteriology 43:651.

Niven, C. F., Jr. 1944. Nutrition of Streptococcus lactis. Journal of Bacteriology 47:343-350.

Obayashi, Yoji and Chujo Cho. 1957. Further studies on the adjuvant for dried BCG vaccine. Bulletin of the World Health Organization 17:255-274.

Peebles, M. M., S. E. Gilliland and M. L. Speck. 1969. Preparation of concentrated lactic streptococci starters. Applied Microbiology 17:805-810.

Pont, E. G. and Gwenda L. Holloway. 1968. A new approach to the production of cheese starter. The Australian Journal of Dairy Technology 23:22-29.

Postgate, J. R. and J. R. Hunter. 1963. Metabolic injury in frozen bacteria. Journal of Applied Bacteriology 26:405-414.

Pulay, G., N. Toth and A. R. Bakos. 1959. Die Bedeutung des eisens im staffwichsel einizer in der milchindustrie vorkommenden mikroorganismen. 15th International Dairy Congress. 2:775-781.

Rahn, Otto, C. P. Hegarty and R. E. Geuel, 1938. Factors influencing the rate of fermentation of Streptococcus lactis. Journal of Bacteriology 38:547-558.

Record, B. R. and R. Taylor. 1953. Some factors influencing the survival of Bacterium coli in freeze-drying. Journal of General Microbiology 9:475-484.

Reddy, M. S., E. R. Vedamuthu, G. W. Reinhold. 1970. A differential broth for separating the lactic streptococci. Journal of Milk and Food Technology 34:43-45.

Reiter, B. 1956. Inhibition of lactic streptococcus bacteriophage. Dairy Industries. 21:877-879.

Reiter, B. and J. D. Oran. 1961. A note on the carbon dioxide requirements of Streptococcus lactis ML3. Journal of Dairy Research 28:175-176.

Reiter, B. and J. D. Oran. 1962. Nutritional studies on cheese starters I. Vitamin and amino acid requirements of single strain starters. Journal of Dairy Research 29:63-77.

Reiter, B. and A. Moller-Madson. 1963. Reviews of the progress of dairy science Section B. cheese and butter starters. Journal of Dairy Research 30:419-456.

Reiter, B. and J. D. Oran. 1968. Iron and vanadium requirements of lactic acid streptococci. Journal of Dairy Research 35:67-69.

Rhodes, Mabel. 1950. Viability of dried bacterial cultures. Journal of General Microbiology 4:450-456.

Rogers, L. A. 1914. The preparation of dried cultures. The Journal of Infectious Diseases 14:100-123.

Sandine, W. E., P. R. Elliker, and H. Hays. 1962. Cultural studies on Streptococcus diacetilactis and other members of the lactic streptococcus group. Canadian Journal of Microbiology 8:161-174.

Schultz, Jerome S. and Phillip Gerhardt. 1969. Dialysis culture of microorganisms: Design, theory, results. Bacteriological Reviews 33:1-47.

Scott, W. J. 1958. The effect of residual water on the survival of dried bacteria during storage. Journal of General Microbiology 19:624-633.

Shachell, L. F. 1909. An improved method of disiccation with some supplications to biological problems. American Journal of Physiology 24:325-340.

Simonart, P. 1959. The removal of bacteria from milk by supercentrifugation. The Netherlands Milk and Dairy Journal 13:100-112.

Smith, F. R. 1943. Nutritional studies on Streptococcus lactis I. an unidentified growth factor found in yeast extract. Journal of Bacteriology 46:369-371.

Snell, Esmond E. and Hershell K. Mitchell. 1941. Purine and pyrimidine bases as growth substances for lactic acid bacteria. Proceedings of the National Academy of Sciences 27:1-7.

Snell, E. E. 1952. The nutrition of the lactic acid bacteria. Bacteriological Reviews 16:236-241.

Speck, M. L. and R. P. Myers. 1946. The viability of dried skim milk cultures of Lactobacillus bulgaricus as affected by the temperature of reconstitution. Journal of Bacteriology 52:656-663.

Stadhouders, J., L. A. Jansen and G. Hup. 1969. Preservation of starters and mass production of starter bacteria. Netherlands Milk and Dairy Journal 23:182-199.

Swartz, H. M. 1970. The effect of oxygen on freezing damage. I. effect on survival of Eschenchia coli B/r and Eschenchia coli B_{s-1}. Crybiology 6:546-551.

Terri, A. E. 1954. Effect of D-amino acids on growth of lactobacilli. Journal of Bacteriology 67:686-689.

Webb, S. J. 1969. The effects of oxygen on the possible repair of dehydration damage by Escherichia coli. Journal of General Microbiology 58:317-326.

Williamson, W. T. and M. L. Speck. 1962. Proteolysis and curd tension in milk associated with accelerated starter culture growth. Journal of Dairy Science 45:164-169.

Zarlengo, M. and A. Abrams. 1963. Selective penetration of ammonia and alkylamines into Streptococcus faecalis and their effect on glycolysis. Biochemica et Biophysica Acta 71:65-77.