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Optimisation of Growth and Storage Conditions of Lactic Streptococci

A DISSERTATION FOR THE M.Sc. DEGREE

Presented by

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ABSTRACT

The application of defined strain culture systems in the Irish Cheese Industry required detailed knowledge of culture preparation and preservation. Concentrated starters were prepared for strains of Streptococcus lactis and Streptococcus cremoris in a low lactose medium (2.5% milk solids) with external pH control. Initial studies indicated that doubling the level of yeast extract in the medium did not have a significant effect on specific growth rate or on final cell numbers. For most strains, final cell numbers at 22°C were greater than obtained in 10% milk solids medium. Individual strains showed variable ability to maintain activity once the culture entered the stationary phase.

When optimum conditions for cell harvesting had been established, individual strains were stored at refrigeration and sub-zero temperatures - both with and without cryo-protectants. Samples were assayed at various intervals for cell viability and culture activity. At -80°C most strains maintained activity and viability whether cryoprotectants were added or not. Strains which showed a decline in viability were best protected by glycerol with lactose being slightly less effective.

At -20°C, the spent ferementation medium was not able to protect the strains from freezing or from frozen storage. Some strains lost activity without showing a decline in viability. With others, activity decreased at a rate corresponding to the loss in viability. For the former strains, subculturing at 21°C did not restore full activity but the latter cultures were able to regain full activity on sub-

culture. The addition of glycerol and lactose prior to freezing aided in cell protection as did freezing at low temperature. However, the degree of protection was strain dependent. Some strains retained activity at refrigeration temperatures for up to 73 days, with all strains remaining active at 4°C for at least 14 days.

One S. cremoris strain did not grow well under pH-control. A bacteriophage was detected in the culture, initially at low levels. The phage propagated and eventually lysed the culture. This occurred in milk based media which were pH controlled between pH values 6.0 and 7.3. Growth under pH controlled conditions at pH 5.5 did not cause phage release even though in separate experiments the phage was able to multiply at this pH. Inducing agents, such as U.V. and mitomycin-C failed to release lytic phage, nor were phage detected in broth or in phage-inhibitory media. Single strain isolates also exhibited phage release, even though these isolates differed from the parent strain in phenotype and phage typing.

With the exception of one S. cremoris strain, conditions of culture growth, harvesting and storage were established. These have proved useful for the growth of lactic streptococci for commercial cheese making.

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LITERATURE REVIEW

INTRODUCTION

Milk has been known to man as a wholesome nutritious food since early times. However in warm climates milk tended to perish rapidly, unless "natural" souring occurred in pouches, usually derived from the stomachs of animals. While the involvement of bacteria in souring was not recognised, it was quickly learned that milk could be preserved by inoculation with a portion of already soured milk (starter) giving rise to a product that was much easier to preserve than milk; as well as being more uniform in taste and appearance than if left to sour by chance contamination.

Milk is composed of various solids, including fat, proteins,lactose and minerals suspended in water. The preservation of milk by souring or fermentation involves removal of water, the amount removed and its rate of removal gives rise to a particular characteristic end product. Fermented milk products include cheese, yoghurt, buttermilk and cottage cheese. In each product the formation of lactic acid from the sole carbohydrate source, lactose, is the critical step. The lactic acid along with rennet, a mixture of crude enzymes extracted from the stomachs of calves, aids in the coagulation of the caesin portion of milk proteins, giving the cheese curd. With the formation of lactic acid only trace amounts of lactose remain in the curd. This prevents growth by spoilage micro-organisms as all carbohydrate in the curd has been used up.

As the practice of using a portion of the previous days ferment as "starter" resulted in faster fermentations and

more uniform product, it persisted until the last quarter of the 19th century. Then the discovery of microorganisms by Van Leeuwenhoek and Pasteur and the development of pure culture techniques by Robert Koch led to the separate isolation of lactic acid bacteria in Denmark, the United States and Germany (Biotechnology Vol. 5 1983).

Since the early 1900's consumption of dairy products has increased markedly. Consequently there has been a greater demand placed on the dairy industry to provide fermented food products. World production of cheese was 10.5 million tonnes in 1978 (F.A.O. 1979) while in Ireland, production exceeds 54,000 tonnes p.a. As cheese starter cultures are inoculated at a rate of 1-1.5% (w/w) to cheese milk, total world production of starter organisms is in the region of 1.1-1.65 million tonnes. The large increase in cheese production has led to a rationalisation of the industry in most countries. Most of the cheese being made today is made in large factories using increased mechanization and computer control over the production process. Such large scale production involves increased milk throughput and shorter cheese "make" times. Therefore, this multifilling of vats requires large quantities of active starter with reproducible acid producing ability giving a consistent product that will command a premium price.

In order to maximise culture performance in the large factories precautions have to be taken to prevent bacteriophage attack and to maintain culture activity. Normally cheese factories propagate cultures from liquid stock through to the bulk tank stage. This practice often leads

to undesirable strain dominance in starter blends (Limsowtin et al., 1978) or variation in acid producing ability due to loss of plasmids (Davies and Gasson, 1981). More importantly repeated transfer of culture in the factory environment increases the possibility of bacteriophage contamination, resulting in slow acid production (Whitehead and Cox, 1936; Collins, 1962).

COMPOSITION OF STARTER CULTURES

Bacteria used in the dairy industry belong to the mesophilic or thermophilic classes. The former group, producing the majority of fermented milk products, include Streptococcus cremoris, Streptococcus lactis, Streptococcus lactis subspecies diacetylactis, Leuconostoc cremoris, Leuconostoc lactis and Leuconostoc dextranicum. These mesophiles are used to manufacture cheeses - Cheddar, Gouda, Camembert, Cottage, etc., cultured butter, cultured buttermilk and cultured sour cream. The latter group includes Lactobacillus bulgaricus, Lactobacillus thermophilus, Lactobacillus helveticus and Streptococcus thermophilus. They are used in various combinations for the production of yoghurt, acidophilus milk and high scalded cheeses such as Emmental and Gruyere. Italian type cheeses such as Mozarella also involve the use of thermophilic bacteria in production.

S. lactis and S. cremoris are selected because of their acid production and consistent performance. Strains of S. lactis subsp. diacetylactis also form lactic acid from lactose fermentation but formation of aroma compounds such as diacetyl from citrate fermentation is more important in imparting characteristic flavors to some products, such as buttermilk and cultured sour cream. The contribution of

S. lactis subsp. diacetylactis and Leuconostoc species to the flavour of Cheddar cheese is unknown as they are present in some mixed strain cultures (Cogan and Daly, 1983; Daly, 1983a), even though their presence can give rise to open texture in Cheddar cheese due to the production of CO_2 from citrate (Sherwood, 1939; Galesloot, 1950).

Lactic acid bacteria can be differentiated from each other morphologically and biochemically. All are gram positive, non motile, microaerophilic or anaerobic rods or cocci. The spherical cocci, occurring in chains of varying length, depending on both growth medium and age of culture, (Smith, 1965) belong to either the genus Streptococcus or Leuconostoc. They can be separated by the relative inactivity of Leuconostoc spp. in milk at 21°C or 30°C compared to that of Streptococcus spp. The lactic streptococci can be differentiated biochemically. S. lactis subsp. diacetylactis is the only one that produces diacetyl and CO_2. S. lactis can be differentiated from S. cremoris by the production of NH_3 by arginine hydrolysis by the former. Genetic studies have revealed that the citrate permease of S. lactis subsp. discetylactis is coded on plasmid DNA that is easily lost (Kempler and McKay, 1979) while other workers have revealed that many S. cremoris strains have two of three enzymes of arginine metabolism (Crow and Thomas, 1982). This evidence may suggest that the species S. lactis, S. cremoris and S. lactis subsp. diacetylactis all belong to the one species.

COMMERICAL CULTURE SYSTEMS

In commercial fermented dairy product manufacture three types of culture systems are used. They are "mixed strain", "single strain" and "multiple strain".

"Mixed strain" starter cultures contained undefined mixtures of S. cremoris, S. lactis and to a lesser extent S. lactis subsp. diacetylactis. These starters were acceptable in the cheese industry because of their contribution to the desirable flavour of the finished product as well as their marked phage insensitivity. The characteristic of phage insensitivity was dependent on the method of culture propagation.

One instance involves the use of starters which are not protected against phage (Crawford and Galloway, 1962; Galesloot et al., 1966) when transferred in the factory. These so called "practice" starters (P-starters) can be propagated without prevention of contamination by airborne phages. The result is a natural selection process whereby phage sensitive mutants if they arise, are unable to dominate the bacterial flora because phages are present in the factory which prevent this.When this contact is broken by transfer in the laboratory under strictly sterile conditions, the culture becomes dominated by phage sensitive mutants (Galesloot et al., 1966; Stadhouders and Leenders, 1984). On transfer under factory conditions the starter fails due to phage attack.

However with the use of mixed strain cultures propagation under non sterile conditions seems to be the exception rather than the rule. Most modern mixed strain cultures

are tranferred in non-phage environments. This means they have become dominated by phage insensitive strains and must be used in long awkward rotations (Daly, 1983b; Sandine, 1977). While undefined mixed strain starter cultures are difficult to study (Sandine, 1977) a comprehensive study was made in Ireland using two large cheesemaking factories in 1978 and 1979 (O'Brien, 1982). This study and a similar type study carried out in the United States (Daniel and Sandine, 1981) found that the use of undefined mixed strain cultures in rotation adds to the diversity of phage in the factory environment and the components of some mixed strain starters are phage related. These results are in agreement with those obtained by Cogan (1975) when he found different mixed strain starters to be phage related. Many of cultures examined were strain dominated as shown by high plaque titres for phage in some wheys even at the beginning of the season, in agreement with the reports of Reiter (1949), Collins (1962), Daniell and Sandine (1981), Thunnell et al. (1981). While the phages may be present in raw milk (Lembke et al., 1980) or survive pasteurisation (Robertson, 1966; Hull, 1977), the starter culture itself may be the principal source of phage which may either be "carried" (Crawford and Galloway, 1962; Galesloot et al., 1966; Stadhouders, 1975) or may exist in the lysogenic state (Czulak and Naylor, 1956; Delaney, 1978; Huggins and Sandine, 1977).

In the Netherlands proper acid development in large scale Gouda cheese manufacture is achieved without starter rotation. The use of closed cheese vats and clorination

between fills (Stadhouders and Leenders, 1981, 1982) allied with close cooperation between the dairy research centre (NIZO) and the industry has ensured that phage insensitive mixed strain cultures have adapted to modern commercial conditions. In Ireland,however,rotations of cultures obtained from several commercial sources had to be used (Daly, 1983a) resulting in the widespread build up of phage and culture inhibition in both open and closed vats (O'Brien, 1982). This led to different manufacturing times, resulting in variable quality of the finished product.

A similar problem with regard to quality, though not caused by bacteriophage, occurred in New Zealand in the 1930's. Due to the fact that the major market for New Zealand cheese in the early ninieteenth century was the United Kingdom, open texture in cheese was not desirable even though it was common. This was the result of gas production by undefined mixed strain starters. It was recognised that work carried out on such cultures was not reproducible (Whitehead and Hunter, 1939) and any use of mixed strains must involve having knowledge of the individual components.

Thus Whitehead isolated pure cultures of non gas producing lactic streptococci and used them initially as single starters,then as a rotation of non phage related starters (Whitehead and Hunter, 1947) to overcome culture inhibition caused by whey contamination of the cheese milk (Lawrence and Pearce, 1972). Pairing of single strains also evened out differences in the rate of acid production and the tendency by some strains to produce bitterness in cheeses.

The concept of paired single strains was introduced in

Australia (Czulak and Naylor, 1956) involving a four day rotation, thought sufficient to allow phage levels to fall below a critical level and to allow for incompatability of strains (Sharkey and Patten, 1955). However, despite the proven ability of single strain starter culture programs, their use was limited to Australia and New Zealand with the exception of some factories in Scotland (Hynd, 1976) and the United States (Collins, 1962). Failure of the American dairy industry to use single strain starters was due to the fact that they were not under undue pressure to make close textured cheese (Collins, 1962), and also laboratories, such as the Dairy Research Institute in New Zealand, and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia, did not make cultures available to the industry.

An extension of the single strain starter system is the multiple starter concept (Limsowtin et al., 1977; Daniell and Sandine, 1981; Thunnell et al., 1981). This system was developed due to the increase in the size of cheese factories which tended to have multifilling of cheese vats and shorter manufacturing times. The performance of paired single strains was placed under considerable stress during the cheesemaking day as phage invariably appeared (Pearce, 1974) resulting in "dead" vats. However the development of a test (Heap and Lawrence, 1976) which aided in predicting how long a newly introduced starter remained viable before succumbing to phage attack in the factory environment, allowed the selection of phage insensitive strains. These strains instead of being rotated could all be used continuously in the factory resulting in consistent performance in all vats

throughout the cheese making day leading to a uniform high quality final product.

In the multiple strain system all the strains are propagated separately and blended before inoculation into the bulk tank. If a fast replicating phage is encountered for any strain in the factory, that susceptible strain is withdrawn and replaced by a different one. While six strains have been used in New Zealand (Limsowtin et al., 1977), Australia (Hull, 1977), America (Daniell and Sandine, 1981; Thunell et al., 1981) and in Ireland (Cogan and Daly, 1983; Daly, 1983a) with success some other systems have shown significant economic savings using pairs of defined single strains without rotation (Richardson et al., 1980; 1981).

When a defined strain shows susceptibility to phage in the factory it can be replaced using a naturally occurring phage insensitive isolate (Heap and Lawrence, 1976; Daniell and Sandine, 1981) or by a bacteriophage insensitive mutant (B.I.M.) of the original strain (Limsowtin and Terzaghi, 1976; Jarvis, 1981; Czulak et al., 1979; Thunell et al., 1981). The isolation of new strains that are phage insensitive is favoured by some workers (Heap and Lawrence, 1976) as bacteriophage insensitive mutants may have slower acid production, may show reversion to phage sensitivity or be attacked by new disturbing phage in the cheese vat (Hull, 1983). If phage occur to the B.I.M. it may be more virulent with an extended host range (Richardson et al., 1981). The use of lysogenic strains in multiple starters has been questioned (Heap and Lawrence, 1976; Huggins and Sandine, 1977; Park and McKay, 1975). However lysogenic strains have

been used in multiple starters and as long as they are not included with known indicater strains their use seems acceptable.

CULTIVATION OF LACTIC STREPTOCOCCI

I: Nutrition

In order to maintain starter cultures at maximum activity, the conditions under which they grow optimally have to be studied. Lactic streptococci are a fastidious group of bacteria requiring along with a carbon and nitrogen source, inorganic salts, vitamins and a minimum of six amino acids (Reiter and Oram, 1962). Lactic streptococci, while experiencing difficulty in growing in chemically defined media, grow well in milk, even though amino acids and low molecular weight peptides are present at only low concentrations. Growth and acid production therefore require efficient systems for the degradation of milk protein as well as for lactose fermentation.

It has been reported (Reiter and Moller-Madson, 1963) that milk supplies all the essential vitamins and nitrogenous substances for lactic acid bacteria. However growth and acid production can be stimulated by the addition of nitrogen in various forms. Yeast extract (Smith, 1943), peptones (Garvie and Mabbitt, 1956), and corn steep liquor (Kennedy et al., 1955) have been found to stimulate lactic acid production. The nature of the stimulation by yeast extract is two fold (Selby-Smith et al., 1975). Firstly, amino acid material in the form of free amino acids and peptides supplement the available nitrogen. Secondly, a yeast extract fraction has

been shown to stimulate cell growth by decomposing H_2O_2 in conditions which maintain a high O_2 tension in the medium. The addition of free amino acids to milk doesn't alter appreciably the activity of the starter bacteria, unless peptide uptake, which stimulates activity more than free amino acids do (Williamson and Speck, 1962),is inhibited by pH (Selby-Smith et al., 1975) or temperature (Hillier et al., 1975).

Growth in milk is not totally dependent on proteolysis (Williamson and Speck, 1962; Lawrence et al., 1976) as proteinase deficient strains grow at low cell densities at the same rate as the parent strain. These variants become growth limited at 10-25% of the cell densities achieved by the parent. Of the milk proteins utilised by lactic acid bacteria casein seems to be the most important. This is inferred from the poor growth of bacteria in whey from which the caesin has been removed. Some strains have been shown to be unable to hydrolyse casein (Exterkate, 1976) and probably grow due to stimulatory interactions involving variants (Pearce et al., 1974) or other strains (Thomas and Lowrie, 1975).

It has been suggested that the essential amino acids necessary for growth of single strain starters are glutamic acid, valine, methionine, leucine, isoleucine and histidine. Other amino acids including arginine, phenylalanine, proline and tryptophan are required by some strains. Fluctuations in the levels of these amino acids in milk due to season and stage of lactation have been reported to affect starter culture performance (Tevilevich, 1962; Gregory, 1962). This

situation also holds true for vitamins. Biotin, nicotinic acid and pantothenate are essential for growth of S. lactis and S. cremoris (Reiter and Oram, 1962). Riboflavin is required by some strains only, while cultures are stimulated by pyridoxal.

Studies have shown that ashed yeast extract stimulates growth of lactic streptococci (Selby-Smith et al., 1975) indicating the requirement of starters for trace metals. The sequestring of iron in milk reduces lactic acid production (Pulay et al., 1959) but cobalt and zinc can be used to replace iron. Acid production is also stimulated by molybdenum,magnesium and selenium but inhibited by greater than 4 p.p.m. copper, iodine or mercury (Olson and Qutub, 1971). An increase in the concentration of manganese in milk stimulates, Leuconostoc species, but not streptococcal species, to produce more diacetyl and acetoin (DeMan and Galesloot, 1962). Manganese does not affect acid production. The use of phosphates (Hargrove et al., 1961) to complex metal ions in milk, as a method for the prevention of phage growth, results in culture inhibition. Calcium is not important for the growth of single strain starters as cultures grow well in some calcium free,phage inhibitory media (Gulstrom et al., 1979; Galesloot and Hassing, 1962).

The major reason why lactic acid bacteria are used in dairy fermentations is because of their production of lactic acid from the milk sugar lactose. The lactic acid bacteria are either heterofermentative or homofermentative, the latter producing mainly lactic acid, with other end products formed in small amounts. This occurs anaerobically but end products

may be altered dramatically depending on the conditions of cultivation (Gunsalus and Niven, 1942). Lactose is taken into the cell via the phosphoenolpyruvate dependent phosphotransferase system (PEP-dependent P.T.S.). The lactose phosphate is then hydrolysed by phospho-B-galactosidase to glucose and galactose which are then metabolized via the Embden-Meyerhoff pathway in homofermenters. However the enzymes of the PTS are plasmid coded (Anderson and McKay, 1977) as are the enzymes involved in citrate transport (Kempler and McKay, 1979), and proteinases (McKay and Baldwin, 1974; 1975).

While deriving energy for growth from the fermentation of lactose, the lactic streptococci can continue fermentation in the absense of growth. This uncoupling of acid production from growth can occur in the presence of excess carbohydrate (Thomas and Batt, 1969), NaCl (Turner and Thomas, 1975), antibiotics (Breheny et al., 1975), at low pH values (Turner and Thomas, 1975) and at elevated temperatures (Breheny et al., 1975; Turner and Thomas, 1975; Lowrie et al., 1972). Lactic streptococci deficient in proteinase ability have also been shown to uncouple acid production from growth as measured by cell numbers (Pearce, 1974; Thomas and Lowrie, 1975). These stresses, to which the lactic acid bacteria are subjected both during cheese making and in the production of starter cultures may result in non uniform activity, where acid production is used as a measure of starter growth.

CULTIVATION OF LACTIC STREPTOCOCCI

II: Physical Factors

In addition to satisfying the nutritional requirements of the lactic acid bacteria, physical factors involved in production of starter cultures have also to be optimised to ensure activity in the cheese vat. The fermentation tanks have to be secure against bacteriophage infection and designed to maximise biomass yield.

It is known that lactic streptoccal phage survive pasteuriation (Koka and Mikolajcik, 1967) in milk and in artificial media. Sterilization of milk and whey based media cannot be totally achieved as at 121°C for 15 minutes milk is degraded to where it will not support active lactic culture growth (Porubcan and Sellars, 1979). Batch heat treatment at 85-95°C for 40-120 minutes is normally used for heat sensitive media. However, the use of UHT treatment (140-145°C for 4-8 seconds) will sterilize 10% reconstituted skim milk without any deleterious effects. Minor changes in whey proteins which are potentially stimulatory, occur. Sterilization can take place inside the fermentation vessel or in another vessel before being pumped into the fermenter. If the media is sterilized in place, air drawn into the head space during cooling has to pass through filters capable of retaining phage. Modern High Efficiency Particulate Air (HEPA) filters as well as microporous (0.22 μm) filters have been shown to be effective in phage control.

On inoculation of the mother culture into the fermenter, the temperature is adjusted until the optimum is reached. It has been found (Dorn and Rahn, 1939) that the optimum temperature for maximum growth rate, maximum population and

greatest fermentation rate is not the same. In the production of culture concentrates, cell mass not growth rate is the most important consideration. The optimum growth rate for S. lactis has been shown to be at 30°C in whey (Pont and Holloway, 1968). Bergere (1968) has reported a maximum growth rate at 35°C for the same strain in an artificial medium. Cell yield at 35°C was less than at 25°C. It is considered that the optimum temperature for growth is that at which the specific growth rate is fastest, especially for batch cultures. Growth under continuous culture conditions must be carried out at temperatures below the constant temperature characteristic of the strain used, in order to maintain parental phenotype and prevent inhibition of growth due to temperature stress (Cogan et al., 1971). Growth of "mixed" or "multiple" strain starters together is best done at 25-27°C (Peebles et al., 1969; Lamprech and Foster, 1963; Efstathiou et al., 1975; Lee and Collins, 1976) as most mesophilic lactic streptococci grow at the same rate at 27°C. Strain domination tends to occur at 22°C and to a lesser extent at 30°C.

All media used for commercial production of lactic acid bacteria are based on milk or whey. The fermentable sugar in each case is lactose. The use of other carbohydrates such as glucose is not advisable as it may be selective for mutants defective in lactose metabolism (McKay et al., 1972; Davies and Gasson, 1981). The use of glucose may also result in autolysis of some starter strains on cessation of growth (Moustafa and Collins, 1968; Vegarud et al., 1983). As lactic acid is formed from lactose at the rate of 10% of

the bacterial weight per minute, the pH of the medium, unless buffered, drops rapidly until it becomes growth limiting at pH 5.0.

Lactic streptococci can grow over a wide pH range (Davis and Thiel, 1939). Growth can occur at pH 4.8 and in alkaline conditions at pH 9.0, with S. lactis being more tolerant of alkaline conditions than S. cremoris. Harvey (1965) has reported a pH optimum of 6.3 for S. lactis with changes in the growth rate being due to the effects of hydrogen ion concentration on the rate of enzymic reactions. The optimum pH for biomass production may not be the same as for other metabolic activities, such as diacetyl production (Gilliland et al., 1970). Other pH optima reported include pH 6.5 (Bergere, 1968; Stadhouders et al., 1969), pH 6.4 (Cogan et al., 1971), pH 6.3 (Blaine et al., 1970) and pH 6.0 (Gilliland, 1977; Jonas et al., 1977; Richardson et al., 1977). Reports (Pont and Holloway, 1968; Peebles et al., 1969) taking a more realistic approach state that generation times of lactic streptococci do not differ significantly between pH 6.0 and 6.5.

In ordinary batch cultures, starter bacteria only grow under optimum conditions for a short time. Inhibition occurs due to acid buildup causing the cells to die and the survivors to show increased lag times on subsequent sub-culture (Harvery, 1965; Terzaghi and Sandine, 1975). Lactic streptococci at low pH are unable to maintain an energy dependent pH gradient (Lawrence et al., 1976) with consequent exposure of cellular components to high hydrogen ion concentrations causing reversible denaturation of cellular components. In order to prevent cell damage due to acid buildup

and in order to allow cultures to be grown to high cell numbers in the least number of generation times preventing culture alteration, neutralisation of the acid produced is employed. Types of neutraliser used and their effects on cell yield and activity will be discussed later.

During growth of cultures under pH control, agitation of the medium is necessary to allow dispersal of the neutraliser as it is added. This agitation,if it occurs under atmospheric conditions, may result in excessive aeration of the medium and the formation of hydrogen peroxide (H_2O_2) in amounts sufficient to cause inhibition of growth and acid production (Anders et al., 1970). This occurs in milk (Gilliland and Speck, 1969) and in whey (Pont and Holloway, 1968). Other reports (Cogan et al., 1971) indicate that neither stirring nor sparging with air significantly affect the growth rate of S. lactis in an artificial medium. Inhibition of S. lactis due to H_2O_2 accumulation also occurs in an N_2 atmosphere containing trace amounts of O_2 under continuous culture conditions (Keen, 1972). In batch culture conditions (Gilliland and Speck, 1969) H_2O_2 concentrations reach a maximum level in the early period of acid production, followed by rapid breakdown as the acid concentration increases. The inability of aeration to affect growth rates as well as the rapid breakdown of H_2O_2 could be due to components in the media such as yeast extract (Cogan et al., 1971; Selby-Smith et al., 1975) and ferrous sulphate (Gilliland and Speck, 1969). The addition of catalase to the medium also results in H_2O_2 breakdown. Differences in the NADH oxidase and peroxidase enzymes of

lactic streptococci result in H_2O_2 having varying effects depending on the strain (Anders *et al.*, 1970). The addition of a capsular material or whole cells of *Micrococcus* can also stimulate acid production through the removal of H_2O_2 (Nath and Wagner, 1983). While H_2O_2 accumlation is the primary result of aeration, O_2 itself can have a direct toxic effect at higher partial pressures irrespective of the pH at which the culture is grown (Keen, 1972).

Other inhibitors of lactic streptococci have been reported. D-Leucine was found to be produced by mixed strain starter cultures grown in broth under pH control (Gilliland and Speck, 1968). Concentrations as low as 1mg/ml were found to be inhibitory probably by competition with L-Leucine for incorporation into proteins rendering them inactive. Aldehydes and to a lesser extent ketones and thiols inhibit growth and acid production of *S. cremoris* (Kulshrestha, 1970).

There are conflicting results as to the effect of CO_2 on the growth of lactic streptococci. Cogan (1971) reported that CO_2 had little effect on the growth rate of *S. lactis* while other reports (Keen, 1972; Linklater and Griffin, 1971a) show that CO_2 increases growth rates. *S. lactis* has an absolute requirement for CO_2 in milk (Reiter and Oram, 1961) with a level of at least 0.2% (v/v) required for an increase in growth rate (Whitehead *et al.*, 1958). *S. lactis* itself can form sufficient metabolic CO_2 to initiate and maintain growth (Keen, 1972). Agitation, even under an atmosphere of CO_2-free N_2, results in a dissipation of CO_2 from the cellular environment into the CO_2-free medium. N_2 is required only if the culture is adversely affected by

H_2O_2 buildup. The sparging of cultures with N_2 gas results in a decreased growth rate unless the N_2 gas contains trace quantities of CO_2 (Cogan et al., 1971).

PRODUCTION OF STARTER ORGANISMS

Conventional starter culture preparation is a cumbersome and time consuming operation never entirely free from risk of failure. This case led to the replacement of mother culture handling by culture concentrates which are inoculated into the bulk starter tank or directly into the cheese vat. These culture concentrates can be prepared in a centralised laboratory to be used immediately or preserved for later use.

Most of the important environmental parameters affecting the growth of lactic cultures have been studied using batch fermentations. This type of fermentation is used by most commercial culture producing companies nowadays. As an alternative, continuous fermentations can also be employed to produce lactic acid bacteria.

In continuous culture the pH need not be controlled by the addition of neutraliser such as sodium or ammonium hydroxide. Instead fresh medium can be added at a constant rate, thereby maintaining a constant pH. The pH at which the cultures are growing can be varied by changing the volume of medium flowing into and/or out of the fermenter. In a chemostat the cell concentration is controlled by a constant inflow of growth limiting medium. However continuous cultivation of lactic acid bacteria differs from that of the chemostat in that a constant acid concentration is maintained by indirect measurement of the pH.

Continuous culture theory, developed for chemostat

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operation, but equally applicable to continuous lactic culture cultivation, indicates that under steady state conditions, the dilution rate equals the specific growth rate of the cultured organism (Herbert et al., 1956). The productivity of a continuous fermentation, using a milk based medium, is best just above the coagulation point of casein (Linklater and Griffin, 1971a). At these pH values (5.2-5.4), coagulation of the milk is a problem, especially coagulation around the electrode (Linklater and Griffin, 1971b), giving a region of low pH compared to the main body of the culture. This results in increased dilution rates with possible wash out of the culture. Total wash out doesn't occur because bacteria trapped in the curd continue to produce acid. While maximum productivity occurs at low pH values, the specific growth rate increases as the pH is increased. However at higher pH values (> pH 6.0) a steady state equilibrium is not obtained. A decline in the specific growth rate occurs (Linklater and Griffin, 1971a) unless the culture is starved of O_2 by sparging with N_2 gas. These results have been confirmed (Keen, 1972) and the inhibition has been shown to be due to H_2O_2 buildup. H_2O_2 could not be detected below pH 5.45 or above pH 6.35 in the latter case probably due to the high dilution rate.

