AN ABSTRACT OF THE THESIS OF

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Title: PREPARATION, EVALUATION, AND USE OF LYOPHILIZED, CONCENTRATED DAIRY STARTER CULTURES IN CHEESE AND YOGURT MANUFACTURE

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Cultures of Streptococcus lactis and Streptococcus cremoris were grown in sterile 11% nonfat milk (NFM) at 32 C for 17 hr with the pH maintained at 6.3 with sodium carbonate. The cells were harvested and resuspended in a medium of 5% gelatin, 5% sodium citrate, 2% monosodium glutamate and 10% sucrose (GCGS) to 1/20th the original culture volume. Suspensions were then frozen in a dry-ice-acetone bath and lyophilized to a residual moisture of 2%. The lyophilized culture produced was approximately 1/600th the weight of a conventional culture containing the same amount of cells. Vials containing the dry concentrate were sealed under vacuum and stored at -22° and +22° for periodic examination for cell viability and acid-producing activity. Rehydration was accomplished by the addition of a sterile solution of 20% lactose containing 1% protein hydrolysate

(rehydration medium - RM) which permitted maximum activity of the culture. Culture activity was assayed by simulating Cottage cheese and Cheddar cheese making procedures and measuring the final pH obtained. Appropriate dilutions of the lyophilized concentrate were made so a comparison could be made on a per cell basis with an actively-growing conventional culture. The final pH obtained for S. lactis C10 was 5.3 for both the dry and the conventional culture; this activity resulted in a period of 5 1/4 hr from milk inoculation to milling in Cheddar cheese making. Several cultures maintained activity values similar to the conventional culture for periods of up to 3 months. Cheddar and Cottage cheese were made in 800 lb vats with lyophilized concentrates and conventional cultures with results similar to that projected by the activity tests. This procedure was also applied to cultures commercially available as frozen concentrates with good activity retained in the lyophilized concentrate.

Modifications of the above methods required for the production of lyophilized concentrates of the yogurt starter cultures included supplementation of the growth medium with 0.1% Tween 80, growth at 37 C for 14 hr, and the use of 6% malt extract as a cryoprotective agent. With these modifications and proper culture strain selection, approximately 10% survival was observed with Lactobacillus bulgaricus and 25% for Streptococcus thermophilus. Low (30 C for 16 hr) and high temperature (42 C for 5.5 hr) yogurt manufacturing conditions

were simulated for evaluation of culture activity. The activity test consisted of inoculating 100 ml of steamed (30 min) 13% NFM with equal volumes (0.25 ml of culture dried from 2.0-ml volume and rehydrated in 10.0 ml RM) of L. bulgaricus and S. thermophilus and measuring the final pH attained after 5.5 or 16 hr at the appropriate temperature. Lyophilized concentrates provided pH values almost as low (4.4 to 4.7) as those achieved with conventional yogurt cultures (4.4 to 4.6), and required only an additional 15 to 30 min of manufacturing time.

Lyophilized concentrates of flavor-producing cultures which included S. diacetilactis, Leuconostoc and Propioniibacterium retained a high level of viability (90 to 100%) with GCGS as the suspending medium. The Propioniibacterium could be grown either in milk or non-milk media without reduction of viability while Leuconostoc and S. diacetilactis required growth in milk, which in case of S. diacetilactis had to be supplemented with yeast extract.

All lyophilized cultures had reduced viability when vacuum was lost during storage indicating a deleterious effect of oxygen. Rehydration medium was important in the reduction of osmotically-induced cell injury during rehydration and to provide peptides for required nitrogen metabolism. The cryoprotective requirements were not the same for all species studied and varied within strains.

Preparation, Evaluation, and Use of Lyophilized, Concentrated Dairy Starter Cultures in Cheese and Yogurt Manufacture

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INTRODUCTION

The manufacture of fermented or cultured dairy products is often considered an art rather than a science. This segment of the dairy industry depends completely on bacterial cultures to transform milk to many products such as cheese, sour cream, buttermilk, etc. This transformation is controlled by the dominant bacterial population in the milk. In order to assure proper fermentation, the milk is inoculated with a relatively high population of desired organisms called the lactic acid starter culture. Occasionally the starter will fail and not dominate, perhaps due to excessive contamination of the culture, attack by bacteriophages or inhibition by antibiotics present in the milk. In these cases an undesirable microflora will develop and cause spoilage and potential health problems.

Reasons for starter culture problems become more apparent when one examines the culture handling procedures used in dairy plants. A large cheese plant may make a ton of starter per day. Often facilities and personnel are inadequate to give attention to the details necessary to produce excellent starter on a regular basis. Starter cultures are fastidious in their growth requirements and also are very temperature sensitive. They require daily transfer to

maintain a high level of activity and are, in general, difficult to care for. Early attempts to store cultures involved refrigeration and freezing. This caused extensive cell injury and death which required numerous subcultures to correct upon reactivation. Later liquid nitrogen storage and lyophilization provided advantages, but subculturing was still required.

The ideal solution to the culture dilemma would be the elimination of all subculturing within the manufacturing plant. This would reduce contamination problems and minimize human error. The starter could be mass produced in a specialized plant under carefully controlled conditions regulated by trained personnel. The starter could then be distributed to the manufacturing plants and used for direct inoculation of milk.

Steps have been taken in this direction and at present several companies in the United States produce frozen concentrated lactic streptococcal starter cultures. Generally these concentrates require one or two subcultures prior to use in order to achieve maximum activity. They also deteriorate with temperature fluctuations, so special handling conditions such as dry ice packing or liquid nitrogen storage are required.

The present study was undertaken in an effort to eliminate some of these problems by developing an active lyophilized starter culture concentrate. The desired culture would be light weight, require no

special shipping or storage conditions, and be sufficiently active to eliminate subculturing. The culture should maintain this activity for a period of two to three months. The procedures proposed in this study would enable the production of active lyophilized concentrates of all types of dairy cultures and provide for better control of dairy fermentations.

LITERATURE REVIEW

Scope

A study of lyophilized culture concentrates should involve at least three major concerns: growth, freezing and lyophilization. In the present study, the latter two areas have been emphasized, since previous work by Blaine (Ph. D. thesis, Oregon State University, 1972) led to development of growth conditions which were used in this study. Therefore this literature review will cover mainly the freezing and lyophilization of starter cultures; review and recent articles dealing with other aspects such as growth will be cited, but not discussed in detail.

Types of Dairy Starter Cultures

In this study starter cultures have been divided into three groups: the lactic streptococci, thermophilic or yogurt cultures and the flavor-producing bacteria. Starters are comprised of combinations of cultures within a single group or combinations of organisms from different groups, depending on the desired product.

The lactic streptococci are traditional Cheddar cheese cultures and consist of Streptococcus lactis and S. cremoris. These cultures are important for their acid, flavor and texture-contributing properties. Characteristics of these and related organisms have been

described in a review by Sandine et al. (1962).

The second group is characterized by Lactobacillus bulgaricus and Streptococcus thermophilus. These two cultures are used for yogurt production and many of the Italian cheeses. Unlike the lactic streptococci which can be grown either as single or mixed strains, the yogurt cultures must be grown mixed in order to achieve rapid acid production. In this symbiotic relationship, L. bulgaricus liberates amino acids which stimulate the growth of S. thermophilus (Bautista et al., 1966), while the S. thermophilus produces formaldehyde which is required by L. bulgaricus. These organisms are important not only for their lactic acid production but also for synthesis of flavor and aroma compounds such as acetaldehyde, acetic acid and diacetyl.

The flavor producing bacteria occur in several different genera. The Propionibacterium are required for the characteristic flavor and eye production in Swiss type cheese. Streptococcus diacetilactis produces some acid but is mainly used for diacetyl production in products such as ripened cream butter, sour cream and cottage cheese dressings. Leuconostoc species are generally employed for diacetyl production in cottage cheese dressings, buttermilk and sour cream. They also are used to correct the "green flavor" defect in cultured buttermilk by their ability to convert acetaldehyde to ethanol (Sellers and Babel, 1970).

Culture Growth

In order to produce an active lyophilized starter concentrate, certain growth requirements must be met for each group. For some groups these requirements are well known and high numbers of hardy, active cells can be obtained.

The lactic streptococci have been the most intensely studied group of dairy cultures. Recent review articles by Cogan (1970) and Lloyd (1971) cover these developments. The growth parameters have also been studied and reviewed by Blaine (1972) and Lloyd and Pont (1973). More recently, a review by Gilliland and Speck (1974) covered the growth of lactic starters for the production of frozen concentrates.

With an increasing interest in yogurt in this country during the past few years, the thermophilic group of starters has been receiving more attention. A review by Humphreys (1969) outlined important parameters essential for yogurt culture growth. A report by Lundstedt (1969) has cited some factors which could affect strain balance when L. bulgaricus and S. thermophilus are grown together.

Perhaps the flavor-producing cultures have been the least studied group with reference to culture growth for the production of concentrates. Goel et al. (1969) studied single strain growth of Leuconostoc species in skim milk with an interest in achieving maximum populations and Lamprech and Foster (1963) reporting similar

studies of S. diacetilactis in a broth medium. Studies of broth-grown cultures of Leuconostoc used for frozen concentrates have been reported by Gilliland et al. (1970) and Lacrosse (1972), with the latter report also concerning frozen concentrates of S. diacetilactis. Langsrud and Reinbold (1973) and Hettinga and Reinbold (1972) in recent review articles outlined the growth requirements of propionic acid bacteria.

Frozen and Freeze-dried Culture Concentrates

The production of frozen culture concentrates involves three steps: (1) concentration of the cells to population levels higher than normally achieved, (2) addition of a cryoprotective agent and (3) controlled freezing, freeze-drying and rehydration. These steps vary with different groups of cultures. An apparently satisfactory cryoprotective agent for one organism may be unsatisfactory even for other strains within a species. The cryoprotective agents are generally believed to protect against injury to deoxyribonucleic acid, enzymes and structural components of the cells. Because freeze-drying cannot take place without freezing, these two preservation methods will be considered together.

Concentration

The most commonly reported method of cell concentration is

centrifugation. Cowman and Speck (1963) used this method to concentrate cells from a growth medium composed of casein, yeast extract and lactose. It wasn't until Stadhouders et al. (1969b) reported a method for the clarification of milk with sodium citrate that cells could be easily recovered from milk. This enabled milk-grown cells to be used in concentrates. Bergere (1968) reported that mixed strain starter cultures grown in milk were superior in activity compared to those grown in a tryptone, yeast extract, lactose medium. Other methods of concentrating cells such as dialysis culturing (Schultz and Gerhardt, 1969) and filtration (Albrect et al., 1973) have not been widely accepted.

Freezing and freeze-drying

The early work on culture preservation was covered in a review by Foster (1962) and recent progress has been discussed by Gilliland and Speck (1974). Christensen (1972) has reviewed the progress of starter technology in the commercial culture industry and various aspects of frozen and freeze-dried bacteria have been considered by Mazur (1970).

Maqur (1960) reported that survial of bacteria to freezing is dependent on the rate of cooling, temperature of holding and the rate of thawing. Electron photomicrographs showing structural changes have been used in an attempt to elucidate the mode of freeze injury

(Sherman and King, 1967). Strange and Postgate (1964) showed that molecules as large as ribonuclease were able to penetrate cold-shocked Aerobacter aerogenes cells. This "opening" of the cell did not necessarily result in cell death and could be reversed. Cells growing in a chemostat could repair this damage easier than cells in the exponential growth phase, therefore, the degree of "openness" was considered to be dependent on cell age. Daw et al. (1973) have shown that freezing causes leakage of solutes through the membrane. The injury and death of freeze injured Escherichia coli was accompanied by the loss of 260 and 280 nm-absorbing material. Cells also became sensitive to desoxycholate, indicating injury to the cell envelope (Ray et al., 1972).

Freeze injury is usually reflected by the ability of cells to recover at higher rates on a richer medium (Postgate and Hunter, 1963). This may be due to impaired enzyme systems essential for cell maintenance and growth (Cowman and Speck, 1967a). Sherman (1967) has also postulated and demonstrated that this injury may be latent and not readily apparent.

Cryoprotective agents are usually included in the freezing and freeze-drying menstrum to reduce injury. The most common agents include glutamic acid and related compounds (Morichi et al., 1963), non-ionic detergents (Calcott et al., 1971) and carbohydrates (Redway and Lapage, 1974). Nitrogen or in vacuo storage for dry

cultures is usually least deleterious (Marshall et al., 1973) but with sucrose or glutamic acid present, this protection is not as apparent.

The deleterious effect of oxygen has been shown by electron spin resonance studies to result from free radical formation (Lion et al., 1961b). Also, Israeli and Kohn (1972) postulated that oxygen acts on a site involved with initiation of a new round of DNA replication; treatment of lyophilized E. coli cells with colicin E1 prevented the toxic effect of oxygen.

Lactic Streptococci

Starter cultures of the lactic streptococci are usually composed of a mixture of strains. The balance between these strains can be shifted and this may be caused by many factors which must be considered in concentrated culture production. For example, growth of cultures in a broth medium can result in a different balance between strains than a milk-grown culture (Gilliland, 1971) and in one study, commercial mixed cultures transferred daily in milk favored the predominance of S. cremoris (Holzapfel et al., 1973). Freezing and freeze-drying of mixed cultures also favor the predominance of certain strains (Gibson et al., 1965). Reddy et al. (1974) have shown that a proper balance is best maintained when cultures are frozen and held at -196 C, with lyophilization next, and freezing at -20 C

least desirable. These cultures were stored in their growth medium without the addition of cryoprotective agents. The amount of freeze injured cells is usually highest immediately following freezing and decreases with storage, while the number of dead cells increase with storage (Moss and Speck, 1963). In this way the strain balance may continually shift with storage time.

Liquid nitrogen freezing and storage was used by Peebles et al. (1969) for holding cultures 231 days without loss of activity. Similar results were obtained by Keogh (1970). Stadhouders et al. (1971) reported no advantage of liquid nitrogen storage when compared to cells protected with 7.5% lactose and stored at -37 C in air.

Accolas and Auclair (1967) used glycerol to protect lactic streptococcal concentrates frozen at -30 C and reported no loss of activity or viability. L-malic acid and apple juice were effective cryoprotective agents for S. lactis C2 and S. cremoris R1 but did not protect S. diacetilactis DRC-2. These two agents also stimulated acid production while glycerol and dimethyl sulfoxide retarded acid production. The latter two agents did not provide cryoprotection for the cultures studied (Gibson et al., 1966).

Frozen concentrated cultures have been used for the direct inoculation of milk for the manufacture of soft cheeses (Accolas and Auclair, 1967), Edam (Poznanski, 1971) and Cheddar cheese (Keogh, 1970; Lattey, 1968). Lloyd and Pont (1970) reported slightly

longer manufacturing times for cheese made in this manner with frozen concentrates. In addition, there have been several patents issued for the production of frozen concentrates since the first one was issued to Farr (1968); also, they are presently produced by several companies in the U. S.

Freeze-dried mother cultures have been produced by several companies, but viability is usually low and several subcultures are required to achieve an active bulk culture. Studies of freeze-dried cultures usually show poor survival and activity. Stadhouders et al. (1969a) reported only a 50% survival with 7% lactose or sucrose present S. lactis survival was greater than 80% under vacuum or air storage in the presence of sodium glutamate or 10% nonfat milk (NFM) fortified with 0.5% each of ascorbic acid, thiourea and ammonium chloride (Sinha et al., 1972).

Bannikova and Lagoda have been the most successful in the preparation of lyophilized starter concentrates. They first reported preparation of concentrates in the range of 5×10^{10} to $.3 \times 10^{12}$ cfu/gm by spray drying and freeze drying (Bannikova et al., 1964). Cultures of L. acidophilus, S. lactis, and S. cremoris were grown in 90% whey, 10% hydrolysed milk buffered with either sodium citrate or sodium acetate. Cells were suspended in a mixture of 10% sucrose, 5% gelatin, 2% sodium glutamate and either 5% sodium acetate for the lactobacilli, or 5% sodium citrate for the streptococci. The dry cultures had

residual moistures of 2.2 to 3.6% and survival rates of 82 to 92%. Following 6 months of storage at 4 to 6 C, there was a reduction in viable count of 16 to 18% (Bannikova and Logoda, 1970; Logoda and Bannikova, 1970). Shershneva and Lagoda (1970) also reported that freeze drying S. thermophilus and S. lactis in a growth medium of 16% NFM supplemented with 1% sodium citrate and 2% peptone with evacuated storage at -18 C was superior to storage in air.

Yogurt Cultures

In order to achieve maximum acid production, L. bulgaricus and S. thermophilus should be cultured together in a 1:1 ratio (Lundstedt, 1973). Attempts to make frozen and lyophilized mixed cultures have revealed difficulties in maintaining this balance. Jabarit (1969) attempted to freeze yogurt, but found L. bulgaricus to predominate, making up 60% of the culture. Freeze drying helped to maintain a closer balance, but cell viability was further reduced. Freezing at -35 C reduced the population from 10^{12} to 10^{9}.

In frozen and freeze-dried concentrates, S. thermophilus predominates (Stefanova et al., 1972) and is most stable in storage. Using a protective medium of 10% saccharose and 5 to 10% orange pulp in 14% NFM or 20 to 25% NFM, Jabarit (1971b) found the streptococci to predominate. This protective medium was unsatisfactory in that it imparted a sweet orange flavor to the product. A

supplement of riboflavin, folic acid, pyridoxine and calcium was also protective.

Gavin (1969) reported that peptides formed from the proteolytic activity of L. bulgaricus stimulated S. thermophilus upon rehydration of freeze-dried cultures. Balance could be maintained by excluding these peptides and suspending the cells in fresh medium. A medium consisting of skim milk with 10% sucrose, 1 to 2% sodium glutamate and 1 to 2% culture was recommended for long storage but not for direct inoculation due to a salty flavor contributed by the glutamate salt.

Valles and Mocquot (1968) successfully used starters frozen in glycerol supplemented with skim milk for making Gruyere cheese. Gehringer (1968) used freeze-dried cultures of S. thermophilus and L. helveticus to make Swiss cheese. Optimum activity was achieved by reconstituting the dry culture in skim milk supplemented with 0.5% yeast extract, but the acid production was still slower than a conventional culture. L. helveticus is more resistant to freezing and freeze-drying so a balance with S. thermophilus is easier to maintain (Jabarit, 1970). Malt extract (6%) was used as a cryoprotective agent for L. leichmannii when frozen at -196 C (Johannsen, 1972). These cultures retained 99% of their acid-producing ability after three days of storage.

Certain strains of L. bulgaricus are particularly sensitive to

the effects of freezing even with cryoprotective agents. Morichi et al. (1963) reported that glutamic acid did not provide adequate protection in freeze drying these organisms. Tween 80 has been successfully used as a cryoprotective agent for Aerobacter aerogenes by Calcott and Hunter (1971). Smittle et al. (1972) did not find Tween 80 or several other cryoprotective agents effective, but addition of Tween 80 to the growth medium produced cells more resistant to freezing. This protection is thought to result from monooleate being incorporated into the cell membrane.

Flavor Cultures

There have been few reports concerning concentrated flavor cultures. The industry uses a frozen paste of Propionibacterium for Swiss cheese (Reinbold, G. W., 1972, personal communication to W. E. Sandine) but this does not appear in published reports.