For S. cremoris specific growth rates obtained at pH 6.3 under continuous fermentation (0.855 h^{-1}), compare favourably with values obtained from batch culture (0.806 h^{-1}) (Keen, 1972). A specific growth rate of 0.99 h^{-1} at pH 6.3, the optimum for growth, has been reported for S. lactis (Pont and Holloway, 1968). Linklater (1971b) reported a

specific growth rate for the same culture of 0.78 h^{-1} at pH 5.4 compared to 2.00 h^{-1} at 5.5 reported by Wilkowski (1958). The high growth rate reported by Wilkowski could have been due to high dilution rates caused by coagulation in the fermenter as previously described.

Various media have been used in the continuous manufacture of culture concentrates. Skim milk has been used alone (Wilkowski and Fouts, 1958; Shmeleva and Yakovlev, 1966; Linklater and Griffin, 1971b; Keen, 1972) or fortified with yeast extract (Keen, 1972). Pasteurised milk has been used (Berridge, 1966) and the performance of streptococci in it has been compared with their performance in reconstituted skim milk (Linklater and Griffin, 1971a). Lower cell numbers occurred in pasteurised milk at any given pH, possibly due to a lower buffering capacity of the milk or due to the presence of low numbers of contaminants which had not been killed by pasteurisation. As unmodified skim milk presents difficulties with regard to coagulation and harvesting of cells, preliminary enzyme treatment using trypsin has been carried out (Lloyd and Pont, 1973a) to give a medium suitable for continuous culture. Whey based media have also been utilised in continuous fermentation. However, the content of heat precipitable proteins causes a problem in that deproteinised whey is a poor growth medium (Haggett et al., 1968; Lloyd, 1971). Whey fortified with yeast extract (Pont and Holloway, 1968) or whey in which the protein has been digested (Berridge and Wilson, 1970; Lloyd and Pont, 1973b) have also been used continuously in culture production, although whey based media have been reported to

give poor yields of S. cremoris (Lloyd and Pont, 1973b).

The length of a continuous fermentation run compared to that of a batch fermentation allows the build up of contaminants, particularily sporeformers that survive the less than adequate heat treatment of milk based media, especially in pilot and factory scale processes. Continuous culture may lead to the accumulation of variant strains with lower acid producing ability (McKay et al., 1972; Lloyd and Pont, 1973b) and increased phage susceptibility (McDonald et al., 1973). Also, continuous fermentation with some S. cremoris strains results in the detection of phage in the concentrates (Lloyd and Pont, 1973c) even though the cultures were grown in rooms fitted with filtered, pressurized air and germicidal, u.v. lamps. As all cells produced are essentially log phase cells, storage problems may be encountered as these cells are more susceptible to low temperature storage than stationary phase cells (Blaine, 1972). While workers in Australia and Russia have used continuous fermentation to produce cultures (Lloyd, 1971) on a pilot scale it is generally accepted that concentrates are best prepared from batch cultures (Condon and Cogan, 1973).

As simpler techniques and equipment are required most cultures are produced nowadays by batch culture methods. This involves manipulating the growth variables to produce high numbers of uninjured cells, free from detectable phage. These cells are usually used for inoculation of the bulk starter tank but, more recently, developments in culture technology have led to their use as direct vat inoculants. Media and methods used in preparing concentrates differ from those used in the preparation of the bulk culture

which will be discussed later.

The main parameters studied in batch fermentations are media composition (Bergere, 1968 ; Stadhouders et al., 1969; Peebles et al., 1969; Valles and Moquot, 1968; Lloyd and Pont, 1973a; Pont and Holloway, 1968; Stanley, 1977); alkali type required for growth at constant pH (Peebles et al., 1969; Blaine et al., 1970; Blaine, 1972; Lloyd, 1971, Lloyd and Pont, 1973a) and optimum point for harvesting (Peebles et al., 1969; Bergere, 1968 ; Lloyd and Pont, 1973a).

To achieve maximum growth in batch fermentations with either mixed or single strains of lactic acid bacteria it is necessary to formulate the most advantageous medium. This medium may be either simple (milk) or a complex mixture of several ingredients. As the lactic acid bacteria are fastidious in their growth requirements, complex nutrients, (which are chemically undefined), such as skim milk, whey, yeast extract and peptones, have to be used to achieve maximum cell density and activity.

Growth of starter bacteria in broth has been found to have some advantages compared to growth in milk especially in the production of concentrates. Condon (1973) found that 19 out of 27 single strains could produce acid in milk as well as controls even though they were pregrown in broth. Other workers (Peebles et al., 1969; Stadhouders et al., 1969; Lamprech and Foster, 1963; Bergere, 1968) have grown lactic acid bacteria in media composed of tryptone, yeast extract and lactose,with glucose (Peebles et al., 1969) and $Mg_3 (PO_4)_2$ (Lamprech and Foster, 1963) being used as additional growth supplements. These media compared favourably

with skim milk as regards final cell numbers, having 50 to 60 times more cells than skim milk (Bergere, 1968; Stadhouders et al., 1969). Other advantages of broth media include substantially faster growth rates and easier separation of the cells from the medium by centrifugation (Condon and Cogan, 1973). Growth of lactic acid bacteria in a complex nutrient broth containing sugars other than lactose could result in a delay in acid production on subsequent transfer to milk due to a shift down from the rich broth to the nutritionally poorer milk medium (Pont and Holloway, 1968). Also, selection of lactose negative variants may occur (Lloyd and Pont, 1973b) in single strains, while strain imbalance may occur in mixed starter cultures (Stadhouders et al., 1969; Gilliland, 1971). Inhibitory substances such as D-leucine may also be produced in broth (Gilliland and Speck, 1968; Peebles et al., 1969). The high cost of media constituants would make large scale preparation of concentrates uneconomic unless whey and milk were used (Bergere and Hermier, 1968b).

Much work has been done in growing lactic cultures in milk or in milk or whey based media. The milk may be digested before use, by proteolytic enzymes such as trypsin, pepsin and papain (Stanley, 1977; Valles and Moquot, 1968; Bouillanne and Auclair, 1978; Lloyd and Pont, 1973a). This has the twofold advantage of making peptides readily available to the starter and enhancing the efficiency of centrifugation. Cells grown in milk do not have to make any metabolic adjustment when added to cheese milk. Whey based media are also used in starter production (Bannikova and Lagoda, 1970;

Manenkova and Belova, 1965; Lloyd, 1971; Lloyd and Pont, 1973b) when supplemented with yeast extract, milk casein, sodium citrate and manganese sulphate, but it has been found that S. cremoris species grow poorly in whey based media (Lloyd and Pont, 1973b) unless it is supplemented with skim milk solids (Valles and Mocquot,1968). For this reason most applications of commercial technology to concentrate production involve the use of milk based media supplemented with yeast extract, glycerol, dextrose, phosphates and citrates (Lloyd, 1971; Farr, 1969; Miles Lab Inc. 1970; Christensen, 1969; Stanley, 1977; Stadhouders and Leenders, 1984; Turner et al., 1979).

When lactic acid bacteria are grown under pH control in batch fermentations, acid is produced at a rapid rate even though maximum cell numbers have been attained following exhaustion of one or more nutrients (Breheny et al., 1975; Peebles et al., 1979). As there is excess lactose in milk, the resting cells continue to produce acid which results in cell injury and on transfer to milk a lag phase results before growth resumes. To overcome this problem, several workers have used low lactose milk to prepare starter culture concentrates (Lindgren et al., 1974; Hup and Stadhouders, 1978; Bunny and Stanley, 1978). Lingren and coworkers (1974) removed lactose from milk by passing it through a Sephadex column giving a protein filtrate containing 0.2-0.5% lactose. Skim milk was then added back to give a final lactose concentration of 2-2.5%. Hup (1978) has patented a low lactose medium consisting of dilute whey, non-whey protein and an unspecified buffer mixture, while Bunny (1978) has grown concentrates in 6%

(w/v) reconstituted skim milk which has been protease digested and supplemented with yeast extract. In each case cell numbers and acid producing ability of cultures grown in such a low lactose medium compared favourably with cultures grown in ordinary skim milk. Storage at refrigeration or subzero temperatures can be carried out knowing that cells produced in such a medium are less likely to suffer acid injury.

The neutralizer used for pH control also influences the maximum attainable population. Neutralization with ammonium carbonate ($(NH_4)_2CO_3$) or ammonium hydroxide (NH_4OH) results in higher cell populations than with either calcium hydroxide ($Ca(OH)_2$) or sodium hydroxide (NaOH) (Peebles et al., 1969; Blaine et al., 1970; Blaine, 1972; Lloyd and Pont, 1973a; Efstathiou et al., 1975; Stadhouders et al., 1969). Maximum populations are reached more quickly when NH_4OH is used as a neutralizer (Gilliland and Speck, 1974a; Peebles et al., 1969; Lloyd and Pont, 1973a) suggesting that the ammonium may be used as source of nitrogen (Blaine, 1972). While NH_4OH results in increased cell numbers compared with NaOH, the activity of some single strains neutralized with NH_4OH, as measured by acid production, lags behind that of cultures grown in milk or neutralised with NaOH (Gilliland and Speck, 1974a; Lloyd and Pont, 1973a; Peebles et al., 1969). This reduced activity could have been due to low levels of proteinase activity. After one subculture, full acid producing ability and proteinase activity were regained in these cells. In contrast, Efstathiou and coworkers (1975) have reported that mixed strain concentrates, prepared using NH_4OH had a

higher activity than those prepared with NaOH.

In most media, maximum cell numbers are reached before all the lactose is used up. Lactic acid continues to be produced, being neutralized by NH_4OH, NaOH and $Ca(OH)_2$ to form ammonium,sodium and calcium lactates. It has been found that low concentrations of ammonium lactate and sodium lactate, which build up during maintenance of pH, can inhibit cultures (Pont and Holloway, 1968; Blaine, 1972; Osborne and Chubb, 1974; Porubcan and Sellars, 1979). Levels such as 0.5% sodium lactate and 2.0% ammonium lactate are sufficient to cause inhibition. Stadhouders (1969) used $Ca(OH)_2$ as a neutralizing agent because lactic cultures were less inhibited by calcium lactate than by ammonium or sodium lactate.

As lactic acid or lactate salts limit the growth of lactic streptococci, attempts have been made to remove them from growing cultures, in order to increase cell concentrations. One of the first attempts (Bergere and Hermier, 1968) involved the use of a cellulose semi-permeable membrane to separate the growing bacterial culture and a larger volume of nutrient medium. Nutrients from the broth medium diffuse through the membrane as they are assimilated by the culture while the metabolites such as lactate salts diffuse in the opposite direction. However the number of viable cells produced was less than that produced by growing a culture under pH control without dialysis. The failure of Bergere (1968) to achieve high cell concentration was thought by Osborne (1977) to be due to the fact that each ml of fermenter culture was only in contact with $1cm^2$ of dialysis

membrane, insufficient to allow adequate diffusion of lactate away from the culture. Increasing the area of the cellulose acetate membrane to at least $5cm^2$/ml of fermenter culture and having a fermenter/reservoir volume of at least 1:15 overcomes this problem and gives total cell concentrations in enzyme treated milk of 1-3 x 10^{11}/ml depending on the species grown (Osborne and Chubb, 1974; Osborne and Brown, 1980).

The advantage of diffusion culture is that the bacteria are restricted to a small volume compared to ordinary batch culture where values of 10^{11} cells/ml can only be achieved by centrifugation. The yield of 1-3 x 10^{11} cells/ml obtained from diffusion culture compares favourably with values of 1.0 x 10^{10} cells/ml from batch culture (Stanley, 1977). Cultures produced by diffusion culture have been shown to be suitable for both direct vat inoculation and bulk starter propagation, even though acid production lagged, compared to controls when grown at 22°C. On subculture these cultures regained normal activity. Loss of activity could have been due to exposure to ammonium lactate (Osborne and Chubb, 1974). It may be possible to run the system continuously by keeping lactate levels below the inhibition threshold of 2.5% by dilution with fresh medium.

Another advantage of diffusion culture is that it is a one step process compared to the batch process which is two stage involving cultivation and harvesting usually by centrifugation where there is an increased risk of contamination at the centrifugation step. There is also a loss of activity due to inefficiency of harvesting, continued acid

production during centrifugation and disruption of the cells due to mechanical stress (Stadhouders et al., 1969; Osborne and Chubb, 1977; Stanley, 1977).

Ultrafiltration has also been tried as a method of removing metabolic waste products from pH controlled growth of lactic streptococci (Porubcan and Sellars, 1979). However the result was not an increase in cell numbers but an increase in the rate of lactic acid production.

Even though lactic acid has an adverse effect on subsequent activity of lactic streptococci, there are conflicting reports on the optimum point for harvesting the cultures. Continued incubation for up to 12 h after cells have entered stationary phase is shown to be neither beneficial or harmful to the cells (Peebles et al., 1969; Gilliland and Speck, 1974a). In contrast,Bergere (1968) and Lloyd (1973a) reported that the optimum harvesting time, based on subsequent activity tests, was when the cells entered late exponential phase or early stationary phase. From an economic point of view,harvesting the culture at this stage is more practical than allowing the cells to rest in stationary phase without any gain in productivity.

While both diffusion culture and ultrafiltration have been used in cell concentrate preparation, the main technique used in harvesting cultures is centrifugation. The main requirements of the concentration step include, efficiency in extracting cells from the medium, ability to maintain a sterile, low temperature environment and preservation of cell activity by preventing cell damage. The concentrate must be phage-free and must meet standards for microbiological

contamination levels (Stanley, 1977).

The concentration of lactic acid bacteria by centrifugation depends on the properties of the cells themselves and the efficiency of the centrifuge used. The density difference between the cells and the liquid medium is also an important consideration. As milk based media are most widely used in concentrate production, problems occur on concentration of cells grown in milk based media. Milk proteins are present in a colloidal form, with casein, the major milk protein occurring as a calcium caseinate-phosphate particle, up to 300µm in diameter. When the pH of milk is adjusted to 4.6, this particle precipitates. Unless the micellar particles are dispersed, the volume of concentrate is going to be increased. Citrates along with sodium and potassium form undissociated complexes with the calcium, resulting in dispersion of the particles to smaller sizes. Stadhouders (1969) solubilised the calcium caseinate-phosphate by adjusting the pH of the culture to 6.8, the point of minimum insolubility of the caseinate particle, and by adding 1% sodium citrate to aid in further dispersal. A procedure patented by Miles Laboratories (1970) uses KOH to solubilize coagulated protein, before harvesting of cells. Predigestion of the casein with enzymes eliminates the need for citrate treatment (Stanley, 1977).

Various types of centrifuge have been used in cell concentrate production depending on the scale of the operation involved. For small scale production of concentrates a refrigerated Sorvall tube centrifuge is used (Stadhouders et al., 1969; Blaine, 1972; Speckman et al., 1974;

Efstathiou et al., 1975). This centrifuge is capable of removing greater than 99% of cells from the medium (Stadhouders et al., 1969) provided that centrifugation is carried out at 7,000 r.p.m. for at least one hour. Spin times of less than one hour result in less efficient removal of cells (Blaine, 1972). As the capacity of the Sorvall does not exceed 4 litres/hour, larger quantities of concentrates are prepared using Sharples and Cepa tubular rotor centrifuges (Stadhouders et al., 1969; Blaine, 1972; Lamprech and Foster, 1963; Lloyd and Pont, 1973a). These centrifuges are as efficient as Sorvall centrifuges for sedimenting cells. However,a major drawback of these centrifuges is that the rotor must be stopped, dismantled from the machine and opened before the bacterial concentrate can be removed (Porubcan and Sellars, 1979). Removal of the cell paste is difficult, even if the bowl is lined with aluminium foil (Blaine, 1972) or polypropylene inserts (Porubcan and Sellars, 1979), and is subject to contamination. Drying of the cells in the bowl eases removal from the centrifuge but poses problems when the dried cells are added back into milk (Blaine, 1972).

In contrast to the manual centrifuges are the automatic disludging C.I.P. separators, which spin at low (4,500-6,000) r.p.m. and are only slightly less efficient. These are two types of automatic desludging C.I.P. separators. Firstly,there is the closed door type which is opened intermittently during operation to eject the concentrate. Examples of this type are made by De Laval and Westphalia. Secondly, there are the nozzle type machines,an example of which is Alfa-Lavals Hermetic Bactofuge, which have contin-

uously opened ports, for continuous concentrate ejection. When these separators are used for production of lactic acid bacterial concentrates they are run at only 5-10% of their capacity in order to maintain efficiency of harvesting (Stadhouders et al., 1969). To achieve a particular degree of concentration, use of the intermittent desludgers has advantages over the nozzle type machines. The bowl of the former can be precisely opened and closed whenever desired giving the degree of concentration required, between 20 and 100 times the initial concentration (Porubcan and Sellers, 1979).

The production of acid continues in media containing excess lactose during centrifugation, even at refrigeration temperatures (Swartling and Lingren, 1961; Foster, 1962) with a resultant loss of activity (Stanley, 1977). Cooling is usually carried out using a water cooled jacket on the centrifuge. Recently cooling of the separator bowl has been carried out using liquid nitrogen in the supercooled vapour form (Porubcan and Sellars, 1979). This results in a greater degree of cooling efficiency than would be achieved using a water cooled system. Maintenance of low temperature results in retention of activity of the starter concentrate, giving cells with more uniform ability to produce acid on subsequent culturing.

PRESERVATION OF STARTER ORGANISMS

As most starter concentrates are produced in centralised laboratories, from where they are dispatched to factories, methods of storage and protection during transport, to main-

tain cell activity are important parameters in the overall culture production scene.

The ideal preservative method would allow the taking of lactic acid bacteria at the peak of their activity, holding them in a state of suspended animation and on adding them into a favourable environment, having them resume acid production and growth immediately. It is impossible to keep a living organism in such a state. Therefore,preservation involves reducing the metabolic activity of the cells. This is usually accomplished by a combination of methods, involving freezing, drying and storing at a low temperature.

Starter cultures can be preserved by freezing or drying. Dried concentrates can be produced by spray drying or by freeze drying (lyophilisation). The development of these processes made the maintenance of liquid stock cultures obsolete, in most cases.

Spray drying involves the conversion of a solution into a dry powder in a single operation. This is done by atomizing the liquid into a fine spray which then comes into contact with flowing stream of hot air. The best known process involving the use of spray drying, is the production of milk powder. Spray drying of cultures replaced vacuum drying in the 1960's as an effective method of culture preservation (Tamime, 1981), as vacuum drying resulted in only 1-2% viable bacteria which required several subcultures before regaining full activity. Early experiments with spray drying reported survival rates of 10-14% with S. cremoris strains (Foster, 1962). Strains of S. lactis

were more resistant to drying and storage in the dried state. The medium in which the cells were grown, pH at the time of drying, storage temperature and atmosphere all influenced the survival of dried cultures.

Starter bacteria cannot be spray dried by the same method as is used for the production of milk powders, as the temperature used results in a hygroscopic powder with a low number of surviving vegetative cells. Stadhouders (1969) developed a procedure for the production of spray dried cells which had been grown in skim milk followed by neutralization to pH 6.6. The culture was firstly evaporated to a dry matter content of 22% before being spray dried at 70°C to give a powder whose moisture content was 9%. The moisture content was then reduced to 5% in a vacuum drier at 27°C, for good keeping quality. The dried starter produced by this process was as active as a liquid starter. The type of neutraliser used during cell growth has an effect on the powder moisture content and the temperature used to achieve it. The lower the desired water activity (a_w) value of the powder, the higher the final powder temperature will be, which increases the risk of inactivating the bacteria. The use of $Ca(OH)_2$ as a neutralizing agent results in a powder that is less hygroscopic than would be obtained using NH_4OH or NaOH. Thus there is less risk of a rise in temperature affecting bacteria during drying, if $Ca(OH)_2$ is used rather than NH_4OH or NaOH.

The ability of spray dried cultures as produced by the method of Stadhouders (1969) to retain activity was not tested, probably due to the low survival rates i.e. 10% for

mesophilic lactic acid abcteria, with S. lactis subsp. diacetylactis doing slightly better (44%). Another method which has been patented (Porubcan and Sellars, 1975a) involving the production of spray dried yoghurt cultures, has resulted in the product having excellent activity after storage at 21°C for up to 6 months. The addition of ascorbate and monosodium glutamate prior to pH adjustment and drying results in enhanced stabilization to storage at ambient temperatures, probably due to the antioxidant properties of ascorbate and glutamate (Porubcan and Sellars, 1975b). Other yoghurt starters have been spray dried at temperatures up to 80°C in milk fortified with lysine, cystine and cyanocobalamin (Anderson, 1975).

As lactic acid bacteria continue to lose activity if stored in liquid or dried form at above zero temperatures, preservation by means of freezing has been intensively studied. Survival during freezing depends on the temperature to which the cells are cooled, the rate at which they are cooled, the rate at which they are thawed and the type of medium in which they are suspended during the process.

The temperature at which freezing starts is determined by the nature and concentration of solutes in the medium in which the cells are suspended. Cell water remains supercooled until a temperature of -10°C to -15°C is reached. Water in the external environment is frozen at these temperatures. A vapour pressure is then set up between the inside and outside of the cell, which can equilibriate in two ways. Firstly, water can be removed from the cells and frozen extracellularly. This results in cellular dehydration.

Secondly, spontaneous ice-crystal formation can occur within the cell as the temperature drops or the cell membrane may cease to act as a barrier to the passage of ice crystals so growth of external ice occurs within the cell (Mazur, 1970). The means used to establish equilibrium depends on the rate of cooling and the permeability of the membrane to water. Fast cooling rates result in intracellular ice formation while slow cooling results in cell dehydration.

The removal of water from the cell may lead to an increased solute concentration (Mazur, 1970). The resultant drop in pH may increase the rate of chemical reactions and affect the secondary and tertiary structure of DNA, RNA and proteins. Hydrophobic bonds may be weakened in proteins in the cell membrane, leading to interaction of exposed active groups which may damage the membrane due to physical strain. It is known that a decrease in activity of S. lactis during cold storage is related to a proteinase associated with the cell membrane (Cowman and Speck, 1967). This may be due to a change, in the enzyme form, from monomer to dimer due to aggregation, (Speck and Cowman, 1969) which is irreversible and leads to loss of proteinase activity.

Death of bacteria at sub-zero temperatures is not usually caused by external ice formation. Nor do freezing and thawing cause cell lysis. High concentrations of solutes in the external environment are much more lethal (Ray and Speck, 1973). If the cell membrane is damaged leakage of low molecular weight compounds such as amino acids and RNA molecules occurs. This manifests itself as increased nutritional needs on growth after thawing. The presence of

oxygen in the freezing medium can cause cellular injury due to free radical formation (Swartz, 1971). Cultures stored under an atmosphere of nitrogen have greater activities than those stored in the presence of O_2 (Baumann and Reinbold, 1966; Stadhouders et al., 1969), probably due to the inertness of nitrogen and the inhibition of oxygen dependent, lethal biochemical processes.

The temperature to which the cells are frozen, the length of time the cells are maintained at this temperature and the rate of thawing also influence survival. Lactic acid bacteria show higher survival rates as measured by acid producing ability and proteinase activity when frozen at -196°C compared with -20°C (Cowman and Speck, 1965, 1967; Baumann and Reinbold, 1966). Fluctuations of temperature during storage affect survival of bacteria as recrystalization of cell water can occur, leading to larger ice crystals which damage the cell membrane more easily.

Death and cellular injury due to frozen storage as opposed to death from the freezing process is the result of either recrystalization or prolonged exposure to the concentrated extra - and intracellular solutes in the environment. If temperature fluctuation can be prevented, cell death is mainly as a consequence of solute concentration (Ray and Speck, 1973). The death rate is rapid initially but then it levels off with low storage temperature giving a low death rate.

While cell death and injury are caused in the main by physical and chemical means, other factors that influence freezing include the inherent characteristics of the cells

themselves and the environmental conditions to which the culture was subjected,prior to and during freezing and thawing.

It is generally agreed that gram positive bacteria are more resistant to freezing than are gram negative bacteria. The effect of freezing on lactic acid bacteria depends on the storage temperature used. At -196°C, in liquid nitrogen, most lactic acid bacteria have an indefinite shelf life (Porubcan and Sellars, 1979) with the exception of Lactobacillus bulgaricus (Smittle et al., 1972). At temperatures less than -196°C the lactic streptococci tend to lose activity on prolonged storage (Gibson et al., 1965). There are conflicting reports as to whether or not lactic streptococcal species are affected differently by the method of freezing used prior to storage. Blaine (1972) has reported that S. cremoris is more susceptible to lyophilization than is S. lactis, while the reverse has also been reported (Sergeeva et al., 1980). The latter report found no difference between S. lactis and S. cremoris as regards proteolytic ability or acid production.

The ability of strains within the same species to withstand freeze injury has also been shown to vary. Strains of S. lactis, S. cremoris and L. bulgaricus have been shown to survive to different degrees during frozen storage (Cowman and Speck, 1965; Lamprech and Foster, 1963; Speckman et al., 1974; Gibson et al., 1965; Smittle et al., 1972). This variation is especially pronounced at temperatures higher than -196°C. In liquid nitrogen strains of all lactic cultures apart from L. bulgaricus survive equally well

(Keogh, 1970). Mutants of S. cremoris and S. lactis which have an increased ability to hydrolyse bitter polypeptides in cheese, have been shown to withstand freezing at -196°C better than the parent strains from which they were derived (Sergeeva et al., 1980).

As species and strains of lactic streptococci lose activity at different rates during storage at subzero temperatures in a liquid or freeze dried state, the storage of mixed or multiple strain starters, at optimum temperatures for all strains in the culture, is important. Failure to do so could lead to strain imbalance (Gilliland, 1971; Stadhouders et al., 1969; Leach and Sandine, 1976; Gibson et al., 1965). Imbalance in the frozen concentrate could be magnified on subsequent growth of the starter in the bulk tank and in the cheese vat, leading to failure of the starter if the dominant strain is attacked by phage. Undefined mixed strains may become dominated during growth before storage but frozen multiple strain starters are grown separately before being frozen together (Farr, 1969). Therefore, alteration of the culture and strain dominance is due to differential freezing survival not subculturing (Leach and Sandine, 1976). Liquid nitrogen freezing and storage is the best procedure for maintaining component balance in mixed lactic cultures (Reddy et al., 1974) with lyophilization better than freezing at -20°C.

Culture age and cell composition also affect survival during freezing; some reports indicate freezing of cells in liquid nitrogen or at -20°C results in better maintenance of activity if the culture is frozen in the exponential phase

of growth (Lamprech and Foster, 1963; Keogh, 1970). Others (Speckman *et al.*, 1974) maintain that 17 h cells, harvested from milk, withstand lyophilization and freezing better than cells harvested earlier. This is confirmed by Ray and Speck (1973).

Variations in strain ability to survive freezing despite similar growth, harvesting and freezing conditions led to the belief that differences in cell composition may be involved. As *L. bulgaricus* was known to be sensitive to freezing under liquid nitrogen, efforts were made to protect it by supplementing both the growth and freezing media (Smittle *et al.*, 1972). Only the addition of Tween 80 (polyoxyethylene sorbitan monooleate) to the growth medium improved the storage stability of *L. bulgaricus* cells. This led to the possibility of cell membrane involvement in cell protection during freezing (Mazur, 1970). Studies revealed that cells grown in the presence of Tween 80 contained higher levels of octadecanoic and cyclopropane non-adecanoic fatty acids and lower levels of saturated fatty acids than cells grown in its absence (Smittle *et al.*, 1974). There was a corelation between death and the amount of cyclopropane and saturated fatty acids present in the cell membrane.

Similar studies (Gilliland and Speck, 1974b) of the lactic streptococci reported considerable variations in the fatty acid composition of individual strains. None of the fatty acids were related to the resistance of cells to freezing apart from octadecanoic acid. As the cellular content of this fatty acid increased the percentage of survivors decreased. There was no relationship between levels

of cyclopropane and resistance to freezing possibly because levels of this fatty acid were much higher in lactic streptococci than in L. bulgaricus. While Goldberg (1977) confirmed the importance of adding Tween 80 to the growth medium, it was concluded that alterations in the overall composition of the membrane and the relative proportions of the fatty acids were the most important factors in bacterial viability. No single fatty acid had the major role in preventing cell death.

Other cellular components may also be involved in protecting the streptococci from damage during freezing. It has been shown that lactic acid bacteria produce capsular material in broth under controlled pH (Accolas and Auclair, 1967; Peebles et al., 1969). The higher the content of this polysaccharide material around a cell, the greater the degree of survival at -17°C.

Environmental conditions which possibly influence survival of lactic cultures during storage include growth medium composition, pH on freezing, temperature of growth, cell concentration and the use of cryoprotective agents in the freezing medium.