Lacrosse (1972) made frozen concentrates of S. diacetilactis and Leuconostoc cremoris but these were not suitable for direct inoculation. Preparations of active frozen concentrates of L. citrovorum have been reported (Gilliland et al., 1970) and were successfully used for culturing Cottage cheese dressings (Gilliland, 1972). A cryoprotective medium of 10% NFM was used along with liquid nitrogen freezing. The growth medium had to contain added sodium citrate to achieve maximum diacetyl production. Diacetyl production

by frozen concentrates was equal to unfrozen cells grown in the same medium. Frozen concentrates of Leuconostoc are now commercially available.

Frozen and freeze-dried cultures of S. diacetilactis with reduced viability have been reported. Gibson et al. (1966) reported lower viability of frozen S. diacetilactis cultures than S. cremoris or S. lactis. Reddy et al. (1974) reported similar findings in both frozen and lyophilized cultures where survival was less than 50%.

Rehydration

Rehydration of dry cultures must be accomplished under conditions which do not further damage the cell. Osmotic shock during rehydration must be reduced to allow the fragile cell to survive.

Speck and Myers (1946) and Ray et al. (1971a) reported highest cell recovery when rehydration took place at a temperature range of 15 to 25 C. Earlier initiation of cell growth was reported to take place at 35 C. The lower temperature probably reduced the rate of rehydration and extent of osmotic shock, allowing maximum survival.

The use of either 20% sucrose or lactose provided a good rehydration medium for Salmonella anatum, probably due to the osmotic buffer action regulating the rate of rehydration (Ray et al., 1971b). Choate and Alexander (1967) reported using a 24% sucrose solution for rehydration of Spirillum atlanticum. During rehydration

S. anatum is extremely sensitive to EDTA, indicating the site of injury may involve the lipopolysaccharide portion of the cell wall (Ray et al., 1971b).

Other supplements to the rehydration liquid such as yeast extract (Gehriger, 1968) may also be beneficial. These supplements may be required due to impaired enzyme systems which are unable to provide the cell with certain peptides (Cowman and Speck, 1967b).

MATERIALS AND METHODS

Preparation and Maintenance of Stock Cultures

The cultures used in this study are listed in Table 1. Cultures were grown in sterile 11% solids NFM (Galloway West Co., Fond du Lac, Wisconsin). This medium was supplemented with 0.1% glucose and 0.1% yeast extract for growth of the Propionibacterium species.

Cultures of lactic streptococci were incubated at 21 C following a 2% inoculation and transferred daily to fresh medium. Yogurt cultures were incubated at 37 C and transferred twice daily. The flavor-producing bacteria were carried at 30 C and transferred daily to fresh medium.

Following transfer for 5 to 7 days, stock cultures were prepared in screw-capped tubes containing 15 ml of the sterile 11% NFM which was inoculated with 10% active culture. These were then frozen and stored at -22 C. Stock cultures were made in sufficiently large volumes so preparation was required only annually. Activation of stock cultures required thawing at room temperature followed by incubation for 24 hr at the appropriate temperature. Routine transfers for a week or longer were required prior to use in preparing culture concentrates.

Plate Counts

Plate counts were made by serially diluting cultures in sterile 0.1% NFM; lyophilized cultures were suspended in the special rehydration medium (described later) to provide a 1:5 dilution and then further diluted as necessary prior to plating.

The propionic acid bacteria were plated on a medium containing 1% Tryptone, 1% yeast extract, 1% sodium lactate, 0.025% KH_2PO_4 0.0005% $MnSO_4$ and 1.5% agar (Malik et al., 1968). The plate was overlayed with the same agar, incubated anaerobically by flushing an anaerobic incubator (National Appliance Company) three times with nitrogen:carbon dioxide gas (95%:5%) and incubated 48 hr at 32 C.

Lactic agar composed of 2% Tryptone, 0.5% yeast extract, 0.25% gelatin, 0.5% dextrose, 0.5% lactose, 0.5% sucrose, 0.4% sodium chloride, 0.15% sodium acetate, 0.05% ascorbic acid, 0.05% Tween 80 and 1.5% agar (Elliker et al., 1956) was used for other cultures. Incubation was at 32 C for 48 hr except for the yogurt cultures which were held at 37 C. L. bulgaricus cultures also were plated on a modified medium of Rogosa et al. (1951) consisting of 1% Tryptone, 0.5% yeast extract, 0.6% KH_2PO_4, 0.2% ammonium citrate, 0.5% of salt solution (2.8 g $MnSO_4 \cdot 4H_2O$, 11.5 g $MgSO_4 \cdot 7H_2O$ and 0.68 g $FeSO_4 \cdot 7H_2O$ in 100 ml of water), 2% glucose, 0.1% Tween 80, 2.5% sodium acetate and 1.5% agar. The YEPT (Smittle et al., 1972) medium, composed of 0.5% yeast extract, 0.5% phytone, 0.5% trypticase, 0.5% dextrose, 0.4% K_2HPO_4

and 1.5% agar was used for some strains. In some cases, a 72-hr incubation period was superior to 48 hr. LDA agar (Vedamuthu, E. R. 1973 - personal communication to W. E. Sandine) for the differentiation of S. thermophilus and L. bulgaricus also was used. It consisted of 1% Tryptone, 1% yeast extract, 0.5% lactose, 0.5% sucrose, 0.3% $CaCO_3$, 0.05% K_2HPO_4, 0.002% Brom-cresol purple and 1.8% agar with incubation at 37 to 40 C for 40 to 48 hr in a CO_2 atmosphere.

Concentrate Preparation

Cells were grown in a Fermentation Design fermentor using either a one-liter Scientific Products Micro-fermentor or a 14-liter Fermentation Design fermentor. A Fermentation Design pH module with an Ingold pH electrode monitored and maintained the pH by the addition of base. In all studies, the base consisted of a sterile aqueous solution of 25% sodium carbonate; continuous agitation in the growth chamber provided complete mixing of base.

Autoclaved (121 C - 12 min.) 11% NFM provided the basic growth medium for the cultures studied. Tween 80 (0.1%) was added for the growth of yogurt bacteria. Lactobacilli were also grown in MRS broth (Rogosa et al., 1951) - 1% Tryptone, 1% beef extract, 0.5% yeast extract, 2.0% dextrose, 0.1% Tween 80, 0.2% K_2HPO_4, 0.5% sodium acetate· $3H_2O$, 0.2% citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$) for some studies. A broth of 0.5% lactose,

1.0% peptone, and 1.0% Tryptone was also used for propionic acid bacteria; when these organisms were grown in milk, it was supplemented with 0.1% glucose and 0.1% yeast extract. Yeast extract (0.1%) was also added to the milk for the growth of Leuconostoc and S. diacetilactis. Growth media were maintained at pH 6.3 throughout culture growth.

Growth temperatures of 32 C were used for the lactic streptococci and flavor bacteria, and 37 C for the yogurt cultures. Cells withstood freezing best if harvested in the early stationary phase. Using a 1% inoculum, this point was reached in approximately 14 hr for yogurt cultures, 17 hr for lactic streptococci and S. diacetilactis, 20 hr for Leuconostoc and 22 hr for propionic acid bacteria.

Following the specified growth period, the medium was adjusted to pH 6.9 with the sodium carbonate. Broth cultures were then centrifuged 30 min at 7000 RPM (7970 × g) in a Sorvall refrigerated centrifuge (RC-2). The milk was clarified by adding a sterile solution of sodium citrate (44.8 gm in 300 ml water) to the milk giving a final concentration of 4.5%, followed by stirring for 10 min and centrifuging for 60 min under the above conditions.

Sedimented cells were scraped and washed from the sides of the 250-ml centrifuge bottles and placed in a sterile screw-capped tube containing 10 to 20 3-mm glass beads. Suspending medium was added to achieve a final volume of 30 or 60 ml of concentrate from

700 ml of culture. Following vortex mixing, 2.0-ml aliquots were dispensed in rubber-stoppered vials for lyophilization.

Suspending Media

The basic suspending medium used was either GCGS (2% monosodium glutamate, 5% sodium citrate, 5% gelatin, 10% sucrose) for streptococci or GAGS (2% monosodium glutamate, 5% sodium acetate, 5% gelatin, 10% sucrose) for lactobacilli. These were modified slightly by supplementation for certain studies. Malt extract (6%) was used for yogurt cultures where GCGS and GAGS were found unsatisfactory.

Freeze-drying

Vials containing concentrate were suspended in a dry ice-acetone bath (-76 C) for freezing. After 5 to 10 min the vials were wiped dry of acetone, stoppers loosened and placed in the vacuum pan of the lyophilizer. A vacuum of 15 to 25 millitorrs was maintained for 22 to 26 hr with a trap temperature of about -60 C. Vials were sealed under vacuum by automatically closing the rubber stoppers, after which they were sealed by crimping aluminum caps over the stoppers.

The lyophilizer used in this study was an Atmo-Vac model manufactured by Refrigeration for Science, Inc., Island Park, New York. This model utilizes an air-powered piston to force the rubber stoppers

closed, sealing the vials under the same vacuum drawn during lyophilization.

Determination of Residual Moisture

A 4.0-ml sample of concentrate was lyophilized for 4 hr or 24 hr under the above conditions. Net weights before and after lyophilization were recorded. Vials containing the dry concentrate were dried over phosphorus pentoxide in a desiccator for one week at 37 C and the net weight recorded. Weight lost after phosphorus pentoxide drying, divided by the weight of the lyophilized sample, multiplied by 100, was reported as percent residual moisture. Duplicates were run to determine the amount of variation.

Rehydration

Dry starter concentrate was rehydrated with 10.0 ml of sterile rehydration fluid. Rehydration was at room temperature (25 C), aided by a Vortex mixer and considered complete when particles were no longer visible. Rehydration time depended on the suspending medium and was between 5 and 20 min. The basic rehydration medium (RM) was 20% lactose containing 1% Stimilac (Marshall Division of Miles Laboratories, Inc., Madison, Wisconsin). Other rehydration media were also tested and are described in the Results section where appropriate.

Storage

Storage temperatures of -22 C and +22 C were used to simulate dairy manufacturing plant storage conditions. Cultures were maintained under vacuum for most studies except when the effects of oxygen were studied. For these studies the vacuum was broken and vials resealed under an air atmosphere for storage.

Assay of Injury and Survival

Plate counts on the appropriate medium before and after lyophilization indicated survival during freezing and freeze-drying. Viability during storage was reflected by plate counts at monthly intervals. Flavor bacteria survival was evaluated by plate counts alone while lactic streptococci and yogurt cultures were also evaluated by activity tests which simulated product manufacture.

The Cheddar cheese activity test was carried out in dilution bottles containing 100 ml of 11% NFM previously steamed 30 min and chilled in an ice bath. Milk in the bottles was then inoculated with either a 2-ml quantity of a 15-hr conventional NFM culture or 0.2 ml of the rehydrated 2-ml culture. This yielded an approximately equal cell number inoculum for each bottle so comparison of the lyophilized and conventional culture could be accomplished on a per cell basis. Each bottle was then shaken and placed in water bath at

31 C for 20 min at which time 1.0 ml of a 1:50 dilution of rennet was added to each bottle. An additional 50 min of incubation was allowed before the cooking period which consisted of raising the water bath temperature to 39 C slowly over a 30-min period. Following cooking, the temperature was allowed to slowly decrease to 37 C during the next 30 min, and then maintained at 37 C for the next 130 min. Following this period, cold water was added to the water bath to quickly decrease the temperature to 32 C for the final 40 min. At the end of the five-hour test, the curd was macerated by shaking bottles vigorously; they were then placed in an ice bath overnight and the pH measured the following morning.

The Cottage cheese activity test consisted of inoculating 100 ml of steamed milk in the same manner as for the Cheddar cheese activity test and incubating at 23 C for 11 hr. This was followed by maceration of the curd by shaking the bottles which then were placed in an ice bath overnight; the pH was measured the following morning.

Two conditions of yogurt manufacture were simulated in the activity tests. Steamed 13% NFM was prepared as in the other activity test and inoculated with equal volumes of a lactobacilli and streptococci. Inoculation volumes were 0.25 ml of each rehydrated culture or 0.25 ml of each conventional culture; total inoculation was 0.5 ml. The long set incubation was 16 hr at 30 C and the short set was 5.5 hr at 42 C. Measurement of pH was performed as described

for other activity tests.

Cheese Making

Cheese was made in two vats each containing 800 lb of milk. One vat was inoculated with a fresh conventional 15 hr NFM culture while the other vat was inoculated with an equal-cell-number inoculum of lyophilized concentrate rehydrated in RM. Conventional methods of manufacture for both the Cheddar and Cottage cheese lots were used (Van Slyke, 1949).

Table 1. Species and origin of strains used

Culture	Origin[a]
Streptococcus lactis	
C2	C.S.I.R.O.
C10	C.S.I.R.O.
ML8	N.Z.D.R.I.
WM1	N.Z.D.R.I.
AC2	N.C.S.
Streptococcus cremoris	
AC-1	N.C.S.
AC-7	N.C.S.
AC-11	N.C.S.
AM1	N.Z.D.R.I.
AM2	N.Z.D.R.I.
EB1	C.S.I.R.O.
EB2	C.S.I.R.O.
EB4	C.S.I.R.O.
EB7	C.S.I.R.O.
EB9	C.S.I.R.O.
E8	C.S.I.R.O.
P2	N.Z.D.R.I.
HP	C.S.I.R.O.
C1	C.S.I.R.O.
C3	C.S.I.R.O.
C6	N.C.S.
C11	C.S.I.R.O.
C13	C.S.I.R.O.
ML1	C.S.I.R.O.
163	O.S.U.
220	O.S.U.
459	O.S.U.
819	O.S.U.
990	O.S.U.
Lactobacillus bulgaricus	
GA	O.S.U.
NCS-1	N.C.S.
NCS-4	N.C.S.
YB-1	D.R.D.[b]
YB-5	D.R.D.[b]
YB-6	D.R.D.[b]
7993	O.S.U.
12278	O.S.U.

Table 1 (continued)

Culture	Origin[a]
Streptococcus thermophilus	
C3	O.S.U.
MC	O.S.U.
S	O.S.U.
YB23	D.R.D.[b]
YB24	D.R.D.[b]
YB33	D.R.D.[b]
Lactobacillus lactis	Microlife Technics Sarasota, Florida
Streptococcus diacetilactis	
DRC-1	C.S.I.R.O.
DRC-2	C.S.I.R.O.
DRC3	C.S.I.R.O.
3d1	O.S.U.
18-16	O.S.U.
26-2	O.S.U.
Leuconostoc	
Da3	O.S.U.
H56	O.S.U.
L28	O.S.U.
2	O.S.U.
688	O.S.U.
Propioniibacterium	
Sauer	I.S.U.[c]
P. freudenreichii 1	I.S.U.[c]
P. shermanii 59	I.S.U.

[a] C.S.I.R.O., Commonwealth Scientific and Industrial Research Organization, Highett, Victoria, Australia, courtesy of Ms. Barbara Keogh; N.Z.D.R.I., New Zealand Dairy Research Institute, courtesy of Dr. Lindsay Pearce, Palmerston North, New Zealand: O.S.U., Oregon State University, the culture collection maintained in the Department of Microbiology, N.C.S., North Carolina State University, courtesy of Dr. M. L Speck.

[b] Dairy Research Department, Catholic University of Milan, Piacenza, Italy, courtesy of Dr. L. Premi.

[c] Department of Food Technology, Iowa State University, Ames.

RESULTS

Lactic Streptococci

Presently available alternatives to use of fresh actively-growing cultures in manufacture of fermented dairy products are frozen, frozen-concentrated and lyophilized cultures. When cells are frozen or lyophilized in the growth medium, there is considerable cell injury and destruction as illustrated in Table 2. This cell destruction is of such a magnitude that resulting cultures are unsatisfactory for direct inoculation of cheese vat milk. The GCGS suspension medium provided maximum protection for the cells during freezing and lyophilization as shown for the concentrated cultures also listed in Table 2.

Cells harvested from 17-hr NFM cultures grown at 32 C and maintained at pH 6.3 were found to withstand freezing and lyophilization better than cells harvested earlier. Concentrates of cells from cultures grown longer than 17 hr did not show an increase in activity. Therefore, these time, temperature, pH and medium conditions were used for preparing and evaluating S. lactis and S. cremoris culture concentrates.

Attempts to vary the culture medium and methods of harvesting the cells were unsuccessful. A whey based medium was unsatisfactory because centrifugation produced a gel which sedimented with the cells making them impossible to harvest. Reducing the citrate

Table 2. Comparison of the numbers of cells attained by different types of cultures of lactic streptococci

Type of Culture	Growth Results (cfu/ml x 10^9)					
	S. lactis			*S. cremoris*		
	$M1_8$	C2	C-10	HP	C1	E8
1. Conventional culture[a]	2.2	1.8	2.8	1.1	1.6	1.2
2. Frozen culture #1	2.0	1.0	2.6	.79	1.0	.8
3. Lyophilized #1[b]	.25	.17	.20	.28	.19	.4
4. Concentrated culture	180.0	160.0	250.0	90.0	125.0	95.0
5. Frozen #4	170.0	150.0	250.0	90.0	135.0	75.0
6. Lyophilized #4[b]	180.0	160.0	250.0	80.0	125.0	75.0

[a] Grown for 15 hr at 22 C.

[b] cfu/ml of lyophilized culture; 2.0 ml of concentrated or conventional culture were lyophilized and then diluted with 10 ml of RM to provide a 1:5 dilution. Regular dilution procedures subsequently were carried out to provide countable plates.

concentration used for clearing the milk also produced a gel sedimentation upon centrifugation. Enzymatic digestion methods for clarification of the milk gave varied results, and provided no advantages over the citrate method in either time or cost.

Culture populations achieved during cell concentration can be seen from typical data in Table 3. Some loss of cells occurred after citrate addition, but thereafter little loss occurred. The frozen concentrate represented approximately a 100-fold concentration of cells when compared to a conventional 15-hr bulk culture. Furthermore, lyophilization further reduced the weight of a 30-ml concentrate preparation from 32.0 to approximately 5.0 g.

Moisture determination studies of the dry concentrate showed that 4.2% and 2.1% residual moisture remained after 4 and 24 hr of lyophilization respectively. Since the lower moisture level was desirable, 24-hr freeze-drying period was used for all preparations. The moisture determinations were made on 4.0-ml samples. When larger samples were lyophilized, care was taken to insure that the thickness of the frozen material was the same as for smaller samples to insure that the final residual moisture was approximately 2%.