The medium used to prepare concentrates can have an effect on the viability of cells after freezing. A source of oleic acid allows L. bularicus to survive storage in liquid nitrogen (Smittle et al., 1974; Goldberg and Eschar, 1974). Cells grown in non-milk media, while being able to produce protective capsular material, may have impaired proteinase systems due to lack of casein in the medium. Inactivation of remaining proteinases during frozen storage

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may lead to impaired ability to initiate growth on addition to milk. In media containing excess lactose, even after maximum cell numbers have been attained, acid production will continue (Breheny et al., 1975). Freezing of large volumes of concentrate at temperatures other than -196°C is a slow process resulting in a drop in pH with resultant loss of activity (Stadhouders et al., 1969). The delay in freezing large volumes can be partially, not totally, eliminated by blast freezing.

The effect of pH on the survival of frozen cultures depends on the temperature at which the cells are stored. At -196°C there is no significant variance in activities due to differences in pH (Baumann and Reinbold, 1966; Stadhouders et al., 1969). Lactic streptococci survive both freezing and frozen storage better at neutral pH than at acid pH values (Lamprech and Foster, 1963; Baumann and Reinbold, 1966; Stadhouders et al., 1969). Neutralization of acid produced can be carried out anytime before freezing.

Work done with S. lactis seems to indicate that cells grown at 30°C can tolerate freezing much better than cells grown at lower temperatures (Baumann and Reinbold, 1966). This difference in freezing tolerance is more noted at -20°C than at -196°C. This may be an important factor in producing strain imbalance in mixed strain cultures especially those containing strains of Leuconostoc. Growth of cultures at the optimum temperature for Streptococcus species may result in reduced resistance of Leuconostoc species to freezing.

Cell concentration on freezing and the storage atmosphere also influence survival during frozen storage (Shahani et al.,

1974). The protective effect of cell concentration is due to leakage products from injured cells acting as cryoprotectants (Ray and Speck, 1973). Cultures stored at -20°C under nitrogen have greater activity than those maintained under CO_2, O_2 or vacuum (Bauman and Reinbold, 1966). The presence of CO_2 and O_2 could result in cellular compounds reacting biochemically, leading to cellular ageing and death. Storage in air, which contains mostly N_2, has less of an affect than has storage under CO_2 or O_2.

While it has been established that storage at -196°C is the best method of preserving lactic cultures (Peebles et al., 1969) problems also arise from the use of liquid nitrogen. Freezing of starter cultures other than concentrates is not very effective due to the small volumes that can be frozen. Preservation of cultures in liquid nitrogen necessitates the use of special equipment making storage and transport to the factory from the centralised production unit very expensive. Normally cultures are shipped in dry ice (-60°C) and upon arrival at the factory are stored in a mechanical deep freeze at temperatures between -10°C and -40°C.

The temperature of storage of frozen cultures can have a critical effect on subsequent culture performance. Storage at -17.8°C has an adverse effect on some lactic strains compared to storage at -23.3°C (Gibson et al., 1965). Starters frozen at -20°C lose activity rapidly with a low percentage of cells surviving 60 days storage (Cowman and Speck, 1965). Storage at -37°C to -40°C can be carried out without any substantial loss of activity for 6-8 months

Birkjaer et al., 1974; Stadhouders et al., 1969), with -196°C offering no special advantage over storage at -37°C when lactose is added as a cryoprotective agent. Other studies have confirmed that the lower the temperature of storage the greater the ability of the cells to withstand frozen storage (Bannikova and Lagoda, 1974; Efstathiou et al., 1975; Accolas and Auclair, 1967; Lloyd, 1975). However, in order to maintain viability and activity at temperatures higher than -40°C, compounds known as cryoprotective agents are added to the medium after growth and before freezing.

Cryoprotective agents can be divided into small and large molecular weight organic compounds which protect the cells from freeze injury by their presence both inside and outside the cell. Their exact mechanism of action is unknown, but most cryoprotectants contain -OH, $-NH_2$ and other groups which have a tendency to form hydrogen bonds within themselves and with water (Ray and Speck, 1973). These compounds provide protection for cells by interaction with both water and the hydrogen bond generating groups on the surface of the bacterial cell (Mazur, 1970). The resulting improved survival is probably due to the protection of the cell surface rather than protection of the cell interior. This is confirmed by the use of large molecular weight compounds, which are unable to diffuse into the cell, as useful cryoprotectants (Mazur, 1970).

The cryoprotective agents which have been assessed as regards their ability to protect frozen cultures include glycerol (Stadhouders et al., 1969; Baumann and Reinbold, 1966; Lamprech and Foster, 1963; Efstathiou et al., 1975;

Gibson et al., 1965; Accolas and Auclair, 1967), lactose (Stadhouders et al., 1969; Efstathiou et al., 1975) dimethyl sulphoxide (D.M.S.O.) (Stadhouders et al., 1969; Gibson et al., 1965) and yeast extract (Baumann and Reinbold, 1966; Efstathiou et al., 1975). Others which have been used include sugars (sucrose), amino acids (glutamic acid) and compounds of undefined composition such as yeast extract, egg albumin, apple juice and dextran.

Glycerol has been found to protect cells especially those stored at acid pH values (Lamprech and Foster, 1963). At neutral values glycerol is of no benefit for the storage of S. lactis, but it does protect aroma formers. This was confirmed by Efstathiou and coworkers (1975). Stadhouders (1969) found that glycerol had a variable effect depending on the mixed strain culture being tested even at storage temperatures of -37°C. He also questioned the legality of using glycerol as a cryoprotective agent as it is not a normal constituent of milk.

While S. diacetylactis is protected by glycerol, lactose, sucrose and L-malic acid do not provide protection for this aroma producer (Efstathiou et al., 1975). Other workers (Stadhouders et al., 1969; Gibson et al., 1965) have shown that lactose and malic acid are effective in protecting S. lactis, S. cremoris and S. diacetylactis but the degree of protection depends on the concentration used. Best results were obtained at -23.3°C using 2% L-malic acid and at -37°C using 7.5% lactose. The use of 7.5% lactose as a cryoprotectant results in retention of activity at levels equal to storage at -196°C (Stadhouders et al., 1969), a

fact of considerable economic significance.

Dimethylsulphoxide (D.M.S.O.) is also effective as a cryoprotective agent (Baumann and Reinbold, 1966; Gibson et al., 1965; Stadhouders et al., 1969) but is objectionable, imparting an unpleasant flavour to cultures even after one or two transfers (Baumann and Reinbold, 1966). It also delays acid production on subsequent thawing (Gibson et al., 1965). It would seem that careful selection of the cryoprotective agent is one of the most important considerations in ensuring optimum survival and strain balance of frozen lactic culture concentrates.

Freeze drying or lyophilization involves the removal of frozen aqueous material, making it suitable for the preservation of small amounts of starter bacteria. Early workers (Nichols and Hoyle, 1949; Czulak and Hammond, 1953) showed that cultures remained viable under freeze dried conditions but measured activity after several transfers, leaving the activty of the original dried preparation in doubt.

Freeze drying of lactic cultures is usually similar to the method described by Stadhouders (1969). After concentration and neutralization of the cultures a cryoprotective agent such as lactose is added. Ascorbate and glutamate can also be used in freeze drying of cultures (Porubcan and Sellars, 1975a). The concentrate is then frozen in a dry ice/acetone (alcohol) mixture at -65°C or in liquid nitrogen (-196°C). The frozen culture is then dried under vacuum to a moisture content of 1% in 24 h before being packaged as a powder in a vial under vacuum (Speckman et al.,1974) or in

aluminum foil sachets (Anon, 1983). Both moisture and light have a deleterious affect on lyophilized cultures (Porubcan and Sellars, 1979), necessitating their storage in moisture-proof, opaque containers.

There is considerable variation among lactic streptococcal strains in their ability to withstand freeze drying (Speckman et al., 1974; Blaine, 1972) with 40% loss of activity being reported (Stadhouders et al., 1969). One of the factors affecting the death of cells during lyophilization is the nature of the suspending medium. Blaine (1972) found that milk as the suspending fluid resulted in greatest activity loss compared with monosodium glutamate and trehalose. This compares favourably with the results of Lagoda (1970) and Speckman (1974). These compounds have been hypothesized to prevent loss of bound water thus preventing denaturation of cellular enzymes.

The lyophilized cultures can survive equally well at 22°C, refrigeration and subzero temperatures (Speckman et al., 1974; Blaine, 1972) over short time periods. Over longer time periods (greater than 3 months),the rehydration medium used is of importance in aiding the recovery of injured cells (Speckman et al., 1974; Tamime and Robertson, 1976). The temperature at which the cells are rehydrated is also critical. There is enhanced acid production if lyophilized cultures are incubated at 22°C instead of 30°C (Yang and Sandine, 1979). Other workers (Gilliland, 1981) maintain that both lyophilized cultures and conventional starter perform equally well at 30°C.

Lyophilized concentrates compared with liquid concent-

rates maintain high activity without special storage conditions giving a shelf life of up to 5 months (Anon, 1983) and are ready for inoculation into the bulk starter tank within an hour of rehydration. Recently, freeze dried concentrated cultures have been made available by Eurozyme which require no special storage conditions, can be kept at 10°C and retain activity for up to 3 months (Anon, 1983; Robinson, 1981). While these culture concentrates represent a fascinating advance in starter technology the main use for freeze dried cultures will be in the inoculation of bulk starter vessels where acid production has been found to be more consistent (Yang and Sandine, 1979) than at the higher temperatures associated with the cheese vat.

ADVANCES IN BULK STARTER TECHNOLOGY AND DIRECT VAT INOCULATION

Normal procedure for culture propagation in cheese factories up to recently was the successive transfer of culture from stock to the bulk tank stage, to build up a big enough inoculum for use in the cheese vat. This, however, was especially prone to bacterial contamination, strain imbalance due to repeated transfer, and, most importantly, bacteriophage attack.

Most starter cultures are supplied by commercial companies in the United States, Europe, Australia and New Zealand, and to a lesser extent by research and educational establishments. While the source of cultures has changed little over time, advances in the technology of growing, harvesting and storing cultures has led to a wide range of different

types of starter being commercially available (Anon, 1983). These are of two main types - freeze dried and liquid frozen - and both are suitable for inoculation of the bulk starter tank or the cheese vat itself.

The preparation of active bulk starter free from phage contamination is necessary as most starter strains used nowadays are phage sensitive due to their propagation in the absence of bacteriophage (Galesloot et al. 1966). The exceptions are mixed strain cultures used in Holland and Scotland (Galesloot et al., 1966; Crawford and Galloway, 1962). These cultures contain phage at levels that prevent the emergence of phage sensitive strains. In both cases, it is necessary to propagate cultures in an environment free from "disturbing" phage (Stadhouders and Leenders, 1983, 1984).

Phage control procedures both in the cheese vat and in the bulk starter vessel have been described (Lawrence and Pearce, 1972) and the role of the bulk starter tank in prevention of contamination of the starter by phage has been recognised (Lewis, 1977; Wigley, 1980; Robertson, 1966). Very low numbers of phage, surviving cleaning of the starter tank, pasteurizing of the medium or gaining access to the tank on starter inoculation, will completely lyse the susceptible host before bulk starter coagulation or if the starter coagulates and is used in the cheese vat, within a few hours of inoculation for cheese making (Robertson, 1966).

Most modern bulk starter tanks are stainless steel vessels with all inlets and outlets protected by High-Efficiency Particulate Air (HEPA) filters which allow less than 1 phage particle in 10^8 to penetrate. As most tanks

are steam sterilized and the medium is also sterilized in place, air inflow occurs during cooling. Care has to be taken that the air in the headspace is maintained at positive pressure during cooling to prevent unfiltered air entering via the hypochlorite seals (Wigley, 1980). Most media are heat treated to 90°C for greater than 30 minutes, a time considered adequate for phage destruction (Huggins, 1984; Pearce and Brice, 1973). This process is likely to be less effective and phage problems more widespread if the head space volume is unusually large (Robertson, 1966) a problem caused by under utilization of bulk tank capacity which is a common occurrence in many cheese factories (Walker et al., 1981; Pearce and Brice, 1973).

The inoculation of the bulk starter is also a critical step in the protection of the starter culture from phage. Most factories locate the starter production and cheese manufacture rooms in different areas to minimise the spread of phage via whey aerosols (Walker et al., 1981; Wigley, 1980). As low numbers of phage can cause starter failure, inoculation through a blanket of steam or a flame are not perfect in preventing culture contamination (Robertson, 1966). The technique which was most widely used in the 60's and 70's is known as the Lewis method (Lewis, 1956). This involves the use of polythene bottles, fitted with Astell Rubber Seals, containing the bulk tank inoculum. The culture is transferred to the bulk tank via a double-ended hypodermic needle. The seal is usually covered with a hypochlorite solution prior to inoculation. While this is a good method of inoculating liquid cultures,doubts have

been cast on its suitability for the inoculation of frozen concentrates (Walker et al., 1981; Daly, 1983a) which are not stored in containers suitable for use with the Lewis two-way needle. The increasing use of concentrates may lead to problems in phage control, especially in factories with poorly designed layouts and inadequate sanitation systems.

While all milk that is available has to be used in the cheese room, it is necessary to have standard conditions for bulk starter manufacture with medium of an acceptable quality available for use through out the season. For this reason most cheese factories use a top grade skim milk powder made from summer milk. The final density of the mix is usually 12 percent total solids. This medium supports good starter growth giving a high acidity and a high cell count, with maximum acidity of 0.6-0.7 percent lactic acid produced after 16-18 h at 22-23°C (Pearce and Brice, 1973; Lewis, 1977).

Two problems are encountered when milk is used in the preparation of bulk starter. The cell yield and subsequent activity may be limited by the increasing hydrogen ion concentration and if phage contamination occurs, subsequent proliferation will not be prevented.

The use of over ripe starter with developed lactic acid of 1.0% is a major problem in cheese factories especially if the starter is not used immediately (Pearce and Brice, 1973; Walker et al., 1981). Exposure of lactic streptococci to a pH below 5.0 results in a lag period of 30-60 minutes when tranferred to a higher pH (Harvey, 1965). The standardisation of cheese make times means loss of activity will

have to be offset by higher inoculum levels. Higher cell densities have been achieved by neutralization of coagulated bulk starter to pH 7.0 using NaOH (Limsowtin et al., 1980). Neutralization can be carried out as a one step or two step process leading to a doubling of viable cell counts. This system increases the starter capacity of the factory without the need for additional bulk tanks but phage replication will occur if contamination takes place during the neutralization process.

Alternative bulk starter systems have been developed which both increase cell numbers and prevent phage proliferation should accidental contamination occur. Most phage require calcium for growth (Reiter, 1956) and the removal or binding of these ions by phosphates prevents phage infection (Kadis and Babel, 1962; Hargrove et al., 1961). These findings led to the development of phage inhibitory media (PIM) which contain milk solids, growth stimulatory factors, phosphates and citrates (Henning et al., 1965; Christensen, 1969). While these media have been widely used in the United States, Canada and Europe, alone, or in combination with skim milk, doubts have arisen as to their effectiveness in protecting and stimulating the growth of starters. Some reports (Sozzi,1972) indicate that phage can still destroy starter in the absence of calcium. Leuconostoc species do not grow well in PIM (Henning et al., 1965) while phosphates have been found to reduce proteinase activity in some starter cultures (Ledford and Speck, 1979).

Of the 6-8 media available commercially the majority were found to be ineffective in their ability to suppress

phage or were unable to support good growth of cultures (Gulstrom et al., 1979). It was concluded that the ability to support good bacterial growth, while at the same time suppressing phage multiplication,were mutually exclusive properties, of any phage inhibitory media. As a phosphate salt concentration of at least 1.6% is required to protect cultures from phage (Zottola and Marth, 1966), work has been done to try and lower this concentration. Ausavanodom and coworkers (1977) combined continuous pH control between 6.0 and 6.2 with reduced phosphate levels (less than 1.0 percent) in a whey based medium. When used as a bulk starter, it resulted in improved bacterial growth and it had better phage inhibitory properties than previously used phage inhibitory media.

This system has been used in many factories in the United States for bulk starter preparation and can be used to produce both mesophilic and thermophilic cultures (Richardson et al., 1977, 1981; Reddy and Richardson, 1977). The culture produced contains 2-5 times more active cells when compared with a conventional non pH controlled culture and it performs better, with no pronounced lag phase, in the cheese vat, due to the pH of the culture (6.0-6.2) being close to that of milk (6.6). The whey used as a base is diluted with water to a certain level giving a limited lactose concentration in the medium. As all the lactose is used during culture growth the culture may be held at 4°C - 13°C for up to 7 days without significant loss of activity and at 27°C for up to 24 h (Richardson et al., 1979). While strain dominance can occur, the problem can be minimised through

the inoculation of the medium with single strains in known proportions (Hansen and Richardson, 1982) or the strains can be propagated separately (Reddy and Richardson, 1977).

The costs incurred by using pH control equipment are offset by reduced inoculum requirements and a more active culture. Also, phage inhibitory media, if used, account for 70% of the cost of producing bulk cultures. Whey, a bye product of cheese manufacture, if used as a growth medium with the addition of supplements could reduce bulk starter cost by up to 50 percent (Jonas et al., 1977). This is in addition to savings made by lower inoculum levels and slower bulk starter tank capacity.

The necessity of using complicated pH control equipment has led to the introduction of internal pH controlled media (Sandine and Ayres, 1982; Wright and Klaenhammer, 1983) for use in the bulk starter tank. These media have built in control of acidity, with buffer systems which maintain the pH high enough to promote growth without acid injury. The first medium known as "Phase 4" is whey based and contains an initially insoluble buffer system composed of ammonium, alkali metal and trimagnesium phosphates. The buffering ingredients are gradually released as the pH drops to 5.2. At this pH enough buffer is released to prevent the pH going any lower while the rate at which the buffer is released is dependent on the rate of acid production. Thus the starter bacteria are not exposed to levels of buffer or acid that cause injury.

"Phase 4" produces 4-8 times more cells than conventional media, which are more active in the cheese vat. This results in lower inoculum levels, faster cheese make times and

increased cheese yields (Mermelstein, 1982). The necessity for daily bulk starter preparation is also avoidable using "Phase 4" as it can be held for up to 3 days at 4°C without any loss of activity (Birlison and Stanley, 1982). While most phage inhibitory media slow down the growth of phage, "Phase 4" is effective even against non calcium requiring phage (Sandine et al., 1980; Mermelstein, 1982), causing a decrease in phage levels in the starter. Thus the starter will perform to a certain extent in the cheese vat, even if contaminated by phage. "Phase 4" has been used satisfactorily in Cheddar and cottage cheese as well as cultured buttermilk manufacture (Sandine et al., 1980; Birlison and Stanley, 1982) with no adverse affects on the final product. This medium can also be used for growth of different strains together without strain dominance occurring (Khosravi et al., 1984) and cultures can be stored at -40°C in Phase 4" without added cryoprotectants for up to 3 months (Thunnel and Sandine, 1984).

Another internal pH controlled whey based medium has been developed (Wright and Klaenhammer, 1983) which allows comparable growth with other phage inhibitory media giving up to 10 fold increases in cell mass over that produced in milk. The buffer system absorbs 8 times the acid of milk and consists of sodium and ammonium phosphates and citrates as well as magnesium hydroxide.

Advances in bulk starter production and use of phage inhibitory media may be rendered obsolete by the tenency of factories to use Direct Vat Inoculation (DVI) systems in cheese manufacture. DVI systems are only a logical conclusion

to the continual process of culture development as predicted during the development of the multiple starter concept (Limsowtin et al., 1977). The first DVI cultures were freeze dried concentrates, containing at least 5 x 10^{11} c.f.u./g of product, produced by Eurozyme (Chapman, 1978). These were quickly followed by the liquid concentrates known as Direct Vat Set (DVS) and "superstart" produced by Chr Hansens Laboratory Ltd., and Miles Laboratories Ltd., respectively.

The DVS cultures are produced in the form of deep frozen pellets containing a viable count per gram of 1.2 x 10^{11} cells. Superstart cultures contain at least 10^{11} cells per ml of concentrate, which has to be thawed before addition to the cheese vat.

The use of DVI systems is more advantageous than propagation of bulk culture. The system has greater demands placed on it as culture activity has to be predictable irrespective of variations in milk composition and the end product must be comparable to that obtained with the starter system already in use. Acid production by DVI cultures in the cheese vat is comparable with normal bulk starter with no lag phase observed (Chapman, 1978; Robinson, 1981). Cheese make times using freeze dried cultures are slightly longer than for liquid bulk starter but are still commercially acceptable. Similar results have been reported using "Superstart" cultures (Wigley, 1980). This could be corrected by varying the inoculation rate and increasing the temperature of the milk at renneting from 30°C to 32-33°C.

While both DVI systems and bulk starter systems produce

cheese of comparable quality both systems are equally expensive as regards cost per ton of cheese produced (Wigley, 1980), with DVI being used in new factories which don't favour the investment of large amounts of capital in bulk starter equipment. In older factories bulk starter preparation will be retained unless DVI costs decrease and products which can be manufactured in bulk tanks increase in demand.

The developemt of starter culture production has moved further away from the cheese factory with the advancement of technology. The use of defined strain systems will lead to a uniform product with local variations being dependent on manufacturing practices, not on the cultures. The culture success will depend on cell stability, which biological systems subject to mutation find difficult to maintain, unless totally removed from the environment, something that cannot be directly achieved in cheese manufacture, without affecting product quality.

MATERIALS

REAGENTS

All chemicals used were Analar Analytical Reagent Grade obtained from Hopkin and Williams, Chadwell Heath, Essex, England, Sigma Chemical Company, London, England or BDH Chemicals Ltd., Poole, England.

Bacteriological media components were obtained from Oxoid Ltd., London.

GROWTH MEDIA

Milk:

Antibiotic free, dried skim milk powder was obtained from Carbery Milk Products, Ballineen, Co.Cork. It was reconstituted to the desired solids concentration using distilled water and sterilised by autoclaving at 10 p.s.i. for 10 minutes.

Lactose Streptococcal Broth (L.S.B.):

This medium was similar to the M17 medium of Terzaghi and Sandine (1975) and was composed of :

Lactose	10.0g
Tryptone	5.0g
Bacteriological peptone	5.0g
Yeast extract	5.0g
Lab-lemco	10.0g
B-glycerophosphate	7.2g
Distilled water	1000ml

components were dissolved and sterilised at 15 p.s.i. for 15 minutes.

For the preparation of agar plates, 15g of agar was

added to 1000ml of broth, autoclaved and dispensed in 15ml quantities into sterile disposable petri dishes. Plates not used immediately were held at 4°C.

Sloppy agar overlays were prepared by the addition of 0.7g of agar to 100ml of broth and dissolved by boiling. This medium was dispensed in 2.5ml quantities into test tubes and autoclaved at 15 p.s.i. for 15 minutes.

Elliker Broth:

This medium consisted of:

Tryptone	20.0g
Yeast extract	5.0g
Gelatin	2.5g
Glucose	5.0g
Lactose	5.0g
Sucrose	5.0g
NaCl	4.0g
Na Acetate	2.4g
Ascorbic acid	0.5g
Distilled water	1000ml

components were dissolved and sterilised in 5ml volumes in test tubes at 15 p.s.i. for 15 minutes.

Phage Inhibitory Medium (Phase 4):

"Phase 4" medium in powdered form was obtained from W.E. Sandine, Oregon State University, U.S.A. 93g were added to 1000ml of distilled water which had been preheated to 45°C. The temperature was then raised to 85°C ± 2°C where it was kept for 45 minutes, stirring continuously. The "Phase 4" was then transferred to a sterile flask and was

held at 4°C before use.

Fast Slow Differential Agar (F.S.D.A.):

F.S.D.A. was prepared as two separate components.

Component A consisted of:

B-glycerophosphate	19.0g
Agar	10.0g
Bromo-cresol purple soln.	5ml
Distilled water	550ml

Bromocresol purple solution consisted of 1g of powder in 25ml of distilled water.

Component A was autoclaved at 15 p.s.i. for 15 minutes and cooled to 55°C in a water bath.

Component B consisted of:

Skim milk powder	100.0g
Distilled water	400ml

Component B was sterilised by autoclaving at 10 p.s.i. for 10 minutes and quickly cooled to 55°C.

Both components were mixed together by swirling and 15ml volumes were poured into sterile disposable petri dishes. Before solidifying the medium was briefly flamed to eliminate air bubbles. Plates were inverted and allowed to dry for two days at room temperature before use.

Ringers Solution:

Quarter strength ringers salt solution was prepared by the addition of two ringers tablets to 1000ml of distilled water. It was distributed in the required volumes and sterilised by autoclaving at 15 p.s.i. for 15 minutes.

Fermentation Medium:

The fermentation medium was milk based, containing:

Skim milk powder	25.0g
Yeast extract	4.0g
Distilled water	1000ml

The medium was brought to the required pH using concentrated sulphuric acid and distributed in 800ml volumes before sterilisation by autocaving at 10 p.s.i. for 10 minutes, before each fermentation run.

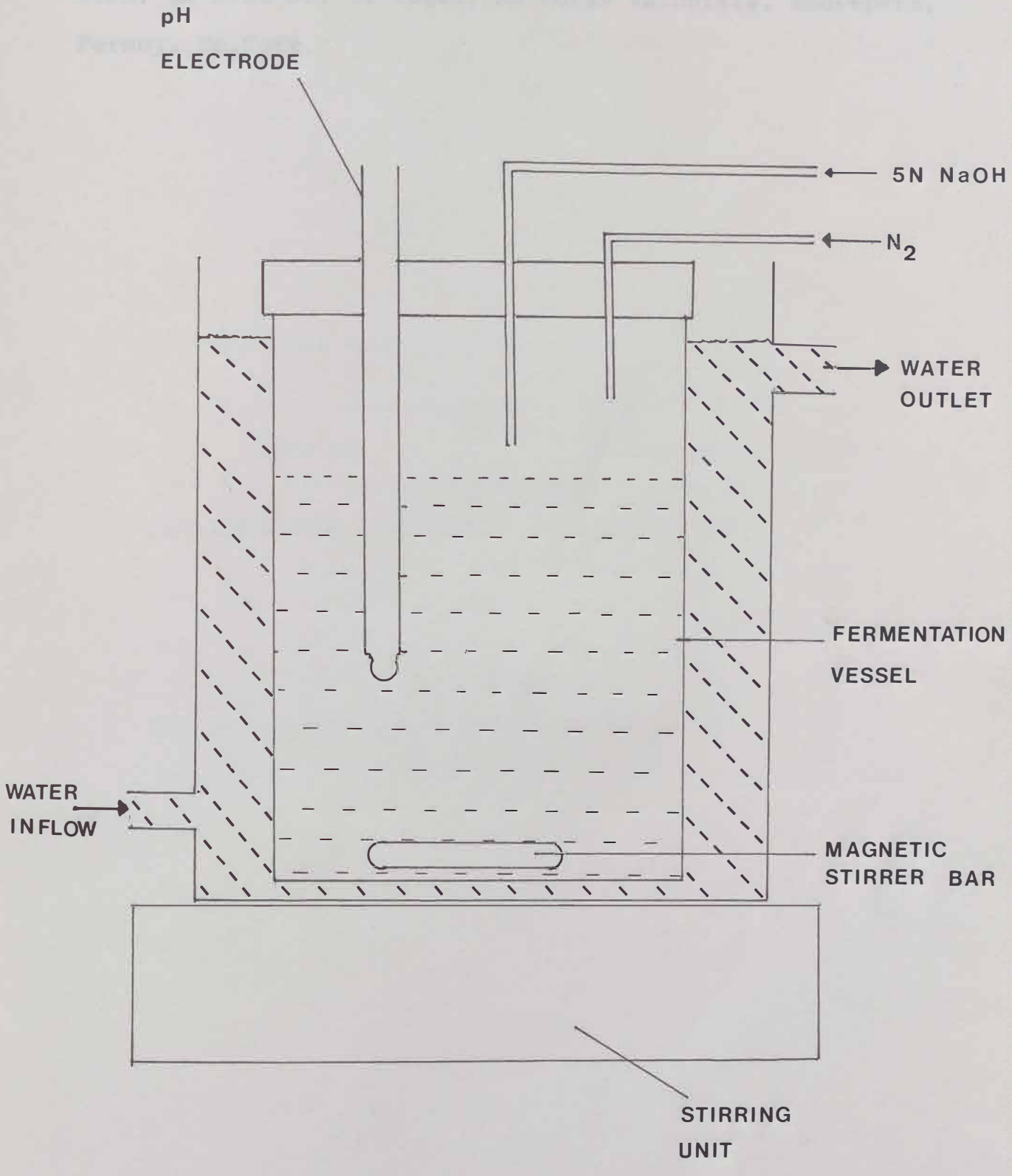
FERMENTATION APPARATUS

The culture vessel was cylindrical of depth 15cm and diameter 11cm giving a working volume of 1 litre, approximately. The screw-on cap housed the inlets for the controls used (Fig. 1). The vessel was heated to the required temperature by water pumped from a thermostatically controlled water bath, to a container in which the fermentation vessel was placed.

The pH of the fermentation was controlled with a combined reference-glass electrode (Radiometer, Copenhagen) to within 0.05 pH units. The pH electrode was attached to an automatic titrator (Radiometer, Copenhagen) which activated an Autoburette (Radiometer, Copenhagen) supplying neutraliser to the fermentation vessel as required. Agitation of the fermentation to disperse neutraliser was done using a magnetic stirring unit.

The pH electrode was sterilised by soaking in 1N HCl overnight. Before use it was washed with sterile buffer pH 7.0. Anaerobic conditions were maintained by using a head of N_2 over the fermentation medium.

Fig. 1. FERMENTATION APPARATUS



BACTERIAL STRAINS

The bacterial cultures studied are listed in Table 1. All cultures were obtained from the Department of Dairy and Food Microbiology culture collection, University College, Cork, or from Dr. T. Cogan, An Foras Taluntais, Moorepark, Fermoy, Co.Cork.

Table 1.

Bacterial strains used in this study.

Species	Strain designation		
Streptococcus cremoris	x	047	107
	091	6	108
	310	320	320/1
Streptococcus lactis	007	021	

METHODS

PREPARATION OF STOCK CULTURES

Each strain was maintained in 10% reconstituted skim milk (R.S.M.). For this purpose 0.3ml of an active culture (in milk) was added to 3.0ml of sterile milk and stored in a frozen state at -20°C or -80°C.

MAINTENANCE OF WORKING CULTURES

Working cultures were activated by transferring twice at 21°C x 16 hours, a 1% inoculum in sterile R.S.M. The clotted culture was transferred at a 1% level to 10ml volumes of R.S.M. and held unincubated at 4°C until needed. This procedure was repeated every 10 days.

GROWTH UNDER pH-CONTROL

A 2% inoculum was prepared from an active culture grown at 21°C x 16 hours in 10% R.S.M. Growth curves were determined by calculating:

1. The percentage Developed Lactic Acid (% D.L.A.).
2. Cell concentration as measured by colony forming units/ml.

The % D.L.A. was calculated as:

$$\frac{\text{Normality of neutraliser x No. ml added}}{\text{Volume of fermentation}} \times 100$$

To determine cell concentration, samples were removed aseptically at specified intervals. Two ml of sample were diluted in 200ml of ice-cold sterile quarter strength Ringers solution. Samples were blended for 30 seconds using a Waring blender and serially diluted before plating on Lactic Streptococcal Agar plates. The plates were incubated aerobically or anaerobically using the Oxoid Gas Generating Kit, for 48 hours at 30°C.

HARVESTING AND STORAGE OF CULTURES

The culture was harvested within 1-2 hours of reaching stationary phase. The temperature of the fermenter vessel was quickly brought to 4°C, while still under pH control. Each grown culture was divided into 200ml volumes, to which various cryoprotective agents were added. Cultures were mixed with glycerol (80% v/v), lactose (7% w/v) and quarter strength Ringers solution to give final volumes of 40%, 7% and 7%, respectively. Some cultures had no added cryo-protective agents.

Each 200ml volume was then divided into 10ml portions and stored at 4°C, -20°C and -80°C. Some culture samples were initially quick frozen in less than 15 minutes at -80°C before transfer to long term storage at -20°C. Storage time varied from 6 to 8 months depending on the culture. For control purposes activity tests were carried out on samples before freezing.

TESTS ON STORED CULTURE SAMPLES

Cultures were sampled at times indicated in the results section for viable cell counts and activity. Each 10ml volume stored frozen was thawed for 15 minutes at 30°C, then held at 4°C, until the following tests were completed.

1. Cell counts
2. Immediate activity test at 30°C x 6 hours
3. Overnight activity test at 21°C x 16 hours
4. Activity test at 30°C x 6 hours on cultures grown overnight at 21°C.

1. Cell count:

Two ml samples were added to 200ml volumes of quarter

strength Ringers solution and blended for 30 seconds in a Waring blender, before serial dilution in Ringers solution and plating on LSB agar plates. Plates were incubated aerobically or anaerobically at 30°C for 48 hours, after which colony forming units per ml of original sample were calculated.

2. Immediate activity test at 30°C:

Tubes of R.S.M. (10ml) were inoculated at a 1% level, taking into account the dilution effect of the cryoprotective agents, and incubated at 30°C for 6 hours. At the end of incubation, the tubes were placed on ice until they were titrated against N/9 NaOH to the phenolpthalein end-point of pH 8.3. Results were expressed as % Developed Lactic Acid compared to an uninoculated control.

3. Overnight activity test:

This test was carried out at 21°C on a 10ml volume of R.S.M. inoculated at a 1% level. After incubation for 14 hours, pH values were determined and Δ pH was calculated compared to an uninoculated control.

4. Activity test on cultures grown at 21°C:

A 1% inoculum of each sample which had previously been incubated at 21°C for 16 hours was added to tubes with 10% R.S.M. (10ml) and incubated at 30°C for 6 hours. This test was carried out on samples regardless of whether or not they had clotted the milk at 21°C. Results were expressed as % Developed Lactic Acid compared to an uninoculated control.

In all activity tests results obtained with stored

cultures were compared with ordinary fully active milk cultures.

GROWTH MEASUREMENT IN NON MILK MEDIA

Growth in broth media was measured as an increase in optical density of the culture at 600nm (OD_{600}) using a Cecil Instruments C.E. 343 Single Sample Spectrophotometer.

Growth in "Phase 4" phage inhibitory medium was measured as % Developed Lactic Acid compared to an uninoculated control.

HYDROGEN PEROXIDE MEASUREMENT

Hydrogen peroxide accumulation by growing cultures was measured by adding excess catalase to 3ml samples in the chamber of a Ranks Bros. oxygen analyser and recording any immediate increase in dissolved oxygen. The oxygen electrode was calibrated to give a reading of 21% oxygen in air. The chamber contents were maintained at 30°C and were continuously stirred using a magnetic stirrer.

Hydrogen peroxide concentrations were calculated by comparison with the amount of dissolved oxygen produced from known concentrations of hydrogen peroxide on catalase addition.

BACTERIOPHAGE DETECTION DURING FERMENTATION

Samples were collected at specified intervals and centrifuged at 5000 x g for 10 minutes to remove milk solids and bacteria. Residual bacteria were removed by filtering through a 0.45 micron cellulose acetate membrane. The presence of phage active against the strain in the fermenter

was determined using an activity test at 30°C in 10% R.S.M. and by plaquing on the culture itself.

In the activity test, the acid producing ability of the culture in sterile milk was compared with the same culture grown in milk with added fermenter filtrate. The % Developed Lactic Acid was measured after 6 hours growth at 30°C.

Fermenter filtrates which showed inhibition of culture activity were plaqued on the starter cultures using the soft agar overlay method of Adams (1959). The following additions were made to 2.5ml of L.S.B. agar overlays held at 45°C:

0.1ml of 0.185M $CaCl_2$ giving a final concentration of 5mM Ca^{++}.

0.1ml of an 14-16 hours broth grown, host culture.

1.0ml of the filtrate, diluted to give isolated plaques.

The overlays after gentle mixing were poured onto L.S.B. agar plates and incubated at 30°C for 14-16 hours in an upright position.

BACTERIOPHAGE STOCK PREPARATION

Fermenter filtrates and factory wheys were plaqued on their homologous hosts.Single plaques were picked using a sterile Pasteur pipette into 1ml of L.S.B. containing 5mM $CaCl_2$. The phage was resuspended in the medium by vortexing for 2 minutes, 0.1ml of an active host was then added and left to lyse at 30°C. Then 1ml L.S.B. and 0.2ml culture were added to allow further lysis. The contents were then filtered to remove any bacterial debris and stored at 4°C.

When large volumes of phage were required, the host culture was inoculated (5%) into L.S.B. and incubated at 30°C

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for 1-1½ hours. Then 0.6ml $CaCl_2$ and 0.1ml of plaque purified phage were added. Incubation was continued until lysis was complete. The lysate was then filtered and stored at 4°C until needed.

EFFECT OF INDUCING AGENTS ON SELECTED STRAINS OF LACTIC STREPTOCOCCI

The effects of both Mitomycin C (M.C.) and Ultraviolet light (U.V.) were examined.

Mitomycin C. (M.C.)

Cells were grown in M17 broth at 30°C and 37°C to an OD_{600} of 0.1 approximately. The culture was then treated with M.C. of final concentration 4ug/ml or 10ug/ml. Incubation was continued, turbidity monitored and compared to a control culture without any added M.C.

Ultraviolet Light (U.V.)

Cells were inoculated (1%) into M17 broth and incubated at 30°C for 3-4 hours, reaching an OD_{600} of 0.3 to 0.5, approximately. The cells were then harvested by centrifuging at 5000 r.p.m. for 10 minutes. The pellet was resuspended in 5ml 0.1M $MgSO_4$, transferred to a sterile disposable Petri dish and irradiated for 30 seconds with constant swirling. Irradiated cells were added at a 1% level to fresh M17 broth, incubation was continued and growth was followed by measuring the OD_{600} at hourly intervals.

After treatment with the inducing agent and subsequent incubation, the cultures were filter sterilised following centrifugation at 5000 r.p.m. for 10 minutes.

Filtrates were checked for the presence of phage by

plating on the homologous host using the soft agar overlay method of Adams (1959).

PROCEDURE FOR PLASMID ISOLATION

The plasmid profiles of selected strains were examined using the procedure of W de Vos (personal communication) based on rapid alkaline extraction method of Birnboim and Doly (1979).

1. An active overnight culture was inoculated (10%) into 5ml of Elliker broth and grown at 30°C for 2-3 hours.
2. 1ml of culture was centrifuged using on Eppendorf centrifuge for 2 minutes.
3. The supernatant was discarded and the pellet was re-suspended in 100ul of the following solution (20mM Tris. HCl, 100mM NaCl, 5mM EDTA, 20% w/v sucrose pH 8.0).
4. 100ul of ice cold lysozyme solution (5mg/ml) was added, the suspension mixed gently and incubated at 37°C for 5-10 minutes.
5. 200ul of SDS-NaOH (1% SDS, 0.2N NaOH) was added, mixed by inversion of the tube and placed on ice for 5 minutes.
6. 150ul of ice cold Na Acetate (3M) pH 4.8 was added, the contents were mixed by inversion and kept on ice for an additional 5 minutes.
7. The precipitate was removed by centrifugation for 3 minutes.
8. The supernatant was removed and put into a fresh tube on ice.
9. To fill the Eppendorf tube approximately 1ml of cold (-20°C) ethanol (95%) was added and mixed with the supernatant by inversion, before leaving at room

temperature for 2 minutes.

10. Precipitated nucleic acids were collected by centrifugation for 5 minutes, followed by removal of all residual alcohol.
11. The pellet was dissolved in 35µl of TES buffer (50mM Tris.HCl, 5mM EDTA, 50mM NaCl pH 7.4) and 5µl of RNA-ase (100µg/ml) and incubated at 37°C for 10-15 minutes.
12. Electrophoresis was carried out using a 1% agarose vertical gel. The electrophoresis running buffer was a Tris-acetate buffer containing 40mM Tris.HCl, 5mM Na-acetate, 1mM EDTA and 15ml glacial acetic acid, pH 7.9. Tracker dye (15µl) was added to the sample before loading on the gel.

Electrophoresis was carried out at 100 volts for 3 to 4 hours. Staining was done in an ethidium bromide (5µg/ml) solution for 15 minutes. The gel was then illuminated by a shortwave (254nm) transilluminator and photographed using a Polaroid MP-4 camera.

SINGLE COLONY ISOLATION OF SELECTED STRAINS OF LACTIC STREPTOCOCCI

Single colony isolates were obtained by two methods:
(i) Freshly coagulated milk cultures were diluted 100-fold in $\frac{1}{4}$ strength Ringers solution. The culture was then blended for 30 seconds using a Waring stainless steel blender, further serially diluted and 0.1ml of the appropriate dilutions was spread plated on Fast-Slow Differential Agar (F.S.D.A.). These plates were incubated anaerobically for 48 hours at 30°C.
(ii) Coagulated milk cultures were blended and diluted as previously described. Appropriate dilutions were plated on

L.S.B. agar and incubated anaerobically at 30°C for 48 h. Single colony isolates were picked at random and sequentially restreaked, on L.S.B. agar plates, up to ten times.

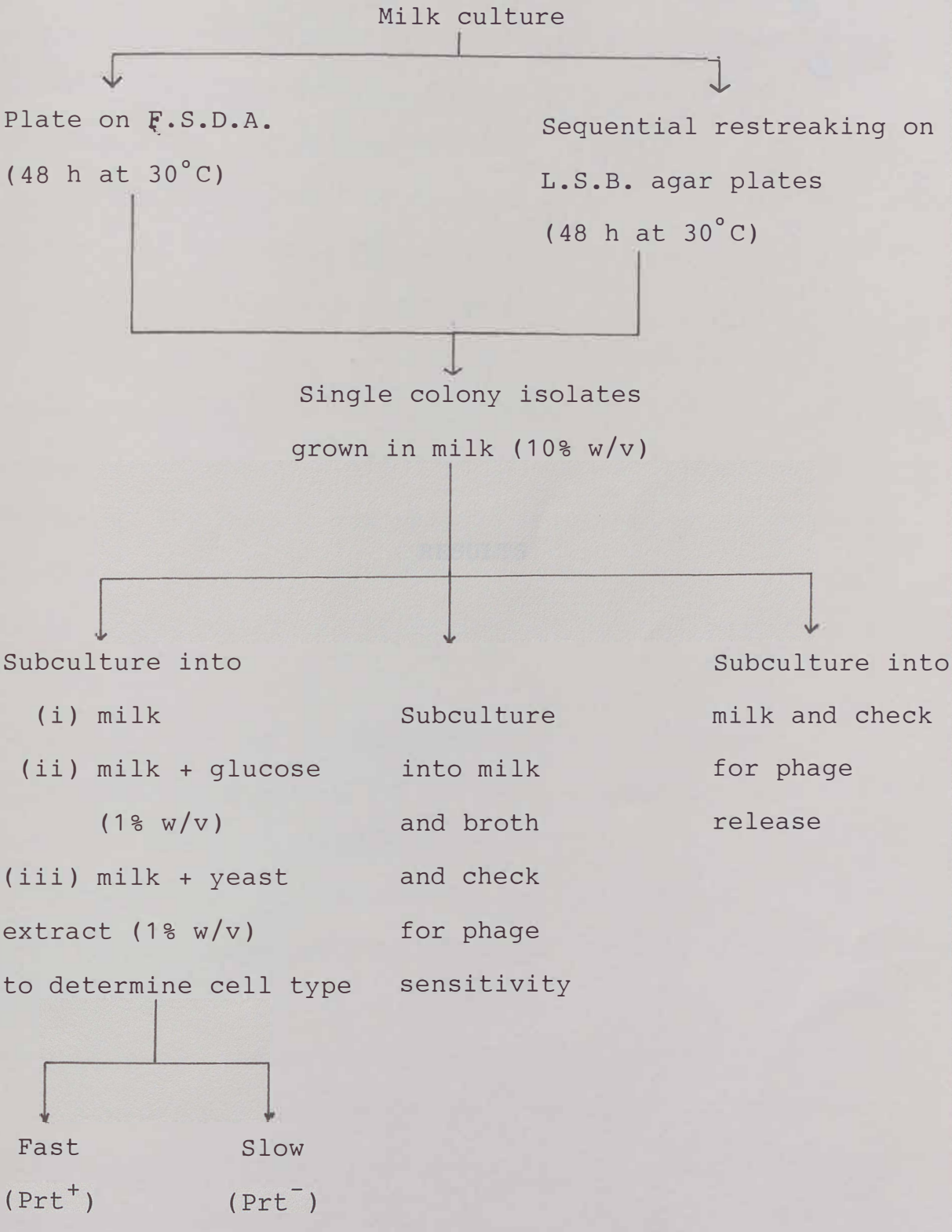
Isolates from both L.S.B. and FSDA agar plates were then picked and transferred to milk. After incubation and coagulation, the isolates were checked as shown in Fig. 2.

REPETITION OF EXPERIMENTS

In general experiments relating to growth performance and storage of individual strains were repeated at least twice.

Fig. 2.

Outline of procedure used to obtain single colony isolates of selected lactic streptococcal strains.



RESULTS

Examination of Growth of Selected Streptococcus cremoris and Streptococcus lactis Strains in a Low Lactose (2.5% milk solids) Medium Under pH Control.

The suitability of a low lactose (2.5% skim milk solids) medium with added yeast extract to grow strains of lactic streptococci was examined. The milk solids concentration was chosen on the recommendation of Dr. T. Cogan, An Foras Taluntais, Moorepark, Fermoy, Co. Cork. Also, the low lactose content was desirable to avoid continued acid production in the culture once growth had ceased.

Each strain was grown at 21°C for 16 h in 10% Reconstituted Skim Milk (R.S.M.) and inoculated (2%) into the growth medium which was maintained at 30°C under anaerobic conditions. The pH of the medium was maintained at 6.5 using 5N NaOH. Growth was measured,by titratable acidity and viable cell counts under anaerobic conditions at 30°C,at hourly intervals.

Growth performance of Streptococcus lactis 021 and Streptococcus cremoris 310 in media containing 0.4% and 0.8% yeast extract as growth stimulants.

In preliminary experiments the suitability of 0.4 and 0.8% yeast extract as supplement to the 2.5% skim milk solids medium, for the growth of lactic streptococci was examined. S. lactis 021 and S. cremoris 310 were used as test strains.

The overall conclusion was that there was no major difference on either culture due to the yeast extract concentration used (Figs. 3 and 4). Some minor differences were noted for each strain.

In the case of S. lactis 021 there was a slight lag of

Fig. 3.

Growth of Streptococcus lactis 021 as measured by colony forming units per ml (c.f.u./ml) in 2.5% Milk Solids Medium under pH-control with 0.4% (▲——▲) or 0.8% (△——△) added Yeast Extract.

Fig. 3.

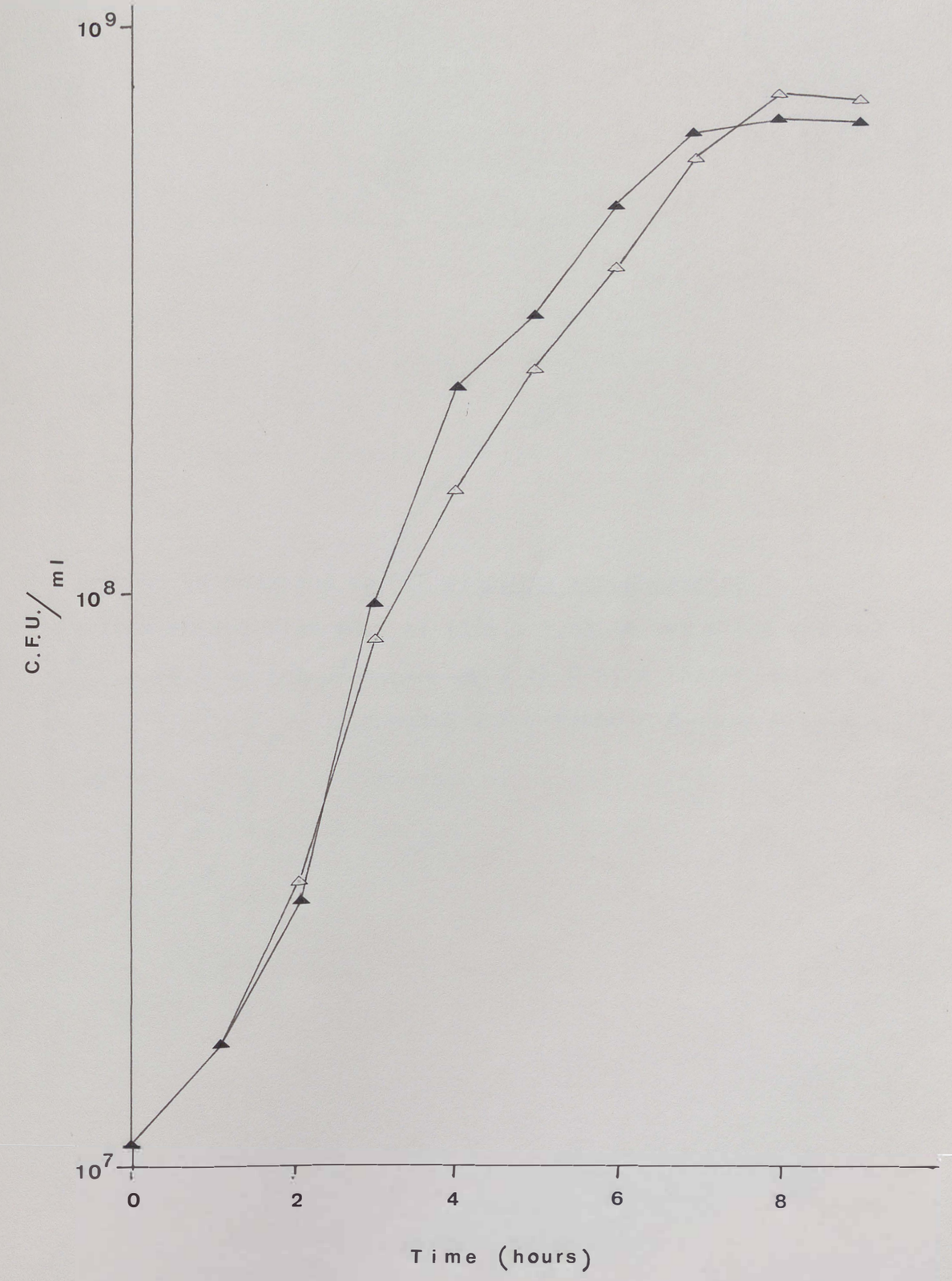
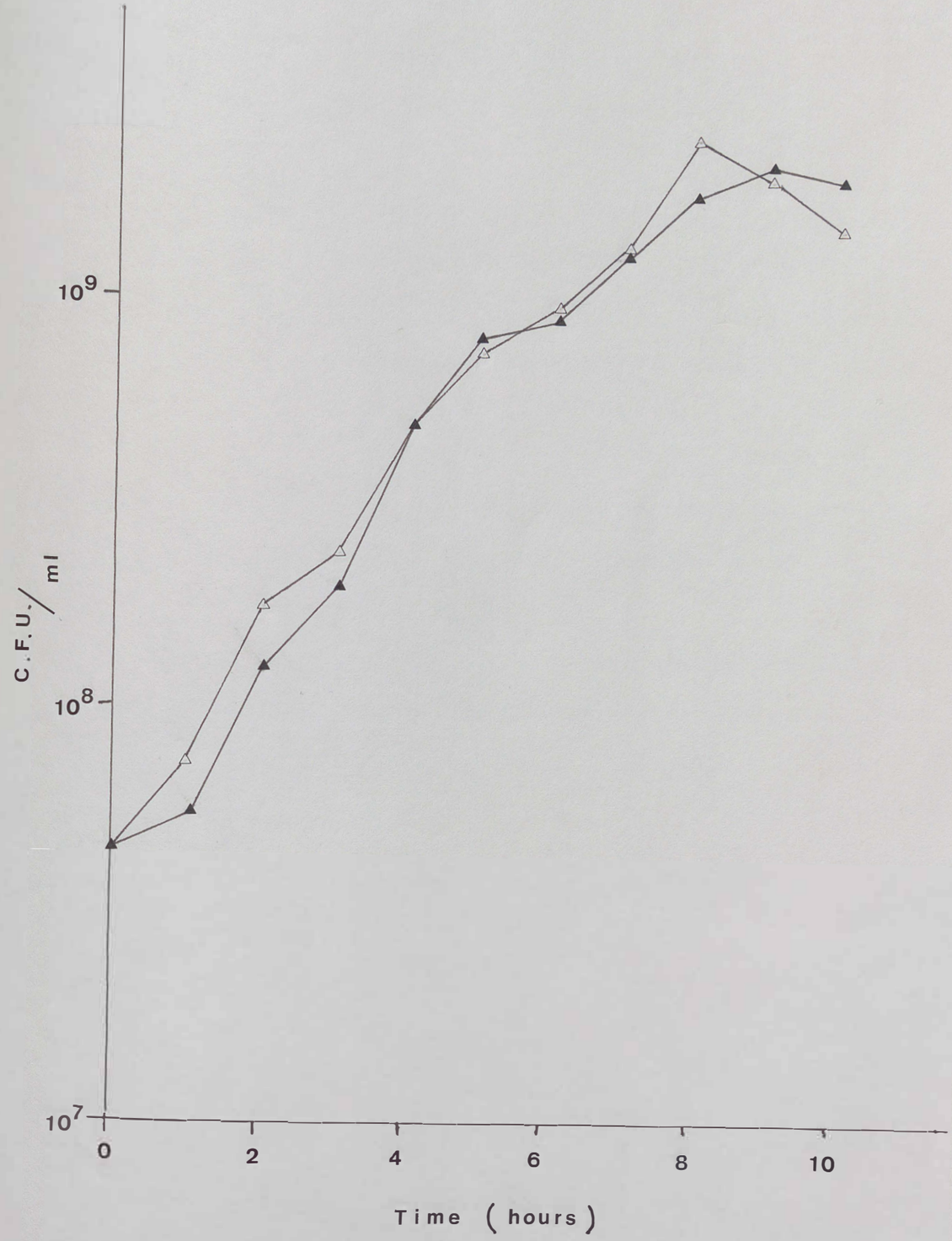


Fig. 4.

Growth of Streptococcus cremoris 310 as measured by colony forming units per ml (c.f.u./ml) in 2.5% Milk Solids Medium under pH-control with 0.4% (▲——▲) or 0.8% (△——△) added Yeast Extract.

Fig. 4.



half an hour as measured by titratable acidity in the medium containing 0.8% yeast extract (Fig. 5), even though inoculum levels were similar (Figs. 3 and 5). The overall growth rate and final cell numbers (7-8 x 10^8 c.f.u./ml) were the same in both media although the stationary phase was entered one hour earlier in the medium containing 0.4% yeast extract.

In the case of S. cremoris 310 growth was the same in both media (Fig. 6). While the maximum cell numbers reached were slightly higher in 0.8% yeast extract, there was a slight decline in viability on continued incubation (Fig. 4).

Acid production in both media for both S. lactis 021 and S. cremoris 310 was the same with final levels on entering stationary phase being 1.60 and 1.69% lactic acid for the respective cultures (Figs. 5 and 6).

On the basis of these results it was decided to use 0.4% yeast extract as an additive in all further growth experiments, involving strains of S. lactis and S. cremoris.

Fig. 5.

Fig. 5.

Growth of Streptococcus lactis 021 as measured by % Developed Lactic Acid (D.L.A.) in 2.5% Milk Solids Medium under pH-control with 0.4% (▲——▲) or 0.8% (△——△) added Yeast Extract.