Storage stability of the lyophilized concentrate (Table 4) was provided by maintenance of vacuum since any leak during storage permitted extensive cell death regardless of storage temperature. The temperatures used (22 C and -22C) in the storage tests were

Table 3. Numbers of cells found at different steps in the process of making a starter culture concentrates of S. cremoris C1

Step	Volume (ml)	Counts (cfu x 10^9/ml)	Total Counts (cfu x 10^{11})	Cells Remaining (%)
Milk culture at 17 hr	750	7.0	66	100
After citrate addition	1050	4.3	45	68
Supernatant	1040	0.4	4	6
Suspended cells[a]	30	125	37	56
Frozen concentrate	30	135	40	61
Rehydrated[b]	30	125	37	56

[a]Suspended in GCGS media

[b]Rehydrated in RM

Table 4. Final pH values found when lyophilized lactic streptococcal starter concentrates were tested by the Cheddar cheese activity test

Culture	pH After Cheddar Cheese Activity Test[a]					
	1 Month		2 Months		3 Months	
	-22 C	+ 22 C	-22 C	+22 C	-22 C	+22 C
S. lactis C2	5.6	5.6	5.7	5.7	5.7	5.8
S. lactis ML8	5.5	5.6	5.7	5.7	5.7	5.8
S. lactis C10	5.3	5.3	5.3	5.3	5.4	5.4
S. cremoris EB2	5.6	5.6	5.7	5.7	5.7	5.7
S. cremoris EB9	5.6	5.6	5.7	5.7	5.7	5.7
S. cremoris C1	5.0	5.0	5.0	5.0	5.1	5.1
S. cremoris E8	5.8	5.8	5.8	5.8	5.8	5.8

[a]pH value of 5.4 or lower insured sufficient activity for Cheddar cheese manufacture by direct vat milk inoculation within 5.5 hr; pH values were determined with a Corning Model 12 research pH meter and were reproducible to within 0.1 unit.

chosen because of practicability for industry. Stability of the activity of cells stored at the two different temperatures was approximately the same, provided the organisms were reconstituted with the RM. This was not the case noted in earlier work when rehydration was accomplished by use of sterile 0.1% NFM, because under these conditions there was a decrease of cell activity with age, indicating increasing injury with storage. It also was found that strains sensitive to lyophilization as concentrates (e.g., strain ML1) were not improved in activity by the presence of an equal number of heat killed cells which were highly active as freeze-dried concentrate. Addition of 0.25% Chlorella protein to the milk used in subculturing also did not enhance acid production.

The RM was found to significantly increase culture activity. This rehydration effect became more pronounced with time during culture storage (Table 5)., indicating that a suitable rehydration environment was required to allow the cells to maintain maximum activity. Only one culture of all the strains studied, S. lactis ML8, did not require RM to maintain maximum activity during storage, though the level of activity maintained was not great as compared to S. lactis C10. Rehydration with RM made it possible to maintain lyophilized cultures for a three-month period with a minimum loss of activity.

The length of rehydration time was dependent on the complete

Table 5. Effect of different rehydration media on the activity[a] as measured by pH of lactic streptococci stored one to 4 months at 22 C

Culture	Storage Time (Months)	Final pH H_2O	10% NFM	RM
S. lactis C2 + S. cremoris ML1[b]	4	6.5	6.3	6.0
S. lactis C2 + S. cremoris EB1	3	6.2	6.1	5.9
S. lactis C2 + S. cremoris E8	1	5.95	5.9	5.85
S. lactis C2 + S. cremoris E8	3	6.25	6.0	5.85
S. lactis C2	3	5.70	5.65	5.60
S. lactis C10	1	5.5	5.4	5.3
S. cremoris E8	1	6.0	5.9	5.8
S. cremoris EB2	1	5.7	5.6	5.6
S. lactis ML8	2	5.7	5.7	5.7

[a]See footnote a, Table 4

[b]Strain ML1 alone was unsatisfactory in activity performance even in RM (pH > 6.5) after storage for 1 week at -22 C

dispersion of the dry concentrate. With concentrates prepared in the manner described, rehydration was accomplished within 15 to 25 min. Longer rehydration periods of up to one hr were examined and found not to significantly affect culture activity upon inoculation into milk. Higher concentrations of the cells produced a matrix that was difficult to rehydrate and often wasn't fully dispersed after 45 min of agitation.

The activity test was designed to evaluate the lyophilized culture concentrate in comparison to an actively growing 15-hr culture on the basis of equal cell numbers in the inoculum. In Table 6 the final pH of the activity test of the lyophilized culture concentrate is compared with the pH of the conventional culture. A difference of 0.1 pH unit represented approximately 10 min of cheese making time (Pearce et al., 1969) and a final pH value between 5.6 to 5.8 was considered good.

The long set Cottage cheese activity test (Table 7) also was designed to compare conventional 15-hr cultures with the lyophilized concentrates on an equal cell number basis under simulated plant conditions. The evaluation of the lyophilized cultures was performed under the long set Cottage cheese conditions rather than the short set method because a smaller amount (2%) of inoculum was required. The large amount of bulk culture (5% v/v) required for short set Cottage cheese is enough to lower the pH of the milk 0.5 pH unit

Table 6. Comparison of the activity of conventional and lyophilized, concentrated lactic streptococcal cultures as determined by final pH achieved after the Cheddar cheese activity test

Culture	Final pH	
	Conventional 15-hr Culture	Lyophilized Concentrate
S. lactis C2	5.5	5.6
S. lactis ML8	5.3	5.5
S. lactis C10	5.3	5.3
S. cremoris EB2	5.5	5.6
S. cremoris EB9	5.7	5.6
S. cremoris C1	5.0	5.0
S. cremoris E8	5.7	5.8

Table 7. Comparison of the activity of conventional and lyophilized concentrated lactic streptococcal cultures as determined by the final pH achieved after the Cottage cheese activity test

Culture	Final pH	
	Conventional 15- hr Culture	Lyophilized Concentrate
S. lactis C2	4.7	4.9
S. lactis ML8	4.8	5.1
S. lactis C10	4.7	4.7
S. lactis C2 and S. cremoris E8	5.0	5.1
S. cremoris EB2	4.7	4.7
S. cremoris EB9	4.7	4.8
S. cremoris C1	4.4	4.4
S. cremoris E8	4.7	4.8

upon inoculation. The lyophilized starter concentrate was at a neutral pH and did not give this initial pH drop; otherwise a false indication of an apparent lag in pH would be given, making an accurate comparison difficult. With the possible exception of S. lactis strains C2 and ML8, activities of the two cultures types compared favorably. The cultures reported in the results generally retained the greatest activity as lyophilized concentrates.

Tables 8 and 9 show typical results of cheese making trials designed to compare the lyophilized concentrated culture with a conventional 15-hr bulk type culture. In the case of Cottage cheese (Table 8) it may be seen that the conventional culture and the lyophilized culture had almost the same activity in the vat. For Cheddar cheese (Table 9) it can be seen that both vats had almost identical increases in titratable acidity at each step; almost no difference was noted between the activity of the lyophilized and conventional bulk cultures. Similar additional evaluations of the lyophilized concentrates were made at the University of Minnesota; a slight lag was noted in Cheddar cheese made with the lyophilized concentrate, the quality of the two cheeses being the same.

In order to determine the suitability of this system to commercial cultures, lyophilized concentrates were made of two cultures from each of three companies, and were evaluated (Table 10). Although good viability was retained during lyophilization only a

Table 8. Comparison of titratable acidities found during manufacture of Cottage cheese from milk inoculated with a conventional or lyophilized concentrated culture of S. lactis C10

Step	Time (hr)	Conventional 15-hr Culture		Lyophilized Concentrate	
		°C	Activity[a]	°C	Activity[a]
Addition of 1% starter	0	23	0.18	23	0.18
Ripening	4	26	0.23	23	0.20
Ripening	5	24	0.28	23	0.24
Cutting	13	23	0.58	23	0.55
Cooking	14.5	32	--	32	--
Cooking	15.3	43	--	43	--
Cooking	16	52	--	52	--

[a]Percent lactic acid as determined by titration of 9.0 - ml samples to the phenolphthalein endpoint (pH 8.3) with 0.1 N NaOH.

Table 9. Comparison of titratable acidities during manufacture of Cheddar cheese from milk inoculated with a conventional or lyophilized concentrate culture of S. lactis C10

Step	Conventional Culture		Lyophilized Concentrate	
	Time	Acidity	Time	Acidity
Starter addition	0	0.18	0	0.17
Rennet addition	1:15	0.19	1:15	0.17
Cutting	1:55	0.11	1:55	0.12
Start cooking	2:10	--	2:05	--
End cooking	2:40	--	2:40	--
Start draining	3:30	0.17	3:25	0.17
Milling	6:00	0.45	5:15	0.46
Salting	6:10	--	5:20	--
pH at 60 days	--	5.20	--	5.20

Table 10. Comparison of lyophilized concentrates made from commercial cultures

	cfu x 10^{11}/ml		Final pH of Cheddar Cheese Activity Test
	Fresh Concentrate	Lyophilized Concentrate	
Flavolac BM#1	0.9	0.8	6.00
Flavolac BM#5	2.3	2.1	5.5
Hansen #70	0.8	0.6	6.3
Hansen #253	2.0	0.9	5.6
Vivolac #5	1.8	1.7	5.7
Vivolac #15	2.8	2.8	5.4

few cultures retained high activity. One of these, Vivolac #15 was selected for evaluation in Colby cheese manufacture. The lyophilized concentrate compared very favorably to the same culture prepared in the conventional manner (Table 11).

Yogurt

Knowledge and experience obtained with the lactic streptococci were applied to the yogurt cultures. Single strains were cultured in milk and grown in the fermentor, harvested and suspended in either GAGS for lactobaccilli or GCGS for yogurt streptococci, and lyophilized. Less than 1% of the cells remained viable under these conditions and almost all acid-producing activity was lost.