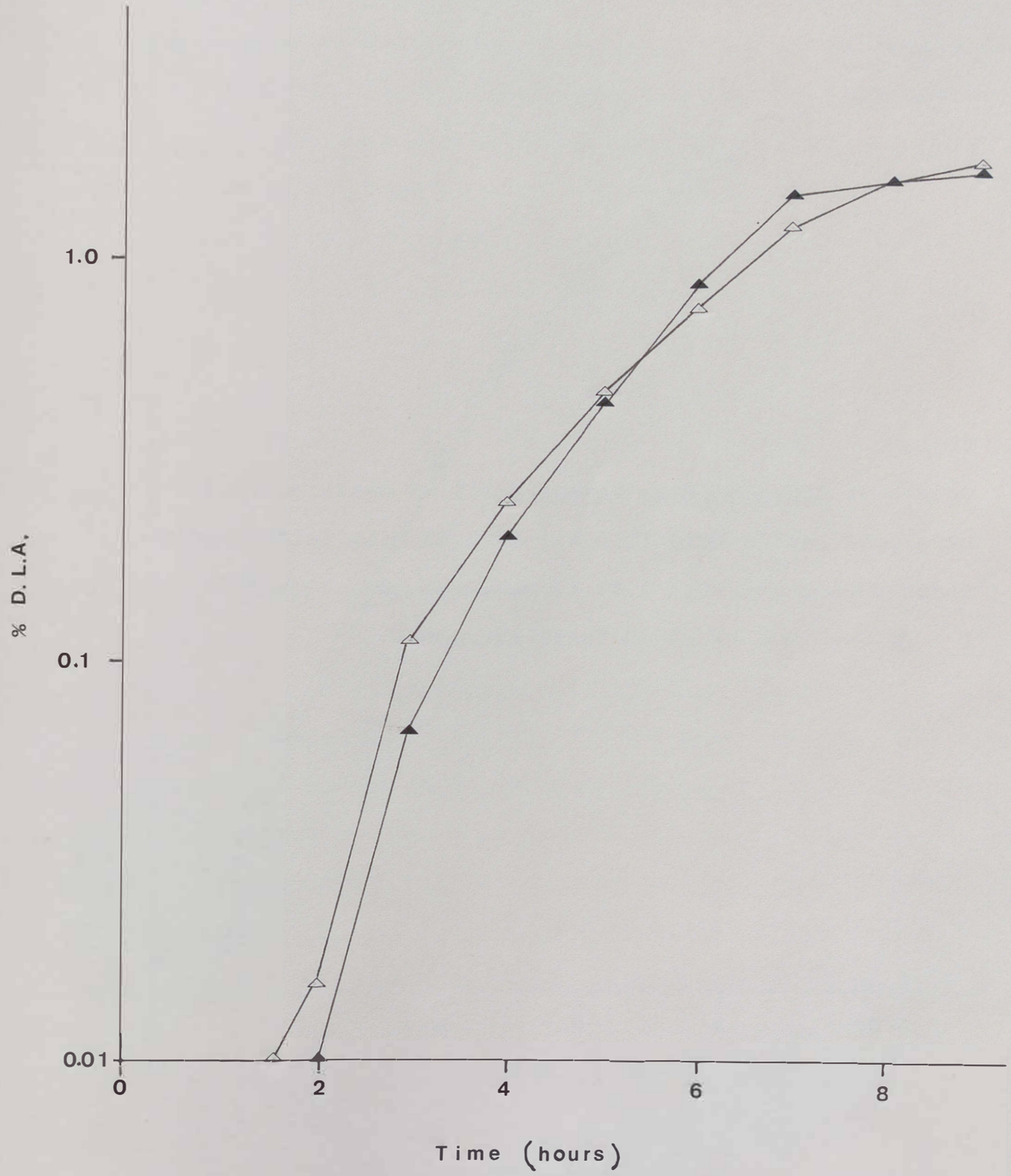
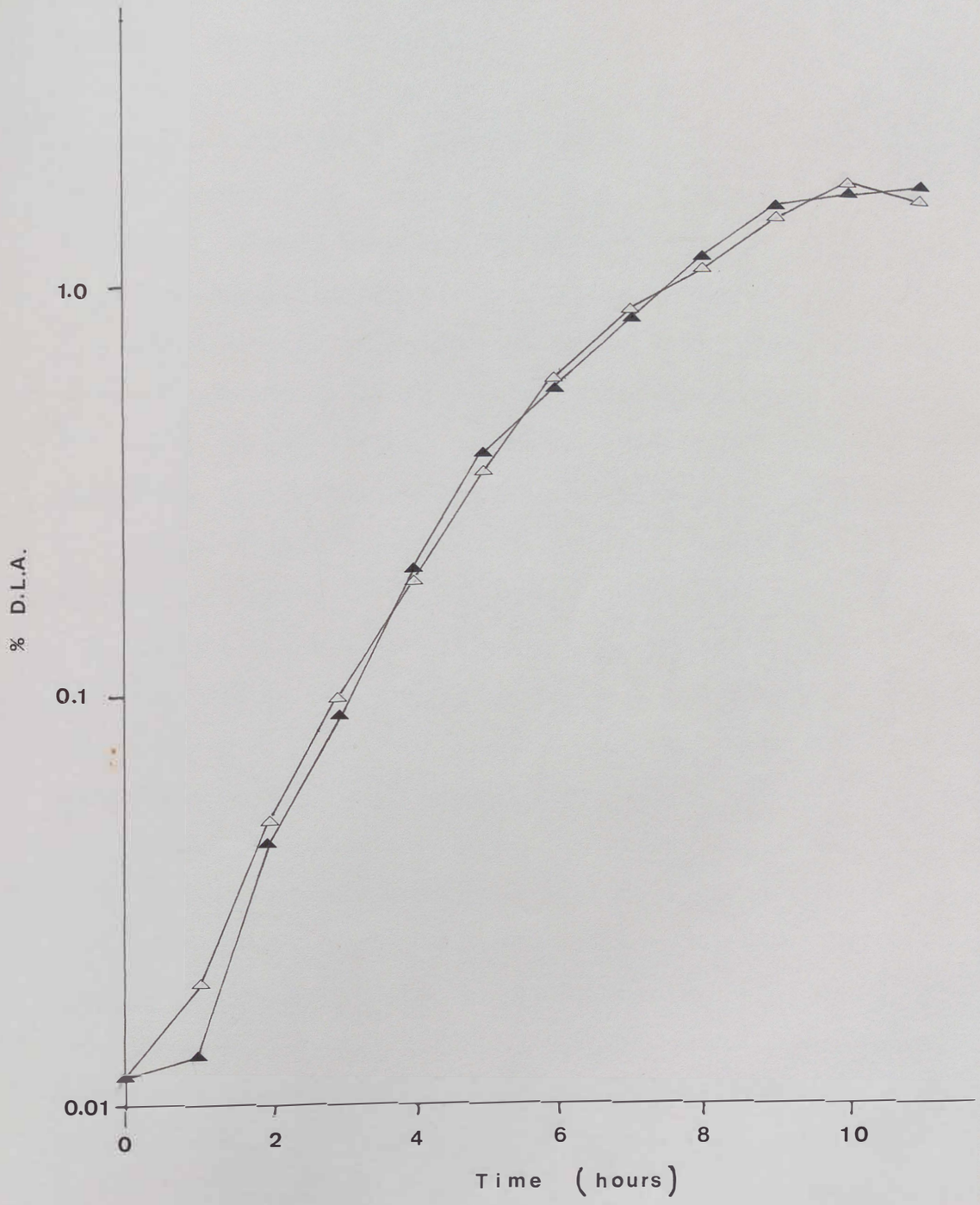


Fig. 6.

Growth of Streptococcus cremoris 310 as measured by % Developed Lactic Acid (D.L.A.) in 2.5% Milk Solids Medium under pH-control with 0.4% (▲——▲) or 0.8% (△——△) added Yeast Extract.

Fig. 6.



Growth performance of other Streptococcus lactis and Streptococcus cremoris strains in 2.5% milk solids medium with 0.4% added yeast extract.

The ability of selected strains of lactic streptococci to grow in 2.5% skim milk solids medium with 0.4% added yeast extract, under pH-control, was studied as previously described. Growth comparisons were made with cultures grown in 10% skim milk solids at 21°C x 16 h without pH-control.

The effect of pH-control on strain growth was compared with non pH-controlled growth. S. cremoris strains 107, and 047 and S. lactis 021 were examined. Without pH-control, due to the low solids content the pH of the medium fell to 4.3 in less than 6 h. This resulted in S. cremoris 047 cell numbers falling by 60%, (3.6×10^8 c.f.u./ml to 1.0×10^8 c.f.u./ml), one hour after maximum cell numbers were attained without pH-control (Table 2). S. cremoris 107 and 021 produced 3-4 times less cells under non pH-controlled conditions than when pH-control was employed (Table 3).

Growth comparisons between 30°C under pH-control and 21°C in 10% skim milk were also made for eight strains of lactic streptococci (Table 3). All the strains studied grew better at 30°C under pH-control than at 21°C, with the exception of S. lactis 021 and S. cremoris 091. With both these strains, cell numbers were approximately equal at both 21°C and 30°C. Cell numbers of other strains were at least $1\frac{1}{2}$ times greater under pH-control than was obtained with 10% skim milk grown cultures. In the case of strains S. cremoris 6 and 310 cell numbers were at least 3 times greater.

Table 2.

Growth of Streptococcus cremoris 047 as measured by Colony Forming Units per ml (c.f.u./ml) in 2.5% Milk Solids Medium with 0.4% added Yeast Extract, with and without pH-control.

Time (hours)	Viable Cell Count (c.f.u./ml)	
	pH-control	no pH-control
0	1.5×10^7	1.5×10^7
1	1.4×10^7	3.2×10^7
2	4.0×10^7	7.5×10^7
3	7.3×10^7	8.4×10^7
5	1.1×10^8	1.2×10^8
6	2.8×10^8	3.6×10^8
7	3.2×10^8	1.0×10^8
8	3.6×10^8	9.2×10^7
10	4.2×10^8	8.1×10^7

Table 3.

Maximum viable cell numbers as measured by colony forming units per ml (c.f.u./ml) of selected S. lactis and S. cremoris strains grown at different temperatures in various milk based media.

Strain	10% Skim Milk (21°C x 16 hours)	2.5% Milk Solids Medium with 0.4% Yeast Extract at 30°C	
		pH-control	no pH-control
X	3.70×10^8	6.00×10^8	-
107	4.80×10^8	6.60×10^8	1.50×10^8
047	2.90×10^8	4.20×10^8	3.60×10^8
007	2.00×10^8	6.00×10^7	-
021	7.80×10^8	7.10×10^8	1.85×10^8
091	2.70×10^8	2.64×10^9	-
6	5.30×10^8	4.51×10^9	-
310	4.80×10^8	2.65×10^9	-

- = not determined

While all the strains examined were grown in 10% skim milk to a pH of 4.6-4.8 at 21°C for 16 h prior to inoculation into the low lactose medium, some responded promptly to inoculation in 2.5% skim milk medium and resumed growth immediately. Others showed a lag phase of 1-2 h depending on the strain.

With some notable exceptions, namely, S. lactis 007 and S. cremoris 108, all other strains grew exponentially for 5-6 h before entering stationary phase. Both S. lactis 007 and S. cremoris 108 grew poorly as measured by titratable acidity and viable cell counts. S. cremoris 107 grown under pH control showed evidence of uncoupling of acid production and growth.

Cultures which grew immediately on inoculation were S. cremoris strains x and 6 (Figs. 7 and 8). Final cell numbers of S. cremoris x were half those obtained with S. cremoris 6 (6.0×10^8 c.f.u./ml vs 3.48×10^9 c.f.u./ml). In both cases, continued incubation at 30°C, after maximum growth was reached resulted in a decline in cell numbers.

As mentioned previously, S. cremoris 310 and S. lactis 021 showed a lag of 0.5 to 1.0 h on inoculation into the low milk solids medium (Results Section 1). In addition, S. cremoris strains 047 and 091 showed similar lag phases. With both these cultures the lag phase lasted for two hours prior to commencement of exponential growth (Figs. 9 and 10). At the end of exponential growth both strains showed evidence of uncoupling of acid production and growth. This was most noticeable in the case of S. cremoris 047 (Fig. 10) where continued incubation did not affect cell viability.

Fig. 7.

Growth of Streptococcus cremoris X as measured by colony forming units per ml (c.f.u./ml) in 2.5% Milk Solids Medium under pH-control with 0.4% added Yeast Extract (▲——▲).

Fig. 7.

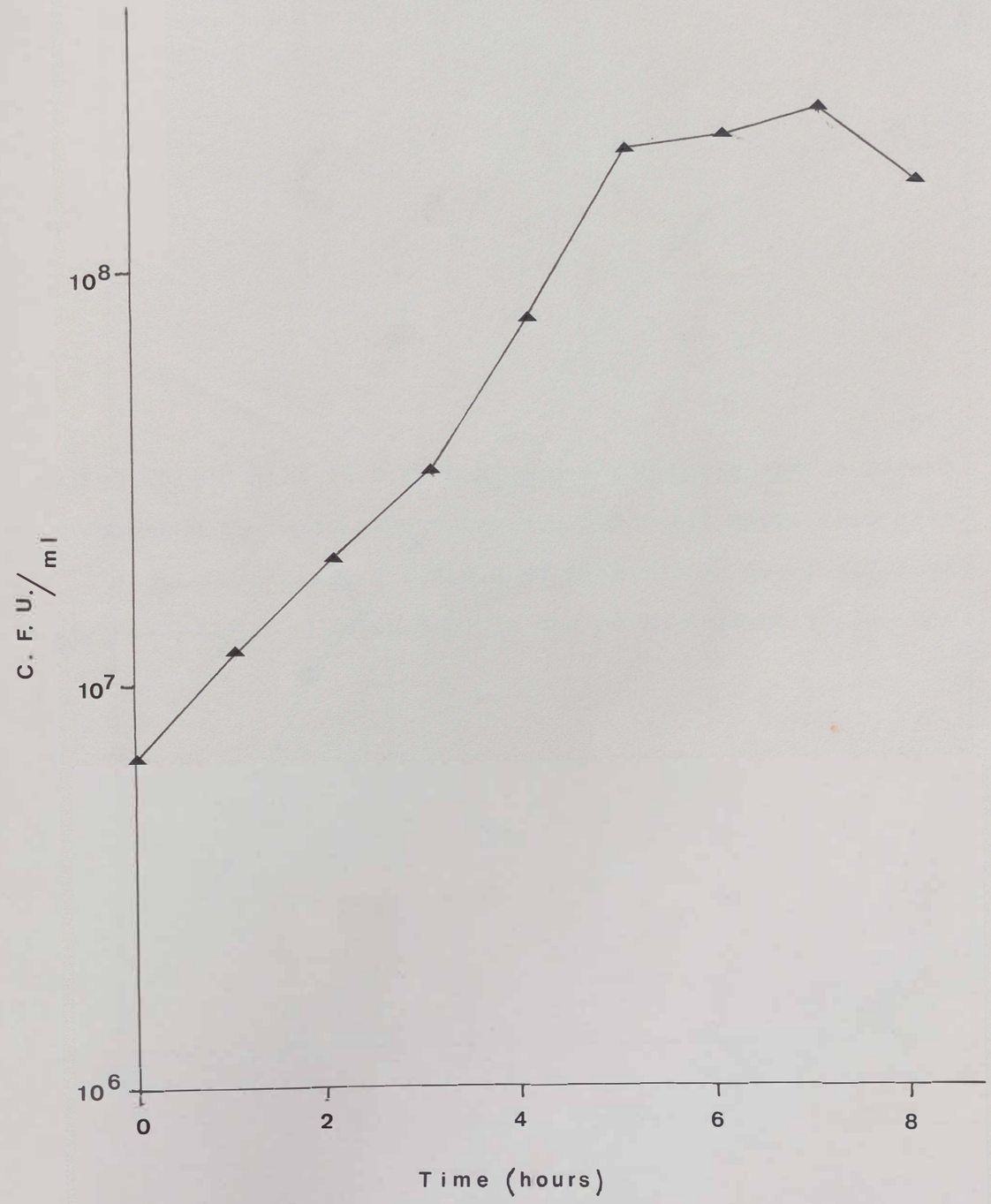


Fig. 8.

Growth of Streptococcus cremoris 6 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid (% D.L.A.) (▲——▲) and colony forming units per ml (c.f.u./ml) (△——△).

Fig. 8.

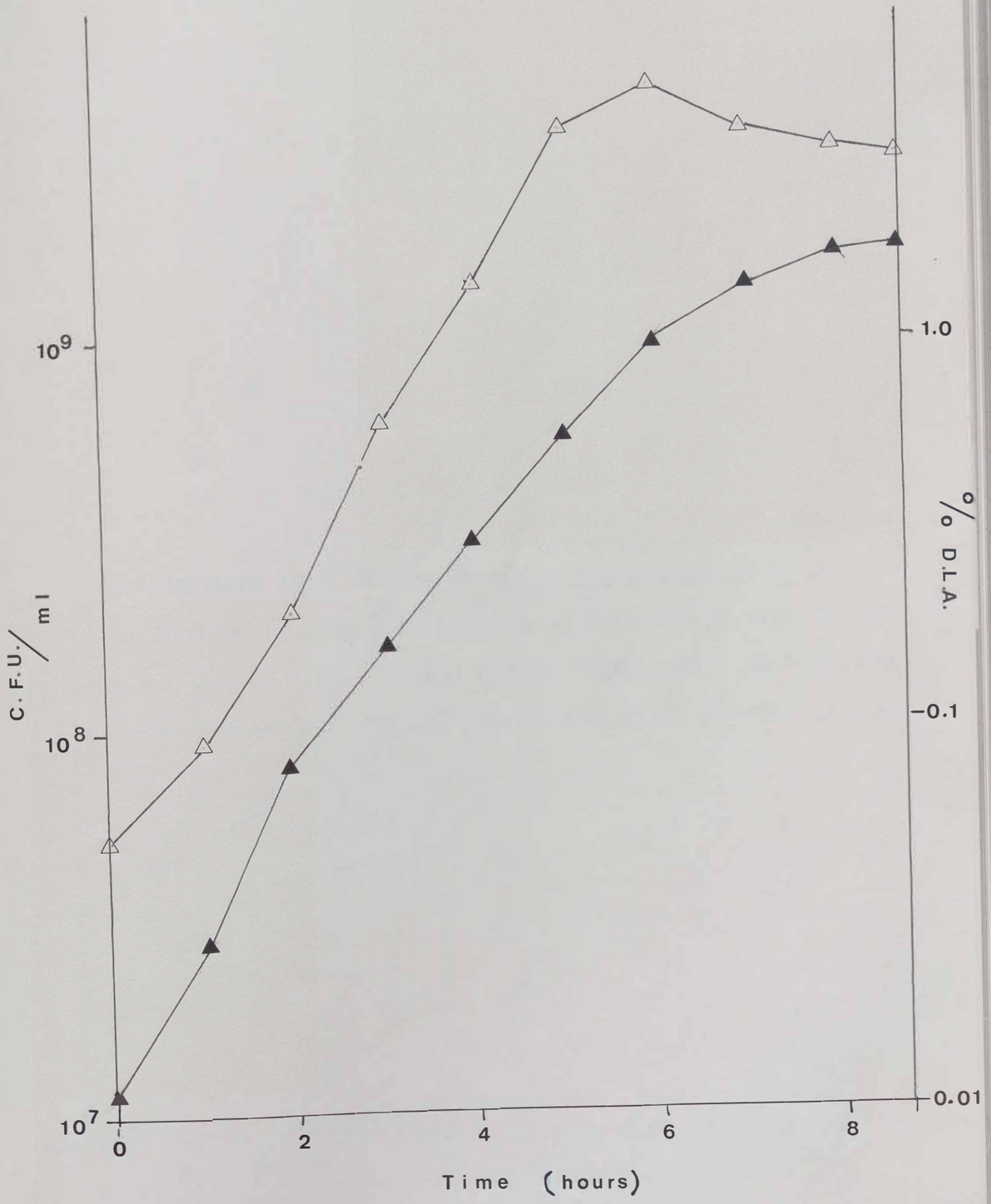


Fig. 9.

Growth of Streptococcus cremoris 091 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid (▲——▲) and Colony Forming Units per ml (c.f.u./ml) (△——△).

Fig. 9.

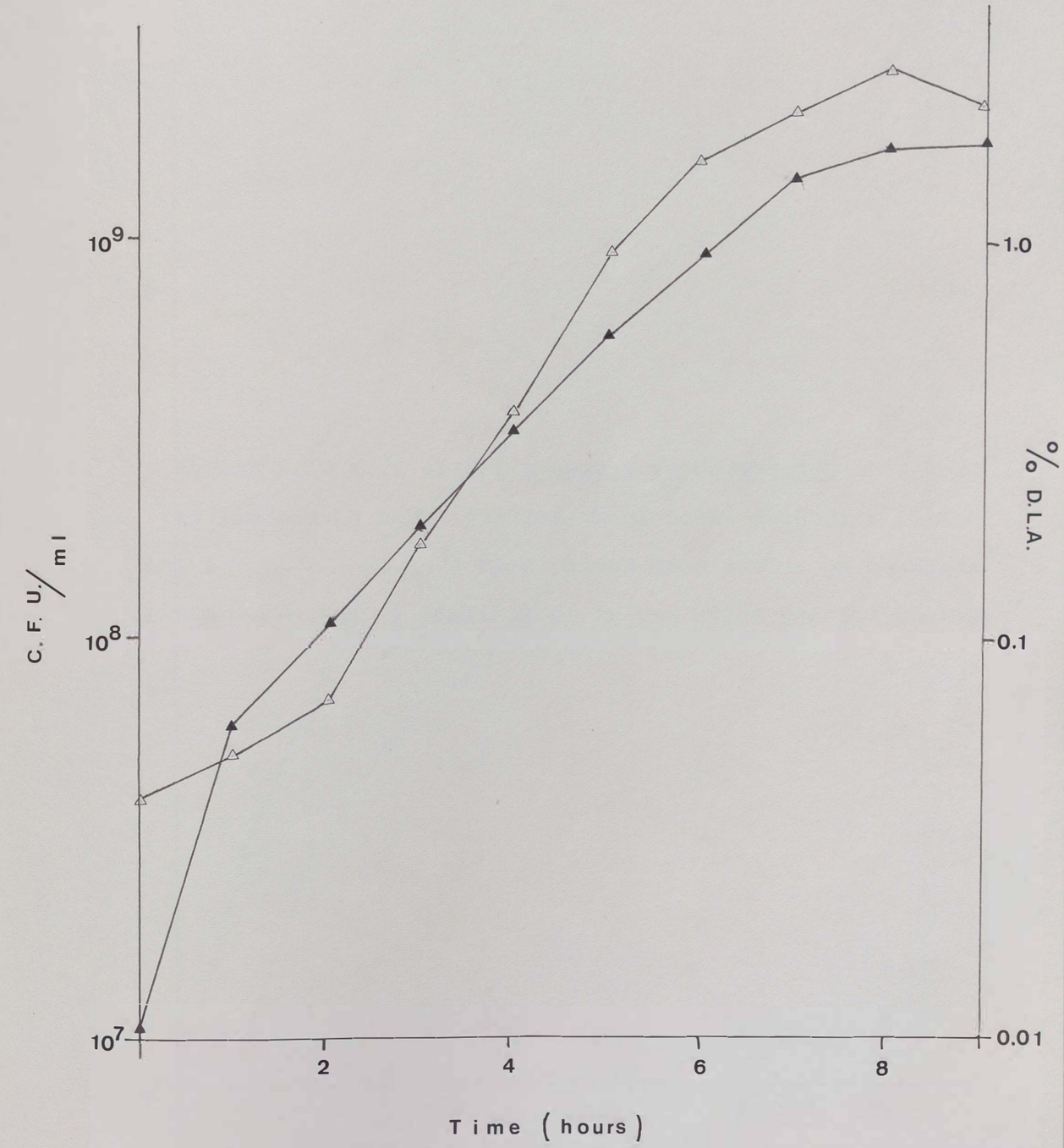
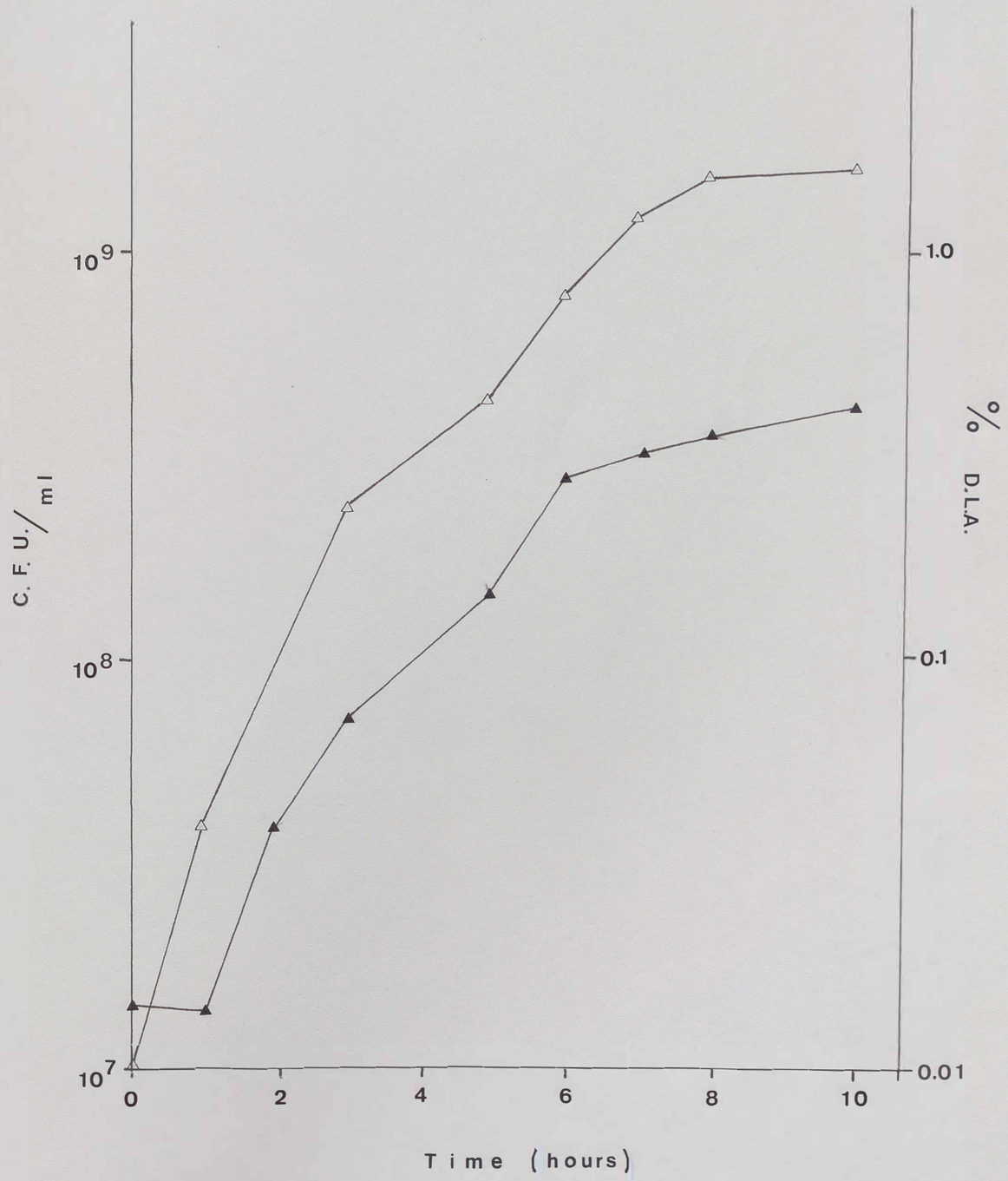


Fig. 10.

Growth of Streptococcus cremoris 047 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid (△——△) and Colony Forming Units per ml (c.f.u./ml) (▲——▲).

Fig. 10.



Similarily, no decline in cell numbers resulted from the continued incubation of S. cremoris 091 at 30°C under pH control (Fig. 9).

S. cremoris 107 exhibited the shortest exponential growth period, as measured by viable cell count, of all the strains studied. Following a lag of two hours, S. cremoris 107 produced acid exponentially for five hours. However, exponential growth only lasted for two hours (Fig. 11), increasing cell numbers ten fold. Further incubation increased cell numbers three fold over a three hour period before the culture entered stationary phase.

Two cultures, S. lactis 007 and S. cremoris 108 grew poorly under pH control. Cell numbers, in the case of S. cremoris 007 increased only from 10^6 to 10^7 c.f.u./ml over eight hours, while a developed acidity of 0.77% was attained (Fig. 12). This compares poorly with other strains which reached levels of 1.6%-1.7% developed lactic acid over the same time period. In the case of S. cremoris 108 a similar inability to grow under pH control was observed (Fig. 13). Following a lag of four hours, 1.0% developed lactic acid was produced at twelve hours incubation.

Both S. lactis 007 and S. cremoris 108 grew well in a similar medium without pH control, both aerobically and anaerobically (Fig. 14). S. lactis 007 reduced the pH of the medium from 6.50 to 4.28 in six hours. In a similar milk based medium without added yeast extract, S. lactis 007 grew, but at a slower rate (Fig. 14). The absence of yeast extract in the low milk solids medium led to a poor growth response of S. cremoris 108. The culture grew in the presence of yeast extract although the lag phase was still pronounced (Fig. 14). S. lactis 007 grew better in 2.5% milk solids medium without yeast extract than did S. cremoris 108 in 10%

Fig. 11.

Growth of Streptococcus cremoris 107 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid %DLA (△———△) and Colony Forming Units per ml (c.f.u./ml) (▲———▲).

Fig. 11.

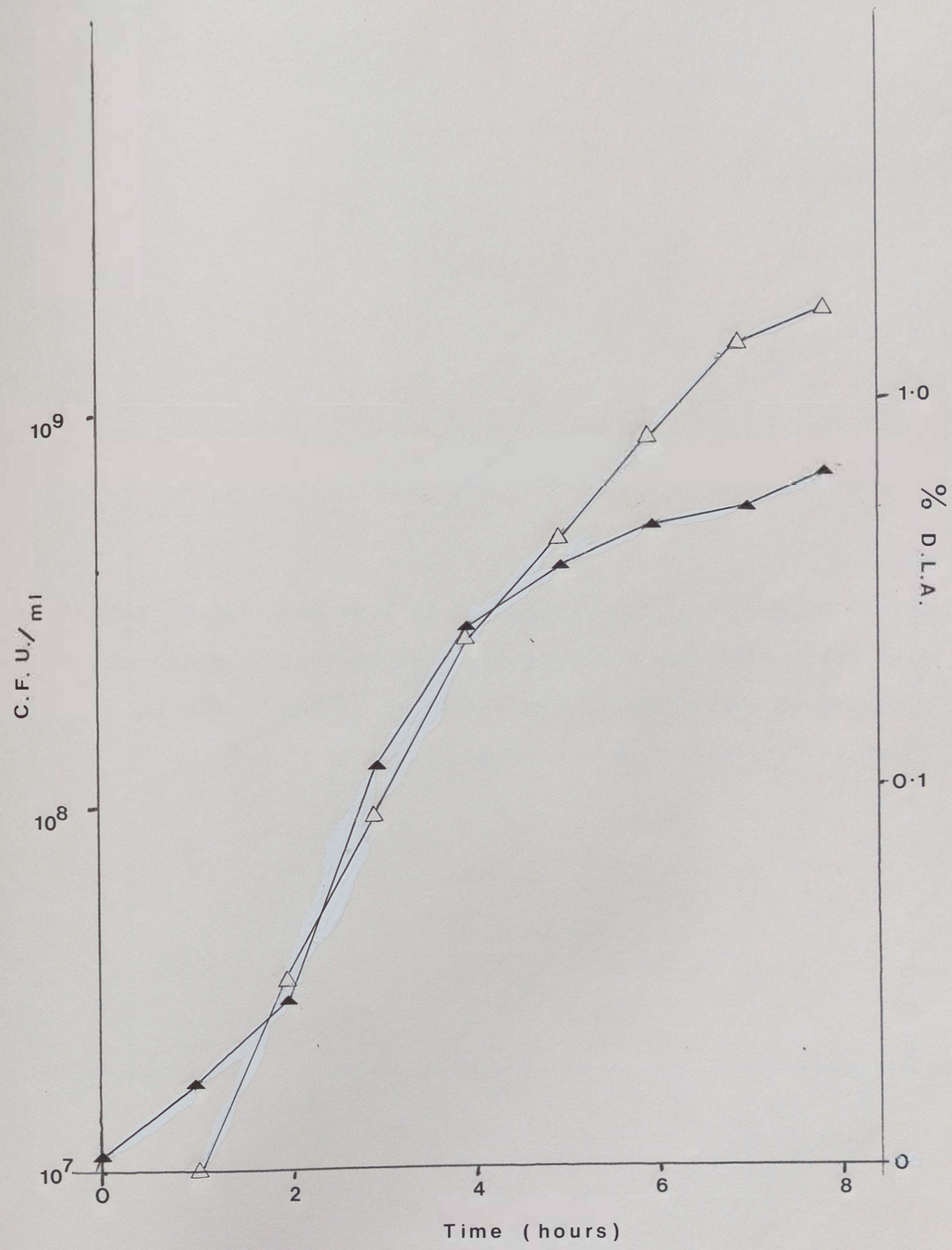


Fig. 12.

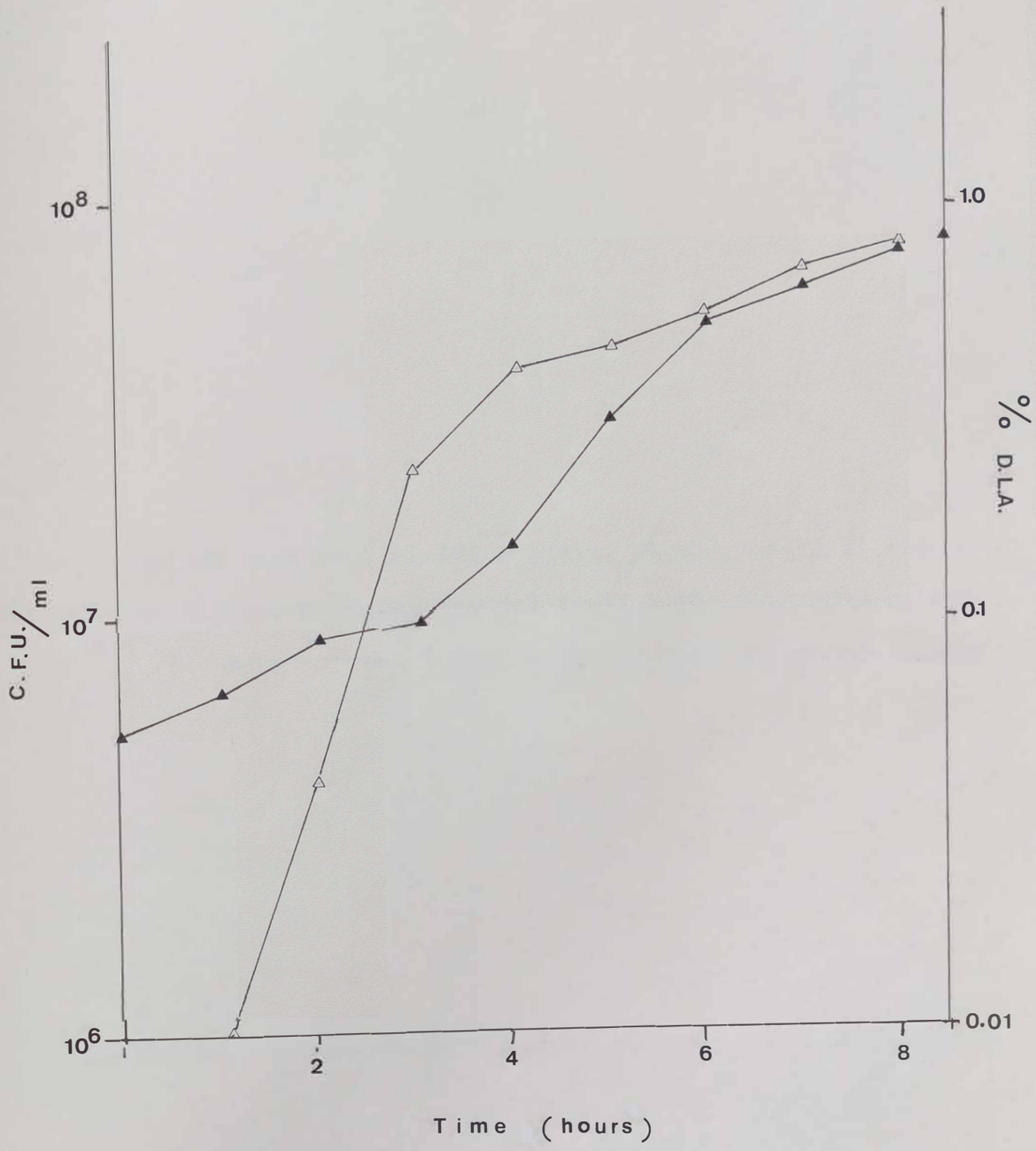


Fig. 12.

Growth of Streptococcus lactis 007 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid % D.L.A.(△——△) and Viable Cell Count (c.f.u./ml) (▲——▲).

Fig. 13.

Growth of Streptococcus cremoris 108 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract under pH-control as measured by % Developed Lactic Acid % D.L.A. (▲——▲).

Fig. 13.

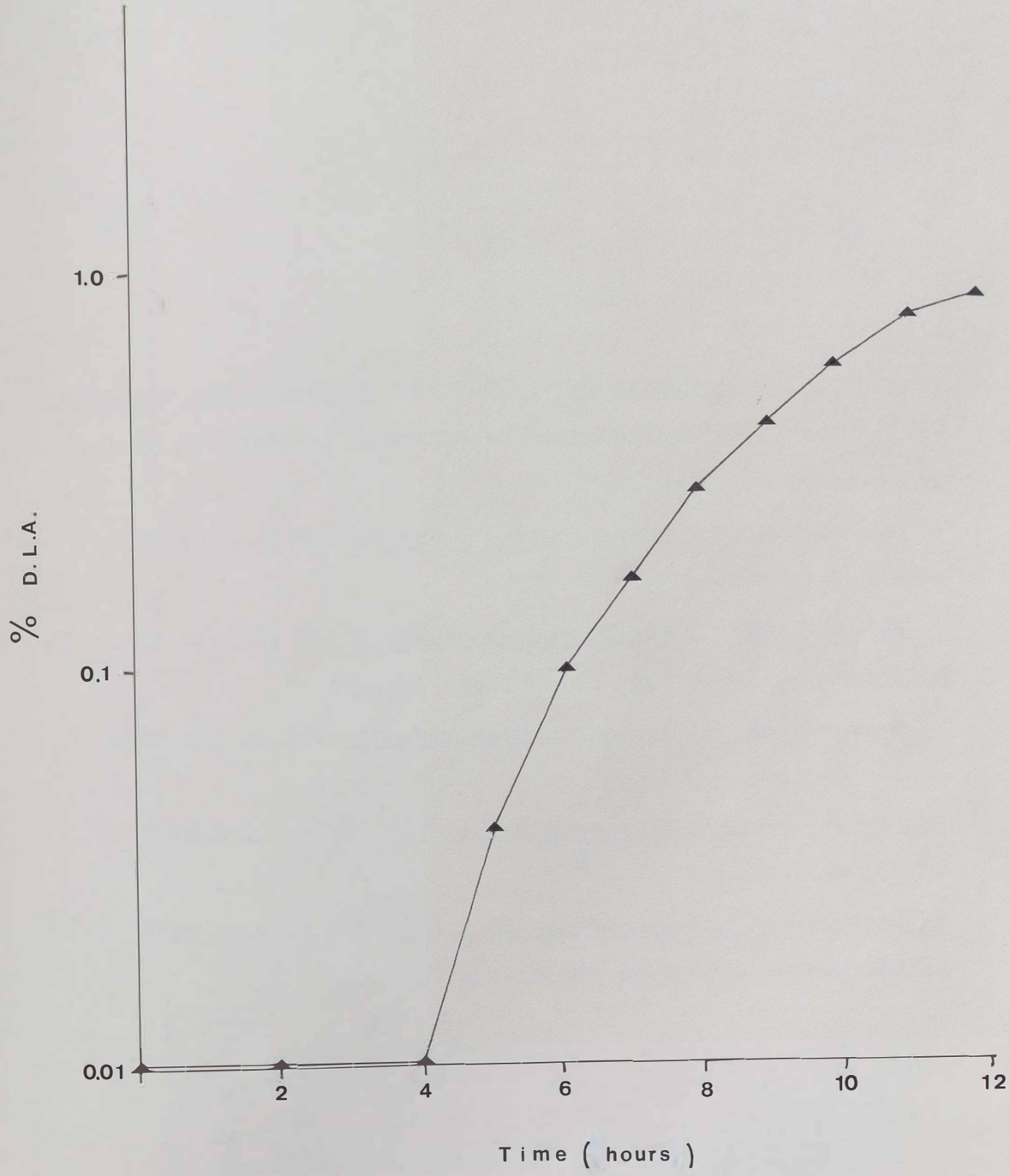


Fig. 14.

Growth of Streptococcus lactis 007 and Streptococcus cremoris 108 in non pH-controlled milk based media as measured by the decrease in pH (△pH).

(△——△) Streptococcus cremoris 108 in 2.5% Milk Solids Medium

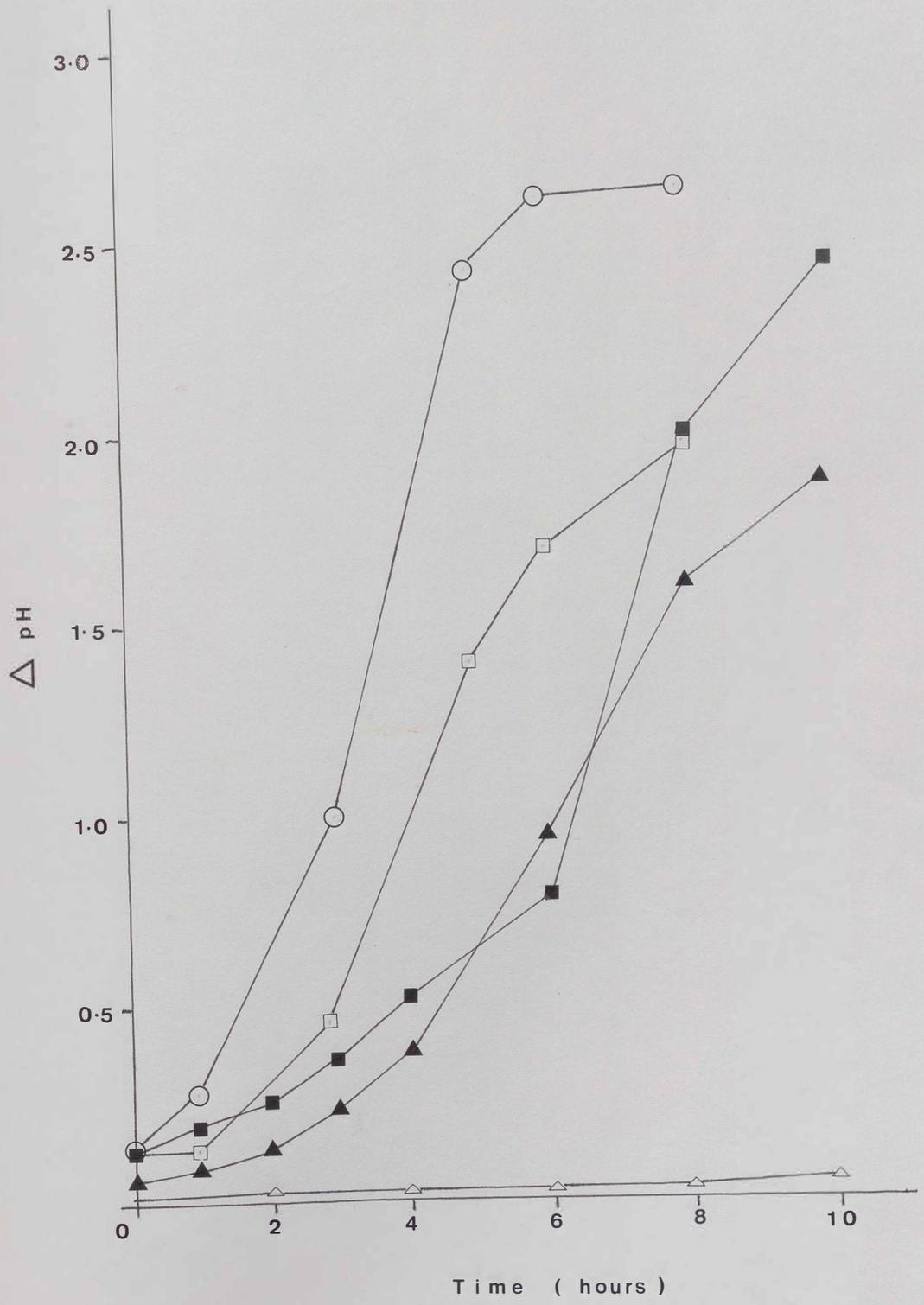
(■——■) Streptococcus cremoris 108 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract.

(▲——▲) Streptocooccus cremoris 108 in 10% Milk Solids Medium.

(□——□) Streptococcus lactis 007 in 2.5% Milk Solids Medium.

(○——○) Streptococcus lactis 007 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract.

Fig. 14.



skim milk (Fig. 14). Also S. cremoris 108 grew better in a low solids medium supplemented with yeast extract than it did in 10% skim milk.

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Storage of Streptococcus cremoris and Streptococcus lactis Single Strains at Refrigeration and Subzero Temperatures.

After optimising growth conditions for the single strains of S. lactis and S. cremoris as shown previously, the strains were harvested and then stored at refrigeration and subzero temperatures. Storage conditions were defined with a view to supplying the cheese industry with frozen concentrates and also establishing the most suitable and economic conditions for culture storage in the factory.

In preliminary experiments it was observed that a rapid fall in pH occurred on removal of pH control at the fermentation temperature of 30°C. This had an adverse effect on culture activity. Therefore, prior to harvesting each culture in lag phase, the fermenter was cooled to 4°C while under pH control. All added cryoprotectants (ringers solution, lactose and glycerol) were ice cold, resulting in all samples being stored at pH values in the range 6.2-6.5, at -80°C, -20°C and 4°C. Samples were stored directly at the temperature stated with the exception of some samples which were frozen at -80°C, then subsequently stored at -20°C.

Culture viability, as expressed by colony forming units per ml of frozen concentrate, was determined at intervals. Culture activity was measured as the ability to produce lactic acid at 21°C and 30°C, in order to assess the suitability of the frozen cultures to be used as inocula for the bulk starter and the cheese vat. Activity at 30°C was examined following growth at 21°C to measure the cultures ability to recover and resume normal activity.

All the strains of S. lactis and S. cremoris studied

showed good retention of activity at -80°C independent of the presence of any cryoprotectants. Activity loss and decline in cell counts at -20°C and 4°C depended both on the strain being studied and on the type of cryoprotectant added to the medium prior to storage. Prior freezing at -80°C had a slightly beneficial effect on storage at -20°C.

The results for each individual strain are now given in detail.

Storage of Streptococcus lactis 021 at refrigeration and subzero temperatures.

S. lactis 021 retained activity and viability at -80°C but at -20°C and 4°C both declined, with lactose having a slightly cryoprotective effect at -20°C.

At -80°C, lactose addition did not result in increased viability or activity. No loss of activity occurred even after 237 days storage (Table 4), as cell numbers had remained constant, at greater than 2.0 x 10^9 c.f.u./ml (Table 5).

Storage at -20°C caused a decline in the viable cell count with a resultant drop in activity. The greatest loss of viability occurred over the first 75 days of storage with cell numbers halving, even in the presence of lactose (Table 5). This decline in viability continued on subsequent storage. The presence of lactose enhanced the ability of S. lactis 021 to produce acid immediately on thawing (Table 4) after as little as 16 days storage, even though there was little difference in cell counts (Table 5). Cells stored at -20°C could recover acid producing ability if grown initially at 21°C x 16 h, dropping the pH of milk to 4.50-4.60 (Table 6). Subsequent activity at 30°C was as good as that of a culture prior to storage, giving final developed acidities of 0.32-0.42% lactic acid (Table 6).

At 4°C, cell numbers appeared to increase from 2.7 x 10^9 to 3.0 x 10^9 c.f.u./ml initially, which resulted in immediate activity after 75 days being 0.39% D.L.A. This was greater than the activity of cultures stored at -20°C (Fig. 15). Cell numbers declined rapidly thereafter with

Table 4.

Effects of low temperature storage on the immediate activity of Streptococcus lactis 021 in RSM at 30°C x 6 hours.

	% Developed Lactic Acid					
	4°C		-20°C		-80°C	
Time	Lactose		Lactose		Lactose	
(Days)	+	-	+	-	+	-
0	0.44	0.44	0.46	0.40	0.46	0.47
16	-	0.40	0.42	0.28	0.46	0.48
75	-	0.39	0.36	0.14	0.46	0.48
116	-	0.01	0.11	0.04	0.44	0.43
168	-	0.00	0.12	0.06	0.44	0.42
237	-	-	0.12	0.05	0.46	0.44

- = not tested

Table 5.

The effects of low temperature storage on the viability of Streptococcus lactis 021, as measured by colony forming ability, at 30°C.

Time (Days)	Colony forming units/ml x 10^8					
	+ Lactose			- Lactose		
	4°C	-20°C	-80°C	4°C	-20°C	-80°C
0	27.10	27.10	27.10	27.10	27.10	27.10
16	0.06	25.50	26.30	26.80	23.90	26.10
75	-	10.70	24.00	30.00	8.00	24.00
116	-	3.90	17.00	0.00	5.10	28.00
168	-	5.20	20.00	-	7.80	23.00
237	-	9.80	20.00	-	4.30	24.00

- = not tested

Table 6.

The effects of low storage on the ability of Str. lactis 021 to reduce the pH of 10% R.S.M. at 21°C x 16 hours and on subsequent activity at 30°C x 6 hours.

	4°C		-20°C		-80°C	
Time (Days)	pH 21°C	%DLA 30°C	pH 21°C	%DLA 30°C	pH 21°C	%DLA 30°C
	+ Lactose					
0	4.60	0.42	4.50	0.42	4.50	0.42
16	6.12	-	4.57	0.38	4.53	0.38
75	6.40	-	4.50	0.37	4.43	0.38
116	6.46	-	5.69	-	4.88	0.34
168	-	-	4.86	0.38	4.68	0.34
237	-	-	4.62	0.41	4.50	0.39
	- Lactose					
0	4.44	0.42	4.40	0.42	4.40	0.42
16	4.55	0.45	4.86	0.29	4.54	0.42
75	4.59	0.40	4.79	0.35	4.44	0.42
116	6.46	-	5.78	-	4.75	0.40
168	-	-	4.63	0.32	4.66	0.42
237	-	-	4.67	0.40	4.50	0.38

- = not tested

Fig. 15.

Effect of storage, at 4°C, -20°C and -80°C in the presence (▲——▲) and absence (△——△) of lactose, on the immediate activity at 30°C of Streptococcus lactis 021 as measured by % Developed Lactic Acid.

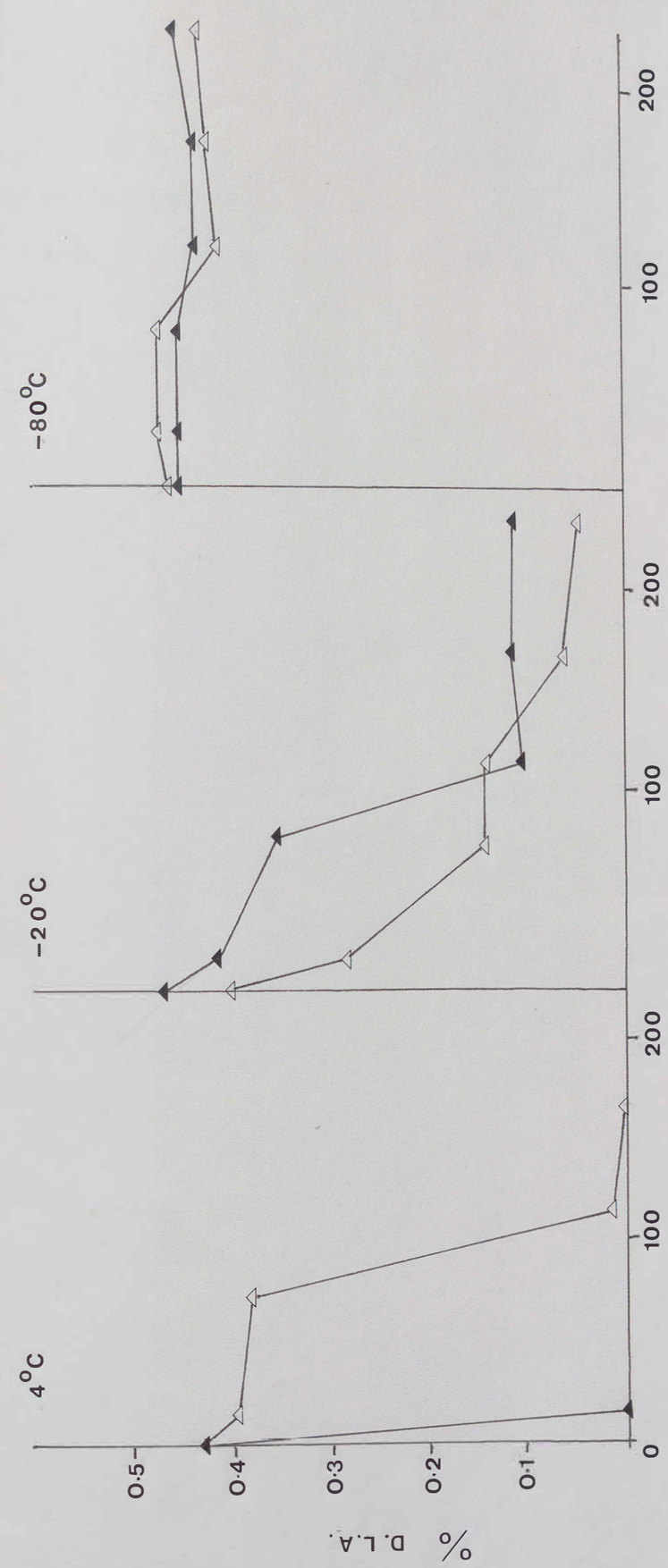


Fig. 15.

no viable cells present after 116 days (Table 5). The addition of lactose to cultures stored at 4°C resulted in acid production, a fall in pH to 4.3 and cell death, in less than 16 days (Table 5).

Storage of Streptococcus cremoris 047 at refrigeration and subzero temperatures.

The addition of lactose to samples of S. cremoris 047 stored at -80°C, had no appreciable effect on viable cell numbers or on activity over a period of 180 days (Fig. 16). During this period cell numbers fell by less than a third, from 3.60×10^8 c.f.u./ml to 2.30×10^8 c.f.u./ml, with activity remaining constant at 0.30-0.40% D.L.A. (Table 7).

While cell numbers remained stable at -20°C in the presence and absence of lactose (Fig. 16), a gradual loss of activity took place in samples stored without lactose. These samples had less than half (0.08-0.09% D.L.A.) the immediate activity of lactose protected samples (0.22-0.26% D.L.A.) (Table 8 and Fig. 16). When the activity of these samples at 21°C was determined, both reduced the pH of milk to 4.40-4.90, with subsequent activity at 30°C of 0.28-0.36% D.L.A. (Table 9).

At 4°C, cell numbers declined rapidly in the sample stored with lactose (Fig. 17), while the sample without added lactose showed an apparent increase in cell numbers from 3.60×10^8 c.f.u./ml to 1.00×10^9 c.f.u./ml. This increase did not manifest itself as an increase in activity. Instead immediate activity fell to 0.07% D.L.A. (Table 8). S. cremoris 047 stored with and without lactose, for 6 and 72 days, respectively, developed acidities of 0.31 to 0.37% lactic acid, at 30°C, after subculturing at 21°C initially (Table 9).

Fig. 16.

Effect of storage, at 4°C, -20°C and -80°C, in the presence (▲——▲) and absence (△——△) of lactose, on the viability of Streptococcus cremoris 047 as measured by Colony Forming Units/ml.

Fig. 16.

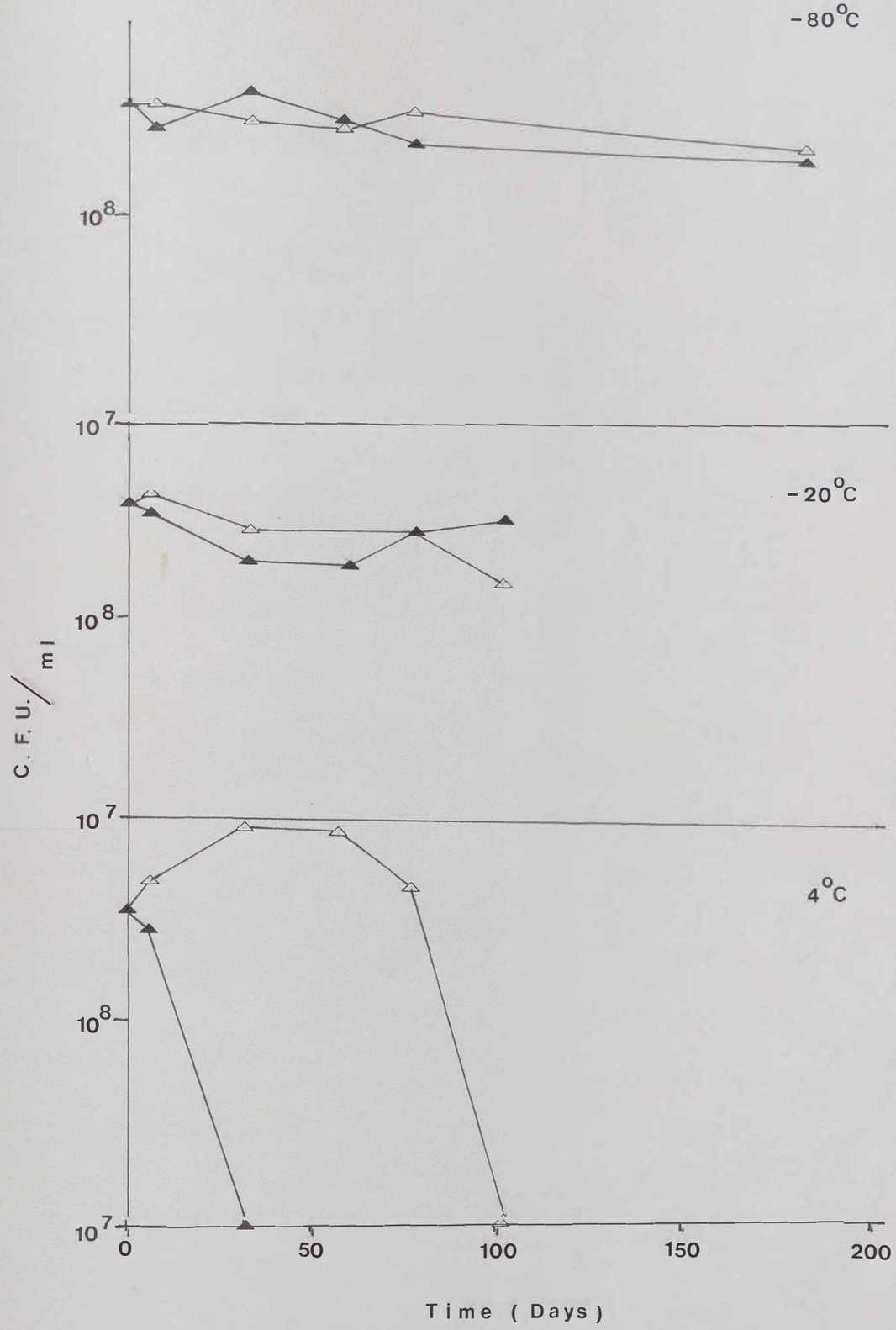


Table 7.

Effects of low temperature storage on Streptococcus cremoris 047 viability, as measured by colony forming ability.

Time (Days)	Colony forming units/ml x 10^8					
	+ Lactose			- Lactose		
	4	-20	-80	4	-20	-80
0	3.60	3.60	3.6	3.60	3.6	3.6
6	2.80	3.20	2.7	5.10	4.0	3.5
33	-	1.90	4.0	10.00	2.8	2.9
56	-	1.84	1.6	8.50	1.8	2.7
72	-	2.80	2.4	4.80	2.8	3.2
96	-	3.10	2.4	0.04	1.5	2.8
181	-	-	2.3	0.00	-	2.6

- = not tested

Table 8.

Effects of low temperature storage on the immediate activity at 30°C of Streptococcus cremoris 047.