Of the two L. bulgaricus strains obtained from Dr. M. L. Speck (Table 1) one (NCS-1) was reported to be highly susceptible to freeze injury, and the other (NCS04) less susceptible. Also, it had been shown by Smittle et al. (1972) that addition of Tween 80 to the growth medium enabled NCS-1 to better withstand freezing. These strains were grown in several different media, harvested and suspended at a concentration of 1 to 2×10^{10} cfu/ml in GAGS for lyophilization. Plating media were YEPT for NCS-1, modified Rogosa agar for NCS-4 and lactic agar for both strains for comparison. Table 12 reports the plate counts observed. These results indicated that growth and plating media affected the colony-forming ability of

Table 11. Comparison of titratable acidities during manufacture of Colby cheese from milk inoculated with a conventional or lyophilized concentrate culture of Vivolac #15

Step	Conventional Culture		Lyophilized Concentrate	
	Time	Acidity	Time	Acidity
Starter addition	0	.175	0	.17
Rennet addition	1:00	.185	1:00	.18
Cutting	1:35	.105	1:40	.105
Start draining	2:45	.125	2:55	.125
pH	1 week	5.05	1 week	5.05

Table 12. Comparing growth and plating media for L. bulgaricus

Strain	Medium		cfu/ml From Lyo. Conc.[a] ($\times 10^8$)
	Growth	Plating	
NCS-1	MRS	YEPT	$<10^7$
	YEPT	YEPT	$<10^7$
	NFM	YEPT	7.5
	NFM + T80	Lactic	1.6
NCS-4	MRS	Rogosa	3.0
	NFM	Rogosa	6.0
	NFM + T80	Rogosa	13.0
	NFM + T80	YEPT	$<10^7$
	NFM + T80	Lactic	15.0

[a] 2×10^{10} cfu/ml before freezing

different strains following lyophilization. Plating media as a factor was explored by including two more cultures and growing these and NCS-4 in 11% NFM with 0.1% Tween 80 added. Plate counts on the various media were made before and after lyophilization and are reported in Table 13.

These results show that supplementing milk with 0.1% Tween 80 protected cells during lyophilization. Also, colony-forming ability was dependent on the plating medium due to a variable response by injured cells in different media. In this regard, the lyophilized concentrates were very slow in activity tests, indicating injured but viable cells accounted for a sizeable portion of the population.

Twelve-hour milk cultures were grown at 37 C without pH adjustment and examined for plating efficiency on the different media. This was an attempt to determine if plating efficiency varied with concentrate preparation. Table 14 reports these counts along with colony morphology. It may be seen that there was some variation in plating efficiency even in the 12-hr cultures. Generally lactic agar provided the best over all medium, with Rogosa agar being as good or better for L. bulgaricus. Due to the fact that S. thermophilus would not grow on Rogosa agar and that future work would involve mixed cultures, lactic agar was used in all further studies. Attempts to use yogurt differential agar (E. R. Vedamuthu, 1973, Personal Communications to W. E. Sandine), for mixed culture work were unsuccessful.

Table 13. Plating medium effect on fresh and rehydrated concentrate

Culture[a]	Medium	cfu/ml x 10^9	
		Fresh	Rehydrated
LB 7993	Rogosa	33.0	38.0
	Lactic	47.0	13.0
ST C_3	YEPT	$<10^7$	$<10^7$
	Rogosa	$<10^7$	$<10^7$
	Lactic	5.6	5.0
LB NCS-4	YEPT	$<10^7$	$<10^7$
	Rogosa	38.0	1.3
	Lactic	14.0	1.5

[a]Grown in 11% NFM + 0.1% T80 @ pH 6.3

Table 14. Plating medium effect on yogurt milk cultures

Culture	Medium	Morphology	cfu/ml ($\times 10^8$)
ST C3	Rogosa	--	$< 10^5$
	YEPT	--	$< 10^5$
	Lactic	S	5.0
LB 7993	Rogosa	S	8.7
	YEPT	R	3.3
	Lactic	R&S	6.7
LB NCS-1	Rogosa	S	6.4
	YEPT	R	6.6
	Lactic	R	14.0
LB NCS-4	Rogosa	S	4.0
	YEPT	--	$<10^5$
	Lactic	S&R	3.5

L. bulgaricus cultures appeared as either smooth or rough colonies, especially with lactic agar. This shift in morphology has been shown to occur when available sugar in a medium falls below 0.5% (DeKlerk and Coetzec, 1962). Lactic agar contains 0.5% each of lactose, sucrose and glucose which should allow smooth colony formation if each sugar were utilized. Modified lactic agar containing 1.5% of only one sugar was therefore used to determine plating efficiency and colony morphology of a 12-hr NFM culture as well as a lyophilized concentrate. The lyophilized concentrate was grown in 11% NFM containing 0.1% Tween 80 and suspended in the appropriate GAGS or GCGS medium. Such a study should indicate if a culture like S. thermophilus C3 would become dependent on a single sugar after lyophilization. The other cultures showed no preference (Table 15) in the 12-hr culture but some difference was noted in the lyophilized culture. L. bulgaricus 7993 retained the highest viability but many colonies were rough when grown on all three sugars. NCS-1 and NCS-4 had reduced viability and showed some selection or preference for certain sugars.

Regular RM was used to rehydrate the lyophilized cultures in this study. Although viability appeared good in some instances, the activity was reduced to a level that eliminated their use for direct product inoculation.

Malt extract (6% w/v) had been reported as an effective

Table 15. Sugar effect on cell recovery - cfu x 10^8

Organism	Sugar[a]	Type Culture	
		12 hr NFM	Lyo. Conc.[b]
ST C 3	S	0.11	$< 10^6$
	G	0.7	$< 10^6$
	L	2.1	0.18
LB 7993	S	9.0	180
	G	8.6	140
	L	8.8	170
NCS-1	S	8.9	2.3
	G	8.8	0.65
	L	8.3	1.2
NCS-4	S	not done	15
	G	not done	10
	L	not done	15

[a] Sugars were used at 1.5% level; S = sucrose, G = glucose, L = lactose

Cultures grown in 11% NFM with 0.1% Tween 80, harvested and suspended in either GAGS or GCGS, lyophilized and rehydrated in RM

cryoprotective agent for lactic acid bacteria (Johannsen, 1972b) and was, therefore, examined in this study. L. bulgaricus 7993 and NCS-1 were suspended in either GAGS or malt extract, lyophilized, rehydrated and plated on lactic agar containing a single sugar. Comparison showed (Table 16) a generally higher count with the malt extract-suspended culture and no increased preference for any sugar.

This phase of the research was expanded and other cultures were grown in 11% NFM with 0.1% Tween 80 and suspended in malt extract at different concentrations. Table 17 summarizes the results of plate counts on lactic agar. Generally, cells suspended in malt extract retained a degree of viability for profitable use in further studies on direct inoculation. Because plate counts can disguise injury, a quick single strain activity test was used to screen for extended lag due to injury; the pH was measured after 4 hr at 45 C and results appear in Table 18. According to these data it appears that 2.0 ml of fresh culture could in certain strains be replaced by 0.5 ml of rehydrated culture (2.0 ml of concentrate rehydrated in 10.0 ml of RM).

In order to obtain a more practical view of acid-producing ability, S. thermophilus and L. bulgaricus must be grown together, thus simulating yogurt production. Table 19 shows that a lag in acid production was evident in such mixed cultures.

Lyophilization under the above conditions appears to injure the

Table 16. Effect of suspending medium

Culture[a]	Medium	Sugar	CFU x 10^9
LB 7993	GAGS	L	17
	GAGS	S	18
	GAGS	G	14
	Malt extract	L	22
	Malt extract	S	25
	Malt extract	G	24
NCS-1	GAGS	L	0.12
	GAGS	S	0.23
	GAGS	G	0.07
	Malt extract	L	2.0
	Malt extract	S	3.2
	Malt extract	G	.8

[a]Lyophilized concentrate

Table 17. Malt extract effect - cfu x 10^{10}

Organism	Type Culture	
	Fresh Concentrate	Lyophilized Concentrate
LB 12278	9.7	21.6
	12.0	8.2
LB 7993	8.5	6.2
	4.6	4.1
	3.7	3.2
LB GA	3.1	0.8
LB NCS-1	0.95	0.04
	1.8	0.34
LB NCS-4	2.4	2.2
	1.6	1.1
ST C 3	0.33	0.11
ST MC	2.9	1.2
	2.6	0.85
ST S	1.9	0.6
	0.6	0.3

Table 18. Activity comparisons - 4 hr. pH @ 45 C

Organism	12-hr. NFM	Lyophilized Concentrate @ 1 Month	
		+22 C	-22 C
LB 7993	5.20	5.90	5.40
LB 12278	5.30	5.45	5.35
LB GA	5.40	5.80	5.60
LB NCS-1	5.90	6.50	6.40
LB NCS-4	5.80	6.40	6.10
ST C3	5.50	6.00	6.00
ST MC	5.70	5.90	5.80
ST S	5.30	5.90	5.80

Table 19. Mixture comparisons - 4 hr. pH @ 45 C

Mixture	12-hr NFM	Lyophilized Concentrate
GA and S	4.50	5.25
7993 and S	4.60	5.40
12278 and S	4.55	5.00
GA and MC	4.50	5.00
7993 and MC	4.75	5.55
12278 and MC	4.60	4.90

cells to the extent of reducing acid production but allowing cell survival. An attempt to determine if injury could be reduced or prevented by different rehydration media is shown in Table 20. It may be seen that no rehydration medium was superior for all cultures examined. It would, therefore, appear that under the conditions used for lyophilization that these cultures were not suitable for direct inoculation of vat milk for yogurt production. While the acid-producing ability was influenced by the rehydration medium, this effect was not uniform. The problem of slow acid production and lack of uniform responses by the cultures prompted a search for new cultures.

Three cultures of L. bulgaricus, YB-1, YB-5, YB-6 and three of S. thermophilus, YB-23, YB-24, YB-33 that are commonly used for Italian cheese and yogurt production in Italy were obtained from Dr. L. Premi (Table 1). These were grown in milk containing 0.1% Tween 80, harvested, suspended in 6% malt extract and lyophilized. Rehydration was in RM and with gentle agitation.

Plate counts on lactic agar shown in Table 21 indicate that S. thermophilus cultures retained a high degree of viability during storage. The loss of viability was not as apparent when activity tests were used to compare them with fresh NFM cultures as seen in Table 22. This activity test compares a conventional culture containing an inoculation of about 3×10^8 cfu to the lyophilized concentrate containing about 7×10^8 cfu.