	% Developed Lactic Acid					
	4°C		-20°C		-80°C	
Time	Lactose		Lactose		Lactose	
(Days)	+	-	+	-	+	-
0	-	-		-		-
6	-	-	-	-	-	-
33	0.01	0.07	0.19	0.10	0.26	0.26
56	0.01	0.07	0.19	0.19	0.36	0.36
72	0.00	0.06	0.22	0.09	0.35	0.32
96	-	0.01	0.26	0.08	0.38	0.30
181	-	-	0.22	0.08	0.39	0.34

- = not tested

Table 9.

The effects of low temperature storage on the ability of Streptococcus cremoris 047 to reduce the pH of 10% R.S.M. at 21°C x 16 hours and on subsequent activity at 30°C x 6 hours.

	4°C		-20°C		-80°C	
Time (Days)	pH 21°C	DLA 30°C	pH 21°C	DLA 30°C	pH 21°C	DLA 30°C
	+ Lactose					
0	4.42	0.36	4.40	0.36	4.42	0.36
6	6.45	0.36	4.40	0.32	4.41	0.31
33	6.40	0.00	4.42	0.28	4.40	0.35
56	-	-	4.63	0.31	4.73	0.34
72	-	-	4.40	0.35	4.40	0.33
96	-	-	4.57	0.35	4.44	0.30
181	-	-	-	-	4.55	0.33
	- Lactose					
0	4.40	0.37	4.40	0.36	4.50	0.36
6	4.43	0.36	4.42	0.30	4.61	0.33
33	4.59	0.37	4.49	0.29	4.66	0.35
56	4.43	0.33	4.55	0.29	4.40	0.33
72	5.84	0.31	4.48	0.28	4.40	0.31
96	6.40	-	4.72	0.28	4.44	0.31
181	-	-	4.90	0.30	4.50	0.35

- = not tested

Fig. 17.

Effect of storage, at 4°C, -20°C and -80°C, in the presence (▲——▲) and absence (△——△) of lactose on the immediate activity at 30°C of Streptococcus cremoris 047 as measured by % Developed Lactic Acid (% D.L.A.).

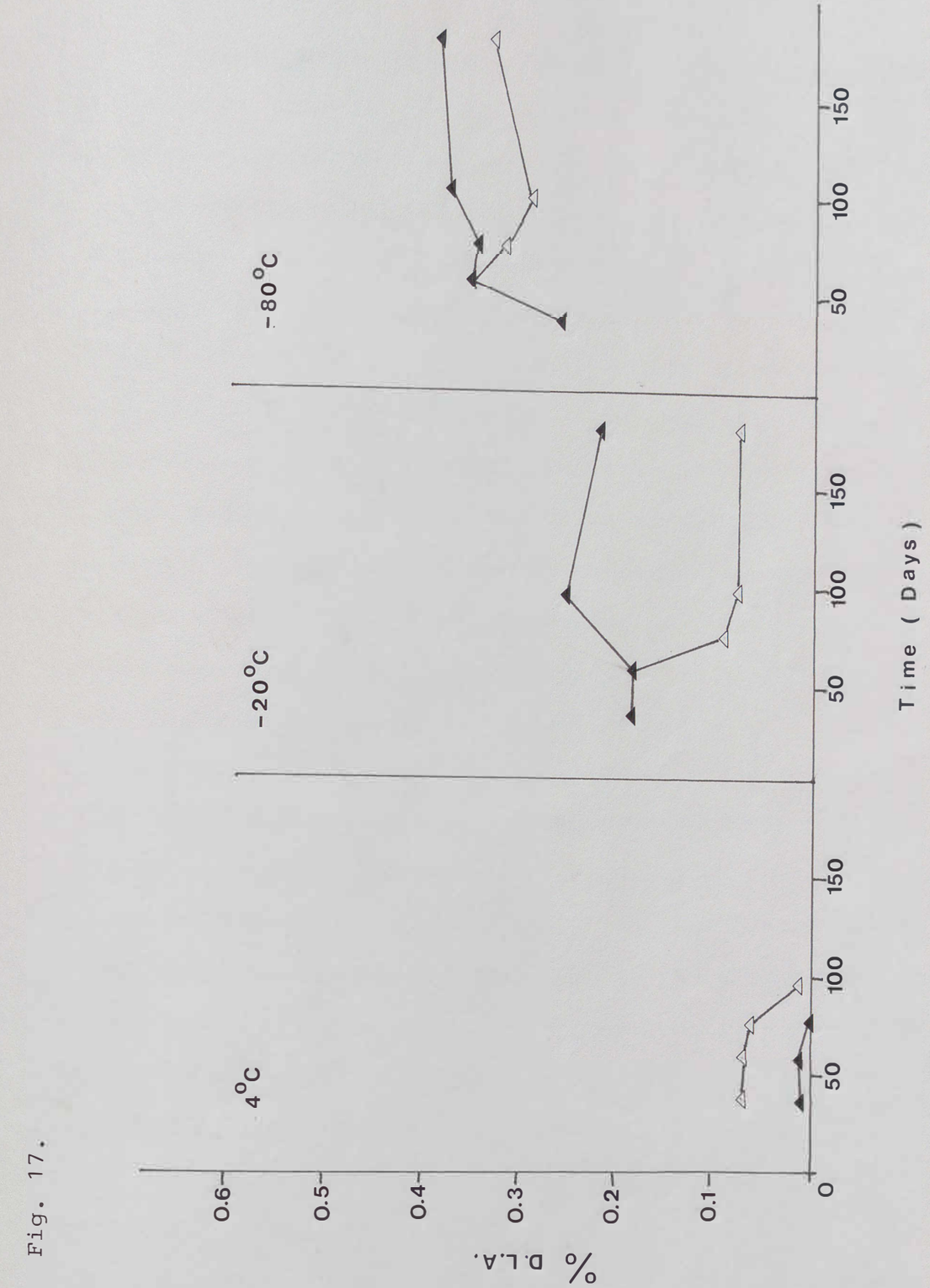


Fig. 17.

Storage of Streptococcus cremoris 091 at refridgeration and subzero temperatures.

S. cremoris 091 samples stored at -80°C lost no viability or activity, with lactose or glycerol conferring no advantage on the culture (Figs. 18 and 19).

In addition, for samples stored for 150 days at -20°C, cell numbers did not fall below 1.69×10^9 c.f.u./ml from 2.00×10^9 c.f.u./ml whether the sample was frozen at -20°C or -80°C (Fig. 18). The activity of the culture immediately on thawing was influenced by the cryoprotectant used, with glycerol being the most protective, followed by lactose, with ringers solution having no protective effect (Fig. 19). Samples stored for 166 days with added glycerol had an activity on thawing of 0.33% D.L.A. before storage. Over the same time period the activity of S. cremoris 091 stored in lactose and ringers solution had fallen to 0.19% and 0.08%, respectively (Fig. 19).

Greatest activity loss with ringers solution stored samples occurred in the initial 15 days (Fig. 19). No loss of activity occurred over the same time period with glycerol or lactose stored samples. Freezing of the culture at -80°C prior to storage at -20°C had a slightly beneficial effect on immediate activity; subsequent loss of activity at -20°C depended on the cryoprotectant used.

Incubation at 21°C x 16 h resulted in recovery of acid producing ability of all -20°C stored samples (Tables 10 and 11). Activities which ranged from 0.37% to 0.44% D.L.A. compared favourably with the activity of S. cremoris 091 prior to storage.

Fig. 18.

Effects of storage at 4°C, -20°C and -80°C, in the presence of lactose (▲——▲), glycerol (■——■) and Ringers Solution (○——○), on viability of Streptococcus cremoris 091 as measured by Colony Forming Units per ml (c.f.u./ml).

Additional samples, stored at -20°C, first frozen at -80°C in the presence of lactose (△--------△) and Ringers Solution (●——●——●), were also checked for viability.

Fig. 18.

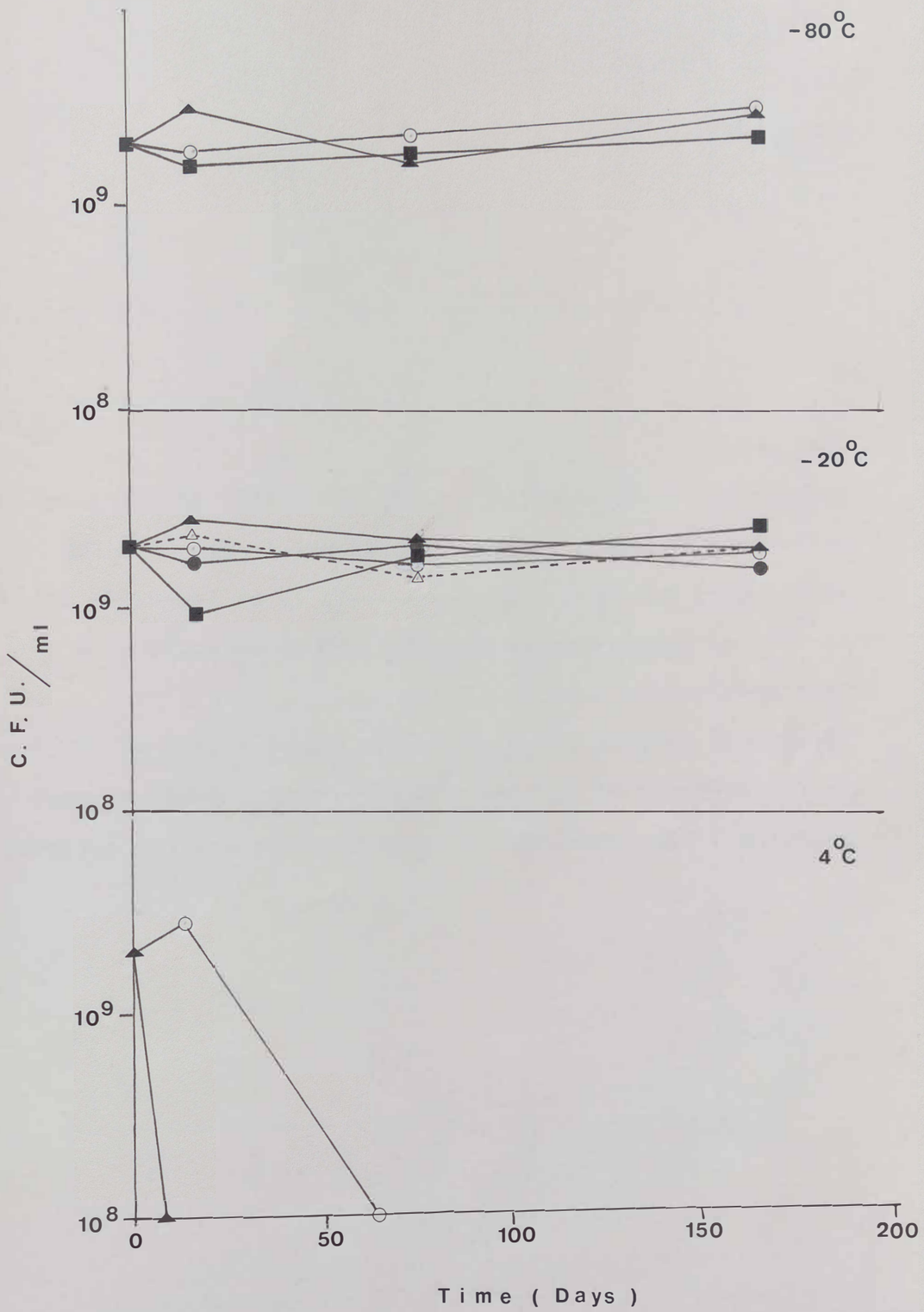


Fig. 19.

Effects of storage, at 4°C, -20°C and -80°C, in the presence of lactose (▲——▲), glycerol (■——■) and Ringers Solution (○——○), on immediate activity at 30°C of Streptococcus cremoris 091 as measured by % Developed Lactic Acid.

Additional samples stored at -20°C, first frozen at -80°C in the presence of lactose (△- - - -△) and Ringers Solution (●——●), were also checked for activity.

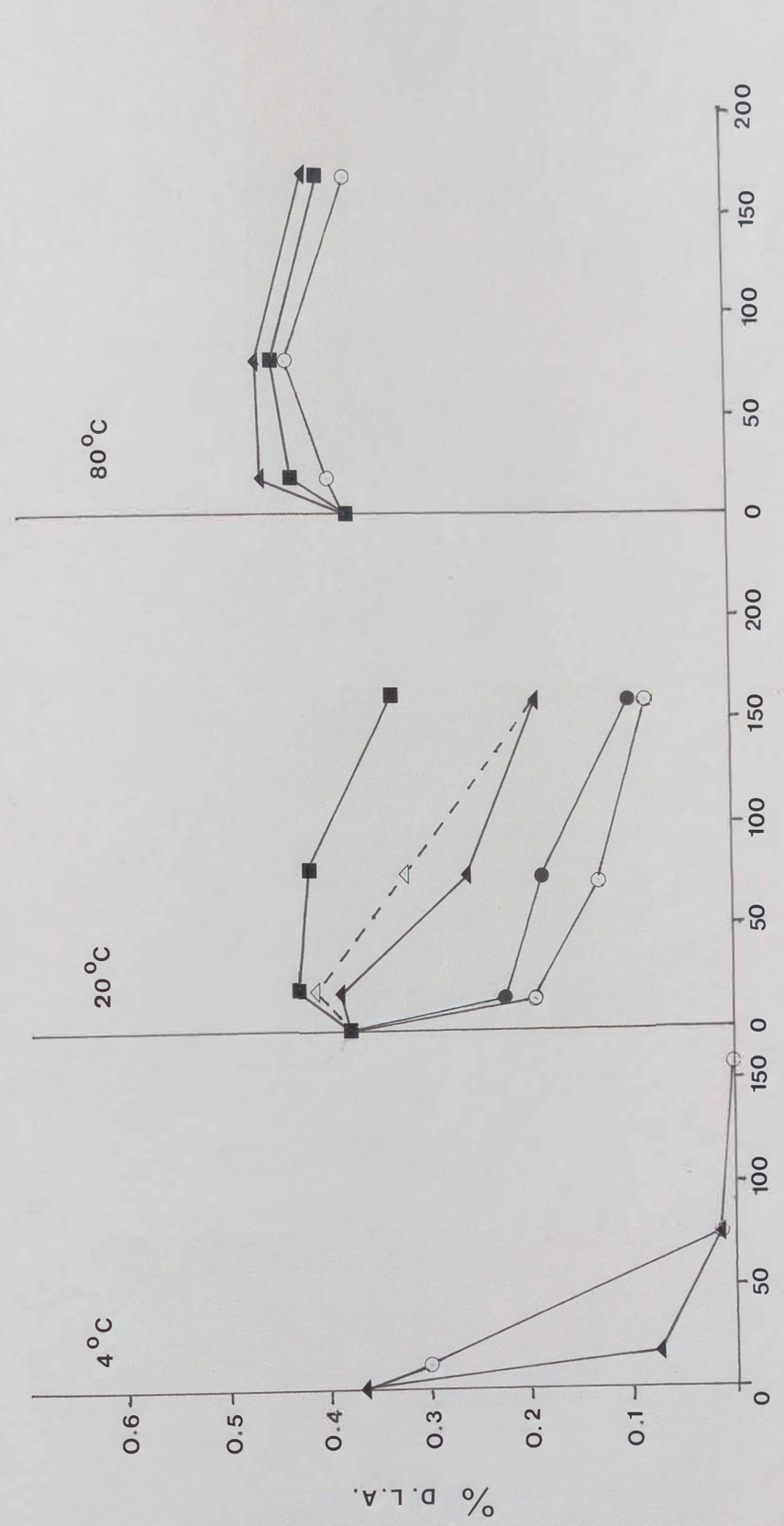


Fig. 19.

Table 10.

The effects of low temperature storage on the ability of Streptococcus cremoris 091 to reduce the pH of 10% R.S.M. at 21°C x 16 hours.

Time (Days)	pH 4°C	-20°C	-20°C Quick frozen at -80°C	-80°C
	+ Lactose			
0	4.44	4.44	4.44	4.44
15	5.17	4.50	4.50	4.50
76	6.48	4.87	4.82	4.66
166	-	4.58	4.68	4.58
	+ Ringers			
0	4.44	4.44	4.44	4.44
15	4.58	4.58	4.50	4.46
76	6.35	4.60	4.70	4.97
166	-	4.53	4.54	4.70
	+ Glycerol			
0	-	4.44	-	4.44
15	-	4.50	-	4.46
76	-	4.70	-	4.65
166	-	4.52	-	4.52

- = not tested

Table 11.

The effects of low temperature storage on the activity of Streptococcus cremoris 091 at 30°C x 6 hours after initial propagation at 21°C x 16 hours, in 10% R.S.M.

Time (Days)	% Developed Lactic Acid			
	4°C	-20°C	-20°C Quick frozen at -80°C	-80°C
	+ Lactose			
0	0.38	0.38	0.38	0.38
15	0.27	0.40	0.42	0.44
76	0.00	0.44	0.40	0.41
166	-	0.38	0.38	0.42
	+ Ringers			
0	0.38	0.38	0.38	0.38
15	0.32	0.37	0.29	0.43
76	0.10	0.35	0.47	0.52
166	-	0.38	0.35	0.36
	+ Glycerol			
0	-	0.38	-	0.38
15	-	0.42	-	0.39
76	-	0.47	-	0.51
166	-	0.40	-	0.39

- = not tested

S. cremoris 091 stored with lactose at 4°C showed almost total loss of viability within 15 days (Fig. 18), with activity at 30°C falling to 0.08% D.L.A. (Fig. 19). Cell numbers having fallen to less than 10^3 c.f.u./ml showed some recovery at 21°C, reducing the pH of milk in 16 h to 5.17 (Table 11). Activity loss of samples stored in ringers solution was more gradual,taking almost 76 days (Tables 10 and 11).

Storage of Streptococcus cremoris 310 at refridgeration and subzero temperatures.

At -80°C, samples of S. cremoris 310 showed a decline in viability and in activity whether stored in lactose, glycerol or ringers solution. After 200 days storage, viable cell numbers had decreased by 25% to 50% (Fig. 20). The most noticeable decline in cell activity took place in samples stored in ringers solution, acidity falling from 0.36% D.L.A. to 0.25% D.L.A. (Fig. 21). Pre-incubation at 21°C did not lead to recovery of acid producing ability at 30°C, for samples stored in both lactose and ringers solution, when compared with glycerol stored samples (Tables 12 and 13).

The decline in culture activity of samples stored at -20°C could be associated with a fall in cell numbers (Figs. 20 and 21). Both were dependent on the cryoprotective agent used. Glycerol had the greatest protective effect followed by lactose, with a 10-fold decrease in cell numbers occurring when S. cremoris 310 was stored in ringers solution. The rate of loss of activity for both lactose and ringers protected cultures was independent of the method of freezing (Fig. 21). While the activities of samples frozen in lactose fell gradually to 0.13-0.14% D.L.A., those stored in ringers solution lost most of their activity during the first 56 days with acidities falling to 0.08%-0.10% lactic acid (Fig. 21).

While subculturing at 21°C aided the recovery of acid producing ability of lactose stored samples, those samples stored in ringers were sufficiently damaged to hinder acid production at both 21°C and 30°C (Tables 12 and 13).

Fig. 20.

Effects of storage, at 4°C, -20°C and -80°C in the presence of lactose (▲——▲), glycerol (△——△) and Ringers Solution (○------○), on viability of Streptococcus cremoris 310, as measured by Colony Forming Units per ml (c.f.u./ml).

Additional samples stored at -20°C, first frozen at -80°C in the presence of lactose (□——□) and Ringers Solution (●——●) were also checked for viability.

Fig. 20.

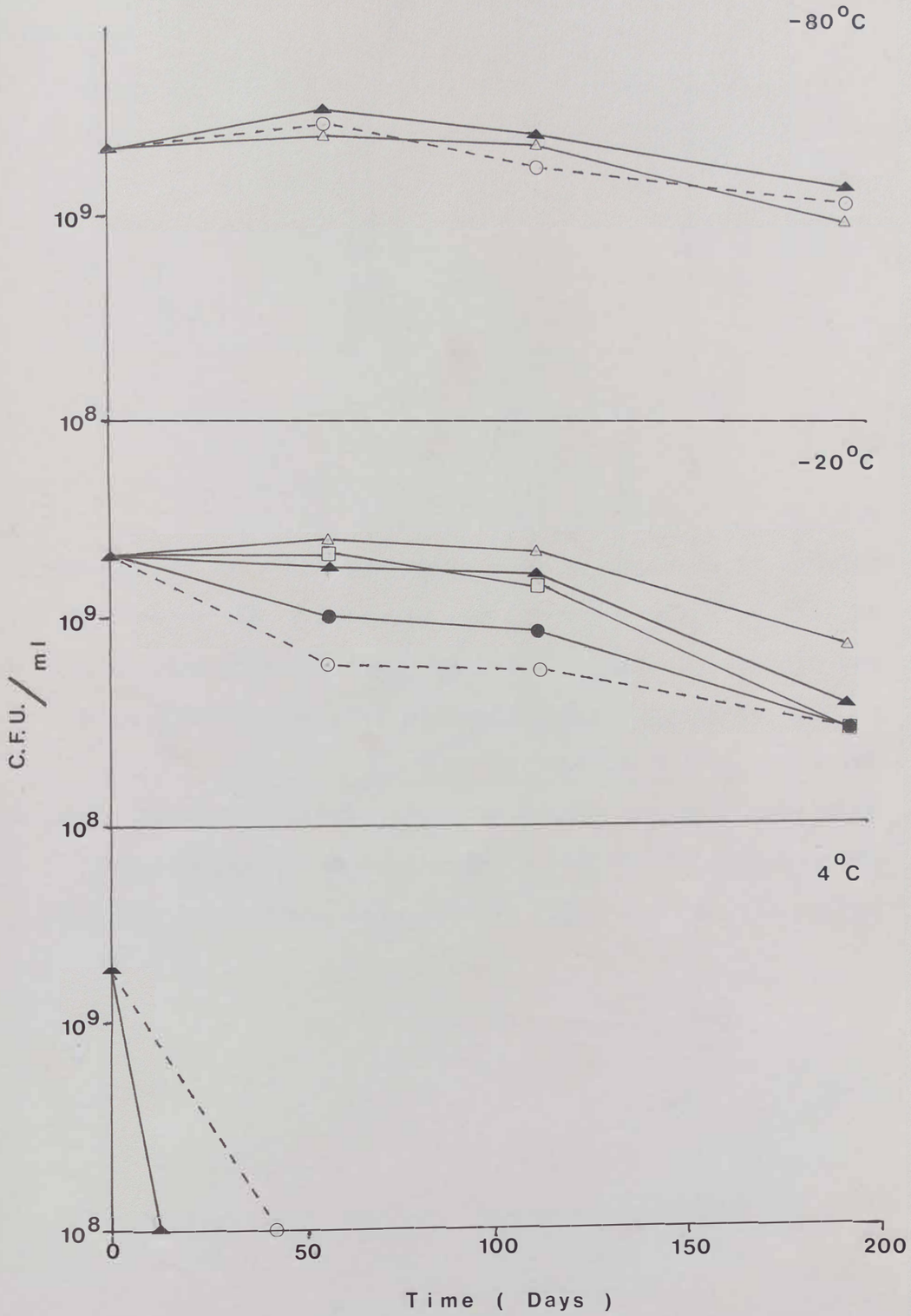


Fig. 21.

Effects of storage, at 4°C, -20°C and -80°C in the presence of lactose (○——○), glycerol (■——■) and Ringers Solution (▲——▲), on immediate activity at 30°C of Streptococcus cremoris 310, as measured by % Developed Lactic Acid.

Additional samples stored at -20°C, first frozen at -80°C in the presence of lactose (●——●) and Ringers Solution (△- - - -△) were also checked for activity.

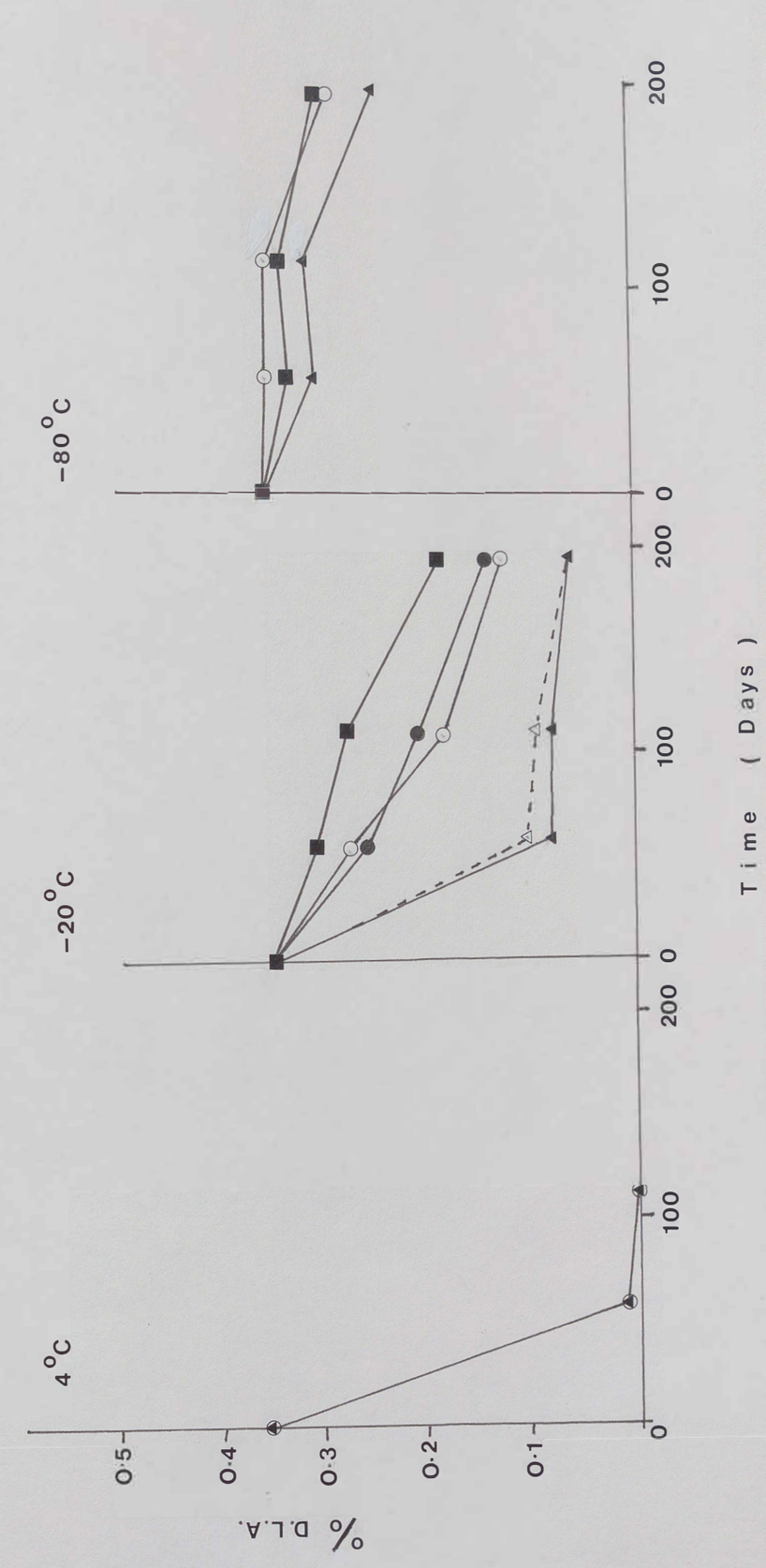


Fig. 21.

Table 12.

The effects of low temperature storage on the ability of Streptococcus cremoris 310 to reduce the pH of milk at 21°C x 16 hours in 10% R.S.M.

Time (Days)	pH 4°C	-20°C	-20°C Quick frozen at -80°C	-80°C
	+ Lactose			
0	4.63	4.63	4.63	4.63
56	6.50	5.23	5.05	4.92
111	-	5.10	5.16	4.85
196	-	5.28	5.28	4.73
	+ Ringers			
0	4.63	4.63	4.63	4.63
56	6.50	5.87	5.78	5.08
111	-	5.78	5.69	5.00
196	-	5.94	6.00	4.97
	+ Glycerol			
0	-	4.63	-	4.63
56	-	4.88	-	4.86
111	-	4.85	-	4.76
196	-	5.03	-	4.82

- = not tested

Table 13.

The effects of low temperature storage on the activity of Streptococcus cremoris 310 at 30°C after initial propagation at 21°C x 16 hours in 10% R.S.M.

Time (Days)	% Developed Lactic Acid			
	4°C	-20°C	-20°C Quick frozen at -80°C	-80°C
	+ Lactose			
0	0.36	0.36	0.36	0.36
56	0.01	0.35	0.44	0.43
111	-	0.36	0.38	0.36
196	-	0.22	0.23	0.30
	+ Ringers			
0	0.36	0.36	0.36	0.36
56	0.00	0.18	0.18	0.41
111	-	0.15	0.17	0.31
196	-	0.10	0.10	0.25
	+ Glycerol			
0	-	0.36	-	0.36
56	-	0.43	-	0.40
111	-	0.36	-	0.38
196	-	0.29	-	0.33

- = not tested

Although the culture was only tested after 56 days storage at 4°C, no activity could be detected at 21°C or 30°C even though samples stored with added ringers contained 5.0 x 10^7 c.f.u./ml (Table 14).