Table 20. RM effect - 4 hr. pH @ 45 C[a]

Culture	Rehydration Medium					
	6% Me	0.5% GEL	10% S + 1% PH	10% L + 1% PH	H_2O	RM
GA	5.05	5.10	5.00			5.35
7993	5.75	5.60	5.30	5.40	5.40	5.40
12278	5.50	5.40	5.40	5.35		5.35
NCS-4	5.90	5.80	5.80	5.90	5.60	6.10

[a]A 4.5 hr. activity test at 45 C using 0.5% inoculum of 2.0 ml of concentrate dried and rehydrated in 10 ml of designated medium

Abbreviations: ME, Malt Extract; GEL, Gelatin; S, Sucrose; PH, Protein Hydrolysate

Table 21. Survival of Italian cultures - cfu/ml x 10^8

Culture	12 hr NFM	Concentrate	Lyophilized Concentrate - Months			
			0	1	2	3
LB 1	6	230	21	8	20	23
LB 5	2	210	27	10	40	40
LB 6	2	110	19	28	75	47
ST 23	15	350	130	130	140	120
ST 24	3	470	40	17	39	45
ST 33	8	930	50	100	57	100

Table 22. Yogurt activity tests

LB + ST Combination	5.5 hr. @ 42 C		16 hr. @ 30 C	
	Normal[a]	Lyo. Conc.[b]	Normal	Lyo. Conc.
1-23	4.9	5.0	4.4	5.1
1-24	4.5	4.7	4.6	4.8
1-33	5.1	4.6	4.4	4.8
5-23	4.5	4.7	4.5	4.7
5-24	4.5	4.6	4.5	4.7
5-33	4.5	4.6	4.4	4.7
6-23	4.9	4.6	4.5	4.8
6-24	4.5	4.7	4.5	4.7
6-33	4.8	4.6	4.4	4.7

[a] 0.25 ml inoculum in 100 ml steamed NFM

[b] 0.25 ml inoculum of lyo. conc. rehydrated to 10.0 ml

Microscopic examination of the milk following activity tests showed optimum activity occurred when the rod to coccus ratio was 1:1. When this balance was not maintained, the rods generally predominated.

With some cultures, the vacuum was broken and the vials resealed and stored at -22 C for one month. In activity tests conducted at 42 C, culture activity was reduced and microscopic examination showed the rods to predominate. This indicated that under the conditions used with these cultures, the lactobaccilli had reduced viability following lyophilization, but were less susceptible to loss of vacuum.

An extension of this study involved a culture which was thought to be Lactobacillus acidophilus (strain Farr) and now classified L. lactis. Because of the importance of this organism in human health (Sandine et al., 1972), a dry concentrate would be beneficial. This culture remained viable during lyophilization in both the 6% malt extract and GAGS media (Table 23) but rehydration was more difficult in the GAGS medium.

Flavor and Aroma Cultures

This group was assayed solely on the basis of plate counts. This criterion was felt sufficient due to the fact that the rate or acid production was not critical both in terms of economics and public health. Uniform cell viability was more important to insure that the

Table 23. Viability and activity of lyophilized concentrates of Lactobacillus lactis grown in MRS

Suspending Medium	cfu/ml x 10^{10}		pH of Activity Test[a]
	Fresh Conc.	Lyophilized Conc.	
GAGS	1.9	1.7	5.6
	2.2	2.1	5.6
Malt extract	2.5	2.0	5.6
	3.6	3.0	5.6

[a] 0.1 ml of concentrate inoculated with 100 ml of steamed 13% NFM and incubated 4 hr. at 45 C

proper rate of inoculation would result in the desired flavor levels.

Propionic Acid Bacteria

The three strains studied were grown in broth because preliminary studies showed that milk grown and broth cultures reached the same populations and maintained viability equally well during lyophilization. An example of this comparison is shown in the top portion of Table 24 with P. freudenreichii 1. Cell harvesting was much easier in the broth culture, therefore broth was used in these studies.

The viability data on Table 24 indicate that GCGS provided protection for all three cultures studied. Cell viability remained fairly constant during the three month study, with P. shermanii 59 the only culture showing a decrease in viability during lyophilization and storage. The use of malt extract as a cryoprotective agent was not encouraging with these organisms, because viability decreased to about 10% after lyophilization.

Leuconostoc

The four strains of Leuconostoc studied were grown in milk without pH maintenance. Viability in the GCGS suspension medium remained at a high level during lyophilization and storage (Table 25). Using different concentrations of cells did not affect the viability, although the higher levels required longer rehydration times. The

Table 24. Viability[a] of propionic acid bacteria during lyophilization and storage - cfu x 10^9

	cfu x 10^9		Storage[b] at -22 C		
	Fresh Concentrate	Lyophilized Concentrate	1 Month	2 Months	3 Months
P. freudenreichii - 1 milk grown	23	24	24	33	20
P. freudenreichii - 1 broth grown	16	13	21	14	16
P. shermanii 59 broth grown	17	5	8	5.5	3
P. shermanii var. Saur broth grown	14	12	12	9.5	14

[a] plate counts on Agar of Malik et al. (1968)

[b] GCGS suspending medium

Table 25. Viability of milk grown Leuconostoc species during lyophilization and storage - cfu x 10^9/ml

Leuconostoc Strains	Fresh Culture Concentrate	Lyophilized Culture	1 Month		2 Months		3 Months	
			+22 C	-22 C	+22 C	-22 C	+22 C	-22 C
2	7.8	6.7	10	9	8.5	11	11	21
2	74	50	--	61	--	50	--	63
Da3	2.8	1.3	2.5	2.0	--	2.0	1.35	2.0
L28	13.3	8.5	16.8	16	13	11	16.3	--
	3.0	3.8	4.9	7.4	5.2	6.2	5.1	5.35
	47	40	--	57	--	40	--	38
LH56	16	18	19	20	27	19	20	17
	17	37	30	19	20	21	19	15

cultures appeared very stable and were not affected by the two different storage temperatures.

Streptococcus diacetilactis

The six strains of S. diacetilactis responded favorably in the GCGS suspending medium. Concentration levels were varied in an effort to determine optimum conditions for viability, but viability remained at a high level for all concentrations studied (Table 26), with the higher levels requiring long rehydration times. The storage temperature was not critical as long as the cultures remained under vacuum.

Table 26. Viability of S. diacetilactis during lyophilization and storage - cfu x 10^9/ml

Strain	Fresh Concentrate	Lyophilized Concentrate	1 Month		2 Months		3 Months	
			+22 C	-22 C	+22 C	-22 C	+22 C	-22 C
18-16	90	72	--	75	--	38	--	58
	7.9	8.7	4.9	8.3	5.9	7.7	3.5	6.4
	1.7	3.7	2.0	2.9	2.9	4.0	1.8	1.1
26-2	62	93	--	56	--	76	--	68
3d	1.8	1.1	1.8	1.7	1.0	1.2	0.2	1.1
DRC-1	14	9	--	12	--	11	--	8
	9.5	8.9	4.1	4	1.2	3.5	4.4	4.0
	11	26	14	12	18	20	6.1	9.2
DRC-2	130	120	--	150	--	135	--	140
	4.0	5.0	2.0	5.2	1.4	5.7	2.2	2.4
DRC-3	20	33	14	19	22	25	35	25
	26	31	14	37	21	26	1.0	12
	290	290	3	300	--	184		

Yeast extract supplemented milk for growth

GCGS suspending medium

DISCUSSION

The objectives of this study were to prepare lyophilized dairy starter culture concentrates with the same activity and viability as conventional, fresh starter cultures, and to maintain this activity for up to 3 months without using special storage conditions. Presently, frozen culture concentrates are being used extensively but they have the disadvantage of requiring storage under dry ice or liquid nitrogen.

In order to produce cells that retained a high level of activity during lyophilization, the nature of the growth medium was found critical. This was also evident from the study of L. bulgaricus by Smittle et al. (1972, 1974) who showed that sodium oleate in Tween 80 was incorporated into the C19 cyclopropane fatty acid component of the cell membrane, thereby imparting greater resistance of cells to freezing. Recently, Gilliland and Speck (1974) reported a lack of relationship between the C19 fatty acid level present in the membrane of lactic streptococci but the data suggested that the fatty acid portion and capsular components present, may aid in protecting the cells during freezing; also, cells grown at pH 6.0 were more stable during freezing. The results of the present study were in agreement with this in that Tween 80 was only beneficial in the L. bulgaricus medium and not for the lactic streptococci.

NFM provided a good basic growth medium for all the cultures studied. This is believed to be beneficial in the ultimate use of the culture because the cells require an active proteinase system to supply needed amino acids (Cowman and Speck, 1967b) and these enzyme systems may not be highly active in other media. For the study of L. lactis and propionic acid bacteria, broth media were used without adverse effect on viability, but acid-producing activity was not considered.

Non-milk media offer an advantage over NFM in harvesting cells. Although the citrate addition method of Stadhouders et al. (1969) was simple and effective, there were occasions when a gel-like material would sediment during centrifugation and prohibit cell concentration. This problem was encountered more frequently in certain cultures such as M18, but did occur randomly. Enzymatic methods of clearing the milk were attempted as well as use of whey-based media, but were abandoned in favor of NFM.

In this study it soon became apparent that strain selection was critical because some strains within a species lacked the ability to withstand the stress of freezing and lyophilization. In commercial practice, this probably accounts for shifts in strain balance in mixed cultures such as that described by Gilliland (1971) and Reddy et al. (1974) in frozen and freeze-dried cultures. Therefore, to collect different lyophilized concentrates for routine use it was necessary to

screen a large number of strains to find those that could withstand freezing and lyophilization. Because of the strain differences only single strain cultures were studied and evaluated. With industrial application, where a mixed strain is desired, the mixing could be accomplished in the vat by adding the desired single strains.

The extent of cellular injury and death in lyophilization can be influenced by several factors. Some factors such as suspending medium, residual moisture, storage conditions and rehydration medium could be controlled, while other such as strain vigor, rate and temperature of lyophilization could not. Because the successful use of these cultures required a maximum amount of activity, each possible level of injury had to be minimized.

The cultures included in the results section were those found most active upon rehydration, while the remainder of the cultures studied either had greatly reduced activity or viability. The cause of these strain differences could not be explained, and the cultures that performed poorly were not included in further studies. Differences in the susceptibility of the cell wall to stress, denaturation of essential enzyme systems, or sensitivity to osmotic shock during freezing and rehydration may account for some differences.

The suspending medium is perhaps the most critical factor. The GCGS medium afforded maximum protection for the lactic streptococci and flavor bacteria during freezing, lyophilization, and

storage, while the 6% malt extract was most effective for the yogurt cultures. The actual mode by which these suspending media work is not clear. This is complicated by the fact that the real cause of injury and death during freeze-drying is not known, therefore the action of the protective agent is controversial. Recently Redway and Lapage (1974) have shown meso-inositol to be the most freeze protective agent they studied followed closely by the higher alcohols (dulcitol, mannitol, sorbitol) and the non-reducing disaccharides (sucrose and trehalose). The reducing disaccharides provided even less protection with the hexoses and pentoses giving poor protection. The protection provided by meso-inositol is thought to result from it being a relatively inert substance fitting into water lattices and forming structures which protect the DNA during storage (Webb and Bhorjee, 1968).

Ribose generally lowers cell viability when present during lyophilization by an unknown mechanism and the greatest loss of viability occurs when the rate of ribose absorption is greatest in the dry cell. The addition of glutamate or sucrose failed to depress the rate or extent of ribose binding even though viability was increased by their presence while semicarbazide did prevent ribose binding and increase viability (Marshall et al., 1974).

Morichi et al. (1963) have shown that the protective effect of glutamate is due to the physico-chemical nature, not its metabolic

activity. Comparison with related compounds have shown the effectiveness due to (1) the presence of a functional group containing the atom of high electronegativity on the α-carbon, e.g. $-NH_2$, (2) presence of two acid groups, e.g. α and γ COOH, (3) close proximity of $-NH_2$ and -COOH. This agrees with findings of Obayashi et al. (1961) who reported the water binding ability to be the mode of protection.

Sucrose has also been reported as an effective cryoprotective agent in lyophilization by Merryman (1966). The hydrophilic characteristic of sucrose has been thought to prevent the loss of bound water, preventing the denaturation of cellular enzymes.

The composition of malt extract syrups containing 75-80% solids has been reported by Heron (1966) as follows: hexose, glucose and fructose, 29.8%; maltose, 33.7%; maltoribose, 5.2%; sucrose, 1.7%; unfermented matter, 18.7%; ash, 1.8%; protein, 7.9% and lactic acid, 1.2%. The presence of high quantities of glucose, fructose and maltose would indicate that malt extract is a poor cryoprotective agent, according to the results of Redway and Lapage (1974). However, malt extract was protective for the yogurt cultures though not others, while GCGS protected other cultures and not the yogurt cultures. Thus different requirements for cryoprotective agents are indicated for different cultures. With the present knowledge it would be difficult to explain the mode of protection afforded by malt extract.

Maintenance of vacuum in the vials was the most critical factor

during storage. Upon opening the vials, if there was not an implosion of air, the vial had lost its vacuum and the lyophilized concentrate had a minimum of activity. This loss of activity was due mainly to cell death during storage as evidenced by low plate counts and failure of the culture to respond to different resuspension media. The small decreases in activity with storage of the culture may in reality be due to slight vacuum losses in the vials. The deleterious effect of vacuum loss was more pronounced in certain cultures like S. thermophilus, as compared to L. bulgaricus, indicating a probable different mode of air-induced injury in these bacteria.

The reason for the deleterious effect of oxygen is not clear, but Webb (1969) has suggested that oxygen inactivates a membrane-bound system necessary for the repair of injury incurred during lyophilization. Lion et al. (1961a) and Dimmick and Heckley (1961) have shown a correlation between free radical formation in lyophilized bacteria measured by electron paramagnetic resonance, and viability. Israeli and Kohn (1972) have postulated that colicin E-1 and oxygen act at the site involved with the initiation of a new round of DNA replication. In an earlier study made in our laboratory, addition of antioxidants such as ascorbic acid and butylated hydroxyanisole were found ineffective in preventing the oxygen-induced damage (Blaine, 1972) but potassium iodide did provide some protection as also noted by Lion and Bergman (1961).

The loss of vacuum became more acute in culture shipment which could create a problem in commercial use of dry concentrates. This could be solved by use of a different closure or perhaps an inert gas atmosphere such as argon or nitrogen which was shown effective by Marshall et al. (1973).

Maintenance of the lyophilization equipment during these studies was given careful attention in order that available free moisture in the lyophilized concentrates be kept as low and uniform as possible. The lyophilizer used for this work did not allow for various temperatures or vacuums to be used during drying so these parameters could not be studied.

Studies by Bannikova et al. (1964) indicated that residual moisture in lyophilized bacteria should neither be too low nor too high if maximum stability is to be expected. Merryman (1966) has suggested 1% residual moisture for maximum stability of dry cultures and Scott (1958) has hypothesized that removal of tightly bound water molecules may reduce the stability of cellular constituents. More studies on this factor where lyophilized starter concentrates are concerned would seem warranted, even though the concentrates prepared as described in this study were quite stable.

The stress of preparing the lyophilized concentrate injured the cells to the extent that rehydration became critical in achieving maximum cellular activity. Rehydration conditions became important

not only from the point of decreasing osmotic shock but also in providing an environment under which cellular damage could be repaired (Sherman, 1967). According to Ray et al. (1971a, b) cell viability and lag of cell growth in Salmonella after rehydration was related to the composition of rehydration media. Cowman et al. (1967a) reported a probable injury to the membrane proteinase system of S. lactis by freezing the cell and in this way preventing the cell from obtaining sufficient peptides after thawing. Realizing also that osmotic shock could cause further injury to the cell upon rehydration (Choate and Alexander, 1967; Sherman and Kim, 1967), a rehydration medium minimizing cell stress was sought. The lactose-stimilac containing medium provided such an environment for the cell and thus enabled the cells to begin optimum activity upon inoculation in the vat. This rehydration medium was successfully used for all cultures except the yogurt cultures suspended in the GCGS and GAGS media. In these two cases there probably was extensive cell damage which could not be aided by the rehydration medium alone.

Plate counts of most lyophilized concentrates showed very little decrease in viability. This was misleading in certain cases where injury reduced cellular activity. Most reports rely solely on viability for evaluation of lyophilization damage and fail to account for injury; therefore, comparison of the lyophilized culture concentrates using simulated fermentation conditions as activity tests permitted

a more realistic evaluation. Under these criteria the lyophilized concentrates of lactic streptococci compared very favorably with the conventional cultures, while the yogurt cultures had a slightly increased lag period.

Actual cheese making trials comparing lyophilized concentrates to a conventional culture were successful. For those experiments 8 lb of conventional culture was compared to 7 g of lyophilized concentrate which was approximately 1/600th the weight of the conventional cultures. Acid development during manufacture and flavor and texture of the final product were comparable.

Use of yogurt cultures for direct inoculation could also be successful because the lag in acid production would only require 20 to 30 min of additional manufacturing time. This is not as critical as lag time in cheese production because yogurt fermentation does not take place in a vat requiring timed processing steps like cheese. Instead the milk is inoculated and cartons filled and incubated until the desired pH achieved and refrigerated. For these reasons it is felt that dry concentrates of yogurt cultures would be very useful, even though viability may be slightly decreased.

The flavor cultures were evaluated only on the basis of viability; but this was considered a reliable criterion. During manufacturing use, these cultures are not relied upon for their acid production, so even if there was a slight lag it would not be critical. Availability

of lyophilized concentrates of these bacteria would be beneficial because large volumes of these starters are not used often, so daily propagation is an inconvenience with conventional cultures. Flavor-enhancing cultures not transferred frequently usually deteriorate and contribute to non-uniform products. Lyophilized concentrates are uniform and require no preparation prior to inoculation, therefore allowing manufacture of products with identical flavor day after day.

Evaluation of this process for industrial use must also include a cost analysis. Attempts to compare the costs of using conventional cultures and dry concentrates was difficult because actual plant costs were unavailable. Approximate cost of material required in growth, harvesting and suspending was determined to be $12.64 per lb of dry concentrate which would replace 600 lb of conventional culture. To this figure must be added overhead costs such as growth facilities, harvesting equipment and lyophilization costs.

Perhaps an easier approach would be to compare costs determined from preparation of commercially available frozen concentrates to costs of preparing dry concentrates. The former are economical to produce and to this cost would be added the cost of suspending medium and lyophilization, but would save the cost of air freighting frozen concentrates packed in dry ice and expensive refrigerated storage. This trade-off could allow lyophilized concentrates to become commercially available in the next few years.

This study has provided the basic procedures and knowledge required to allow further study of lyophilized dairy starter concentrates. Presently, the literature is very limited in this regard, with few thinking that lyophilized concentrates could be practical.

From the results it can be seen that lyophilized lactic acid starter culture concentrates can be expected to perform well under industrial conditions. However, more strains that are able to perform well under these conditions will need to be discovered. In this way, a sufficient number of different strains can be accumulated to allow culture rotation schedules. Lyophilized concentrates should then be able to replace the more conventional culture and eliminate many of the problems of culture handling.

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