Table 14.

The effects of low temperature storage on the viability of Streptococcus cremoris 310.

Time (Days)	Colony forming units/ml x 10^9			
	4°C	-20°C	-20°C quick frozen	-80°C
	+ Lactose			
0	2.10	2.0	2.0	2.0
56	<10	1.9	2.1	3.4
111	-	1.7	1.6	2.6
196	-	0.4	0.3	1.5
	+ Ringers			
0	2.0	2.0	2.0	2.0
56	0.05	0.6	1.0	2.9
111	-	0.6	0.9	1.9
196	-	0.3	0.3	1.3
	+ Glycerol			
0	-	2.0	-	2.0
56	-	2.5	-	2.5
111	-	2.3	-	2.5
196	-	0.8	-	1.0

- = not tested

Storage of Streptococcus cremoris 6 at refridgeration and subzero temperatures.

At -80°C viable cell counts of S. cremoris 6 remained at the original storage level of 2.90 x 10^9 c.f.u./ml for the 240 days studied. Culture activity remained constant for over 140 days, after which a slight fall in the activity of cultures stored in both glycerol and ringers occurred (Fig. 22). Subculturing at 21°C x 16 h led to subsequent activities of 0.40% to 0.51% lactic acid, which were 10% to 20% higher than those acidities of the culture when checked immediately after thawing.

Viable cell counts fell, slightly, from 2.90 x 10^9 c.f.u./ml to 2.30 x 10^9 c.f.u./ml and 1.80 x 10^9 c.f.u./ml for glycerol and lactose protected cultures, respectively, at -20°C after 240 days storage, with the major part of the decline occurring after 140 days. The freezing temperature did not affect the viability of lactose protected cells (Fig. 23). With samples of S. cremoris 6 stored in ringers solution, the greatest decline in cell numbers occurred over the first 13 days of storage (Fig. 23). This decline in viability continued over the entire storage period, with samples quick frozen at -80°C being even more affected (Fig. 23).

Even though cell numbers remained stable, cultures frozen in glycerol and lactose lost activity during storage with acidities falling by 50% (Fig. 22). The decline in activity of ringers stored cultures was even greater (66%). Subculturing at 21°C x 16 h led to full recovery of acid production (Tables 15 and 16), for all samples stored at

Fig. 22.

Effects of storage, at 4°C, -20°C and -80°C, in the presence of lactose (▲——▲), glycerol (○——○) and Ringers Solution (■——■), on immediate activity at 30°C of Streptococcus cremoris 6 as measured by % Developed Lactic Acid (% D.L.A.).

Additional samples stored at -20°C, first frozen at -80°C in the presence of lactose (△--------△) and Ringers Solution (□——□) were also checked for activity.

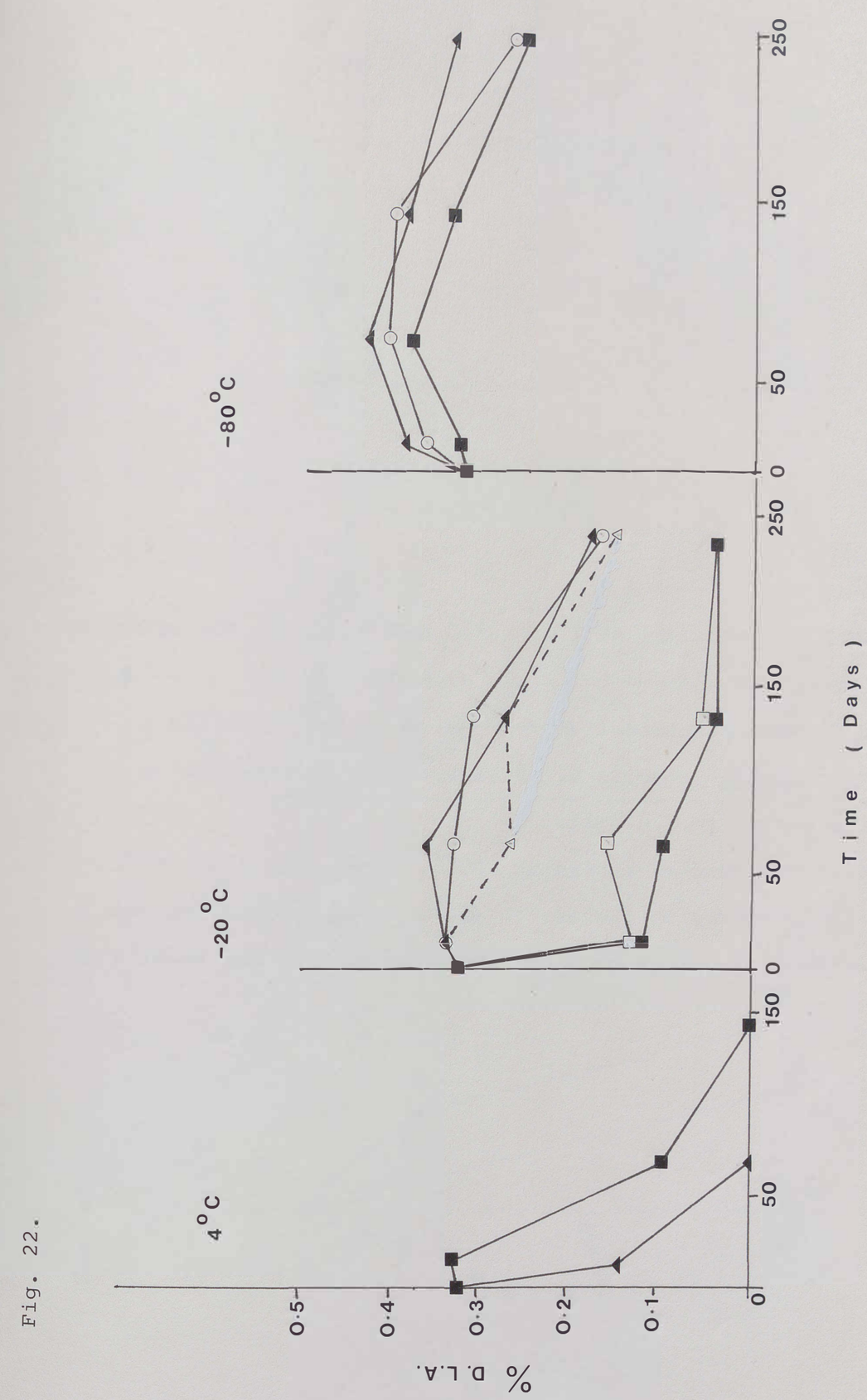


Fig. 22.

Fig. 23.

Effects of storage, at 4°C, -20°C and -80°C, in the presence of lactose (○——○), glycerol (■- - - - - - - - - - ■) and Ringers Solution (▲——▲), on viability of Streptococcus cremoris 6, as measured by Colony Forming Units per ml (c.f.u./ml).

Additional samples stored at -20°C, first frozen at -80°C in the presence of lactose (●——●) and Ringers Solution (△——△) were also checked for viability.

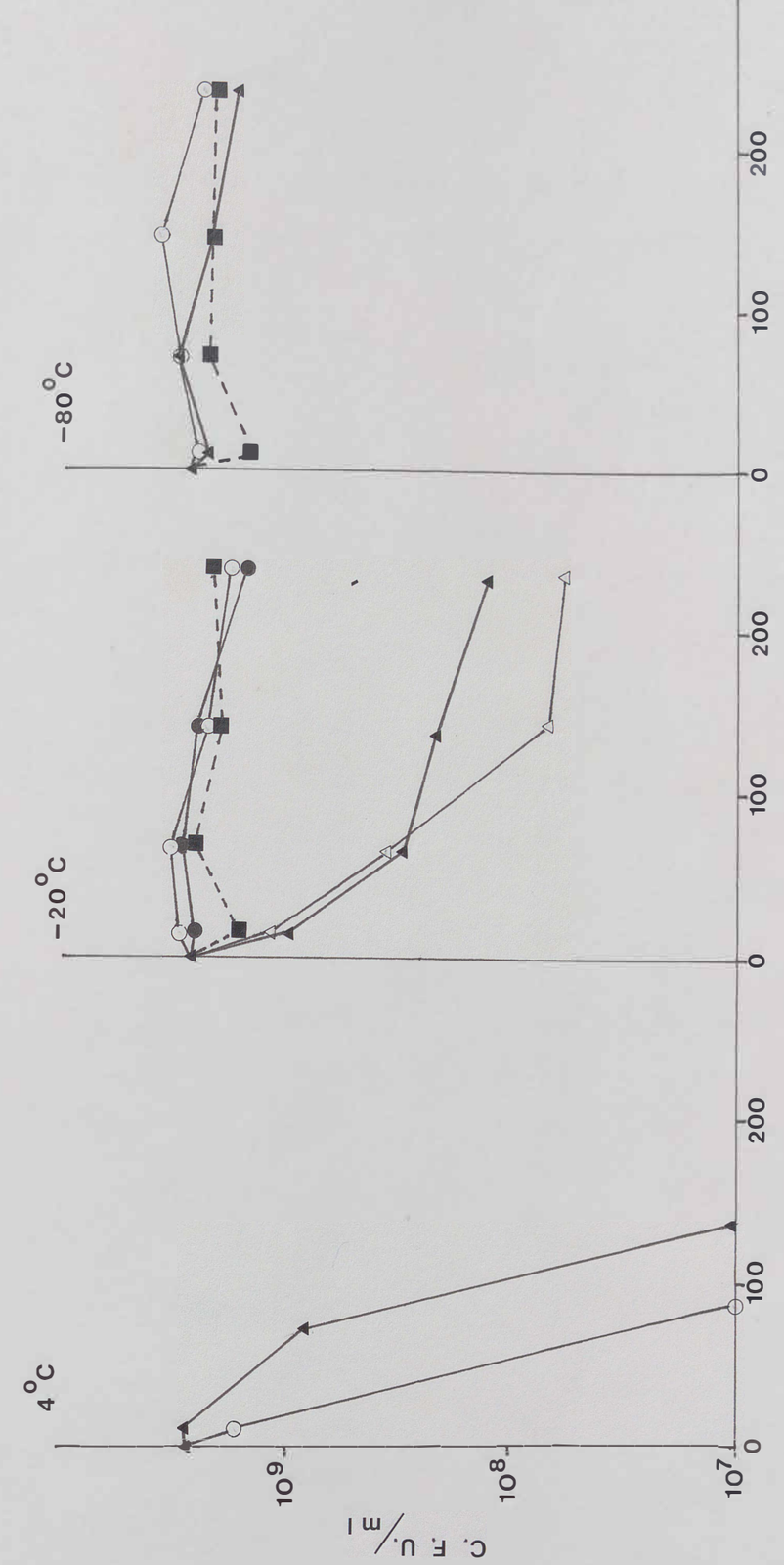


Fig. 23.

Table 15.

The effects of low temperature storage on the ability of streptococcus cremoris 6 to reduce the pH of 10% R.S.M. at 21°C x 16 hours.

Time (Days)	pH 4°C	-20°C	-20°C quick frozen at -80°C	-80°C
	+ Lactose			
0	4.40	4.40	4.40	4.40
13	4.63	4.40	4.40	4.40
71	6.50	4.44	4.48	4.40
141	-	4.56	4.50	4.50
240	-	4.89	4.83	4.68
	+ Ringers			
0	4.40	4.40	4.40	4.40
13	4.40	4.50	4.70	4.60
71	5.75	4.60	4.65	4.96
141	6.50	5.66	5.62	4.50
240	-	5.87	5.86	4.65
	+ Glycerol			
0	-	4.40	-	4.40
13	-	4.48	-	4.45
71	-	4.98	-	4.95
141	-	4.50	-	4.50
240	-	4.69	-	4.63

- = not tested

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Table 16.

The effects of low temperature storage on the activity of Streptococcus cremoris 6 at 30°C in 10% R.S.M. after initial propagation at 21°C x 16 hours.

Time (Days)	% Developed Lactic Acid			
	4°C	-20°C	-20°C quick frozen at -80°C	-80°C
	+ Lactose			
0	0.32	0.32	0.32	0.32
13	0.31	0.40	0.42	0.39
71	0.00	0.49	0.50	0.50
141	-	-	-	-
240	-	0.37	0.38	0.43
	+ Ringers			
0	0.32	0.32	0.32	0.32
13	0.40	0.38	0.32	0.43
71	0.36	0.27	0.33	0.51
141	-	-	-	-
240	-	0.23	0.16	0.43
	+ Glycerol			
0	-	0.32	-	0.32
13	-	0.41	-	0.40
71	-	0.48	-	0.49
141	-	-	-	-
240	-	0.43	-	0.45

- = not tested

-20°C, with the exception of samples stored in ringers solution for greater than 140 days.

S. cremoris 6 held at 4°C with ringers did not lose either activity or viability for 13 days. Cell numbers remained at 2.9×10^9 c.f.u./ml while those in lactose declined by 50% (Fig. 23), with a similar decline in activity. After 71 days storage, samples stored in ringers solution regained full activity on subculturing, with developed acidities of 0.36% (Table 16).

Storage of Streptococcus cremoris 107 at refrigeration and subzero temperatures.

A detailed assessment of storage trends, particularily viability, is not possible, as the initial two viable cell counts were carried out aerobically (Table 17).

For this strain, lactose enhanced activity of cultures stored at -80°C. In its absence, activity fell from 0.50% D.L.A. to 0.38% D.L.A. after 150 days. Subculturing at 21°C led to total recovery of acid producing ability (Table 18).

Lactose also had a cryoprotective effect at -20°C with a 75% reduction in activity occurring after only 15 days storage in its absence. Storage at -20°C for longer than 15 days led to only a partial recovery in acid producing ability both at 21°C and 30°C (Table 18).

At 4°C in the absence of lactose, activity of S. cremoris 107 fell from 0.50% D.L.A. to 0.36% D.L.A. after 15 days (Fig. 24) even though cell numbers remained the same (Table 17). This decrease in activity was less than that which occurred at -20°C in the absence of lactose. While residual activity could be detected in samples stored without lactose for 150 days, at 4°C, subculturing at 21°C did not lead to total recovery of acid producing ability (Fig. 24 and Table 18).

Table 17.

Effects of low temperature storage on Streptococcus cremoris 107 viability as measured by colony forming units per ml (c.f.u./ml).

Time (Days)	Colony forming units/ml x 10^8					
	+ Lactose			-Lactose		
	4°C	-20°C	-80°C	4°C	-20°C	-80°C
*0	6.60	6.60	6.60	6.60	6.60	6.60
*15	2.70	7.14	7.50	7.50	1.43	3.90
82	-	6.00	12.60	5.40	3.00	17.40
150	-	5.70	18.20	3.26	2.18	11.60

* Aerobic counts at 30°C x 48 hours

Table 18.

The effect of low temperature storage on the ability of Streptococcus cremoris 107 to reduce the pH of 10% R.S.M. at 21°C x 16 hours and on subsequent activity at 30°C x 6 hours.

	4°C		-20°C		-80°C	
Time (Days)	pH 21°C	%DLA 30°C	pH 21°C	%DLA 30°C	pH 21°C	%DLA 30°C
	+ Lactose					
0	4.40	0.46	4.40	0.46	4.40	0.46
15	4.96	0.35	4.47	0.43	4.47	0.44
82	6.44	-	5.22	-	4.53	0.49
150	-	-	5.15	0.30	4.52	0.49
	- Lactose					
0	4.40	0.46	4.40	0.46	4.40	0.46
15	4.44	0.44	4.73	0.40	4.42	0.46
82	5.34	-	5.50	-	4.64	0.48
150	5.72	0.17	5.50	0.23	4.60	0.47

- = not tested

Fig. 24.

Effects of storage, at 4°C, -20°C and -80°C, in the presence (△——△) and absence (▲——▲) of lactose, on the immediate activity of Streptococcus cremoris 107 as measured by % Developed Lactic Acid.

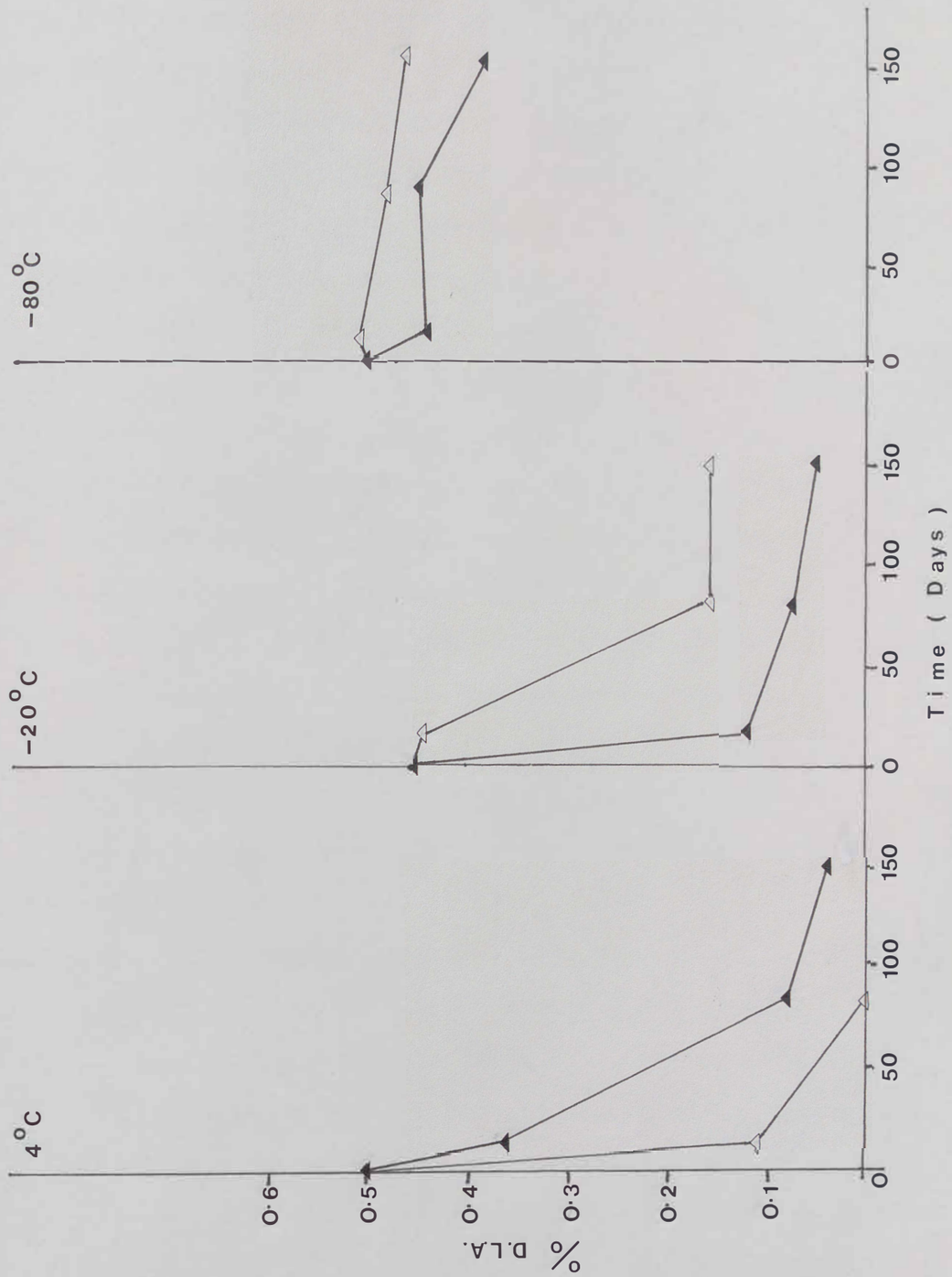


Fig. 24.

Storage of Streptococcus cremoris x at refridgeration and subzero temperatures.

Study of the storage patterns of S. cremoris X is complicated by the fact that the immediate activity was not determined until after 40 days storage.

However at -80°C, it can be seen that lactose had a cryoprotective effect, particularily over the first 140 days of storage (Table 19). Further storage led to a decline in activity from 0.35% D.L.A. to 0.30% D.L.A., with the activity of samples stored without lactose declining even further. Subculturing of samples, stored at -80°C, led to total recovery of acid producing ability (Table 20).

At -20°C, acid producing ability at 30°C had fallen from 0.38% D.L.A. to 0.08% and 0.05% D.L.A. for samples stored with and without lactose, respectively, after 40 days storage. Further storage at -20°C saw activity falling to residual levels (less than 0.02% D.L.A.). Subculturing of samples stored for greater than 40 days did not lead to a recovery in acid producing ability (Table 20).

All samples held at 4°C showed almost total loss of immediate activity after 40 days storage. However, samples stored without added lactose, recovered acid producing ability at 21°C and to a lesser extent at 30°C after 70 days storage (Table 20), with recovery at 21°C being greater than for a sample stored at -20°C for a similar time period.

Table 19 .

Effects of low temperature storage on the immediate activity of *Streptococcus cremoris* x in 10% R.S.M. at 30°C.

	% Developed Lactic Acid					
	4°C		-20°C		-80°C	
Time stored	Lactose		Lactose		Lactose	
(Days)	+	-	+	-	+	-
0	-	-	-	-	-	-
20	-	-	-	-	-	-
40	0.00	0.06	0.08	0.05	0.34	0.37
70	-	0.02	0.04	0.04	0.35	0.31
110	-	0.01	0.02	0.01	0.31	0.28
195	-	-	0.01	0.01	0.30	0.27

- = not tested

Table 20.

The effects of low temperature storage on the ability of *Streptococcus cremoris* x to reduce the pH of 10% R.S.M. at 21°C x 16 hours and on subsequent activity at 30°C x 6 hours.

	4°C		-20°C		-80°C	
Time	pH	%DLA	pH	%DLA	pH	%DLA
(Days)	21°C	30°C	21°C	30°C	21°C	30°C
	+ Lactose					
0	4.40	0.38	4.40	0.38	4.40	0.38
20	6.45	0.00	4.61	0.40	4.45	0.36
40	6.50	-	4.70	0.35	4.41	0.49
70	6.50	-	5.11	-	4.52	-
110	-	-	5.74	-	4.60	0.41
195	-	-	6.23	-	4.71	0.47
	- Lactose					
0	4.40	0.38	4.40	0.38	4.40	0.38
20	4.34	0.35	4.44	0.36	4.40	0.34
40	4.52	0.40	4.82	0.21	4.44	0.42
70	4.81	-	5.33	-	4.62	-
110	5.27	-	5.72	-	4.60	0.39
195	6.50	-	6.36	-	4.63	0.40

Growth of Streptococcus cremoris 320 in milk based media under pH control - Analysis of Inhibition Observed:

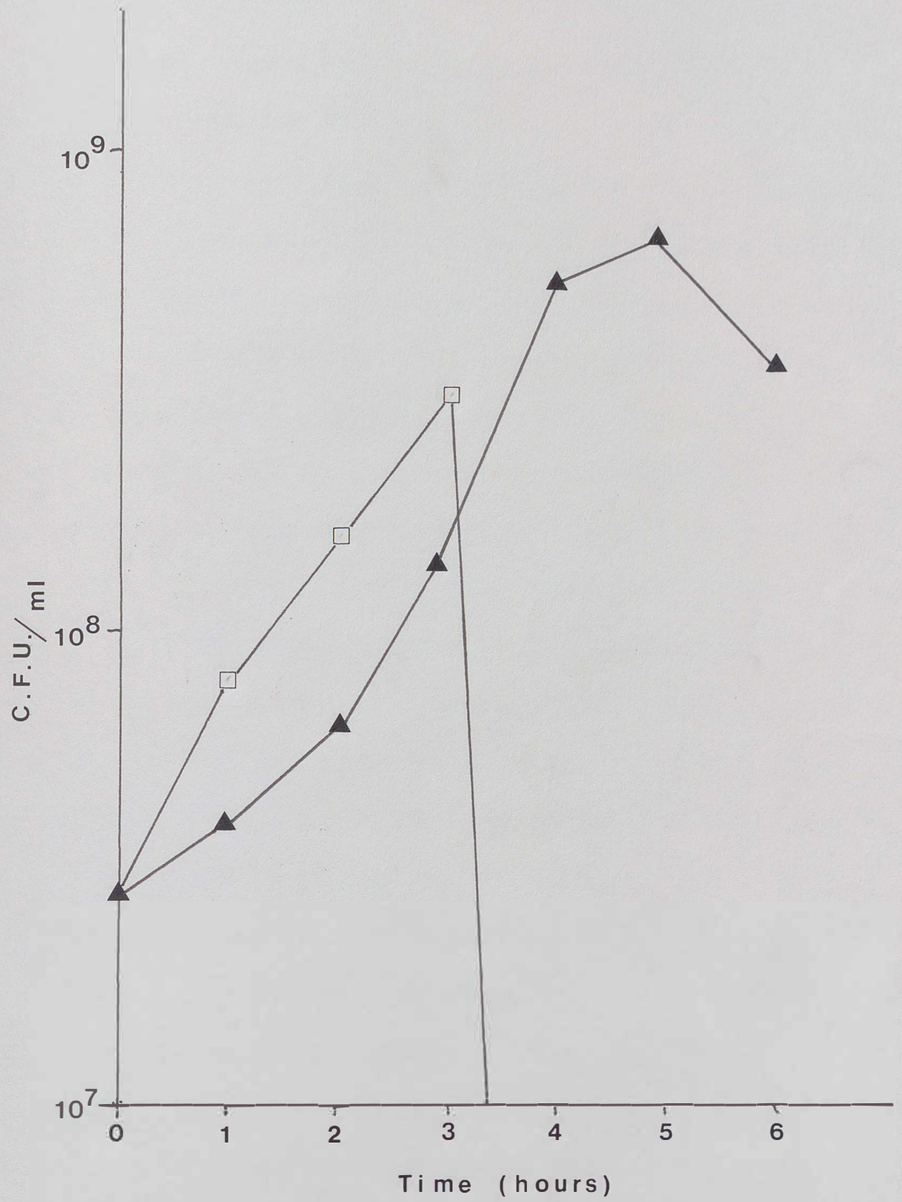
As outlined previously, all the cultures examined for growth in low milk solids media under pH control grew to varying degrees, with those showing optimum growth, being studied for retention of activity at refridgeration and sub-zero temperatures. S. cremoris 320 was studied separately because of the repeated "inhibition" observed when the culture was grown under pH control. Initial studies on S. cremoris 320 were carried out in the 2.5% milk solids medium with 0.4% added Yeast Extract, as previously described.

S. cremoris 320 failed to grow under pH control, while growing normally in a batch non pH controlled fermentation (Fig. 25). Growth comparisons in pH controlled and non pH controlled 2.5% milk solids media with 0.4% added yeast extract revealed, that, in the latter maximum cell numbers of 7×10^8 colony forming units/ml (c.f.u./ml) were reached after five hours growth at 30°C. On further incubation, cell numbers declined to 5×10^8 c.f.u./ml, possibly due to the low pH (4.1) of the fermentation. In a culture pH controlled at 6.5, growth,as measured by lactic acid production, ceased after 3-4 h, having reached a viable cell count of 3×10^8 c.f.u./ml. Viable cell numbers dropped rapidly to between 10 and 50 per ml which on continued incubation at pH 6.5 failed to grow.

Cessation of growth occurred under both aerobic and anaerobic conditions, indicating that hydrogen peroxide (H_2O_2) inhibition of the culture was not a factor in failure

Fig. 25 . Growth of Streptococcus cremoris 320 as measured by viable cell count (c.f.u./ml) in 2.5% Milk Solids Medium with 0.4% added Yeast Extract, under pH controlled conditions at 6.5 (▲——▲) and under non pH controlled conditions (□——□).

Fig. 25.



of the culture to grow under pH control. Even though six times more H_2O_2 was present in aerobically grown, pH controlled cultures compared with anaerobically grown cultures (Fig. 26), growth ceased in both cases at the same time.

As cessation of growth, under pH control conditions was due to a rapid decline in viable cell numbers, not due to the culture entering the stationary phase of growth, culture filtrates were examined for the presence of bacteriophage particles. Sampling of the fermenter vessel at regular intervals of 15 min, indicated that low levels of phage lytic for S. cremoris 320 (the releasing culture), could be detected in the culture supernatant 1-1$\frac{1}{4}$ h after inoculation. This released phage multiplied rapidly giving final levels of 2-5 x 10^{10} plaque forming units/ml (p.f.u./ml) after 5 h (Fig. 27). In media of similar milk solids content, no phage capable of plaquing on S. cremoris 320 were observed when the growth pattern was studied as a batch, non pH controlled fermentation.

Confirmation of the non involvement of H_2O_2 or O_2 as factors in phage release was demonstrated by subjecting a "batch", non pH controlled fermentation to stirring at a rate comparable with that of a pH controlled culture. No phage release was observed and the culture grew in a manner similar to a batch fermentation (Fig. 28). Final cell numbers were lower in the stirred unit possibly due to the combined effects of H_2O_2 and the increasing lactic acid concentration.

pH control, not the presence of inhibitors was found

Fig. 26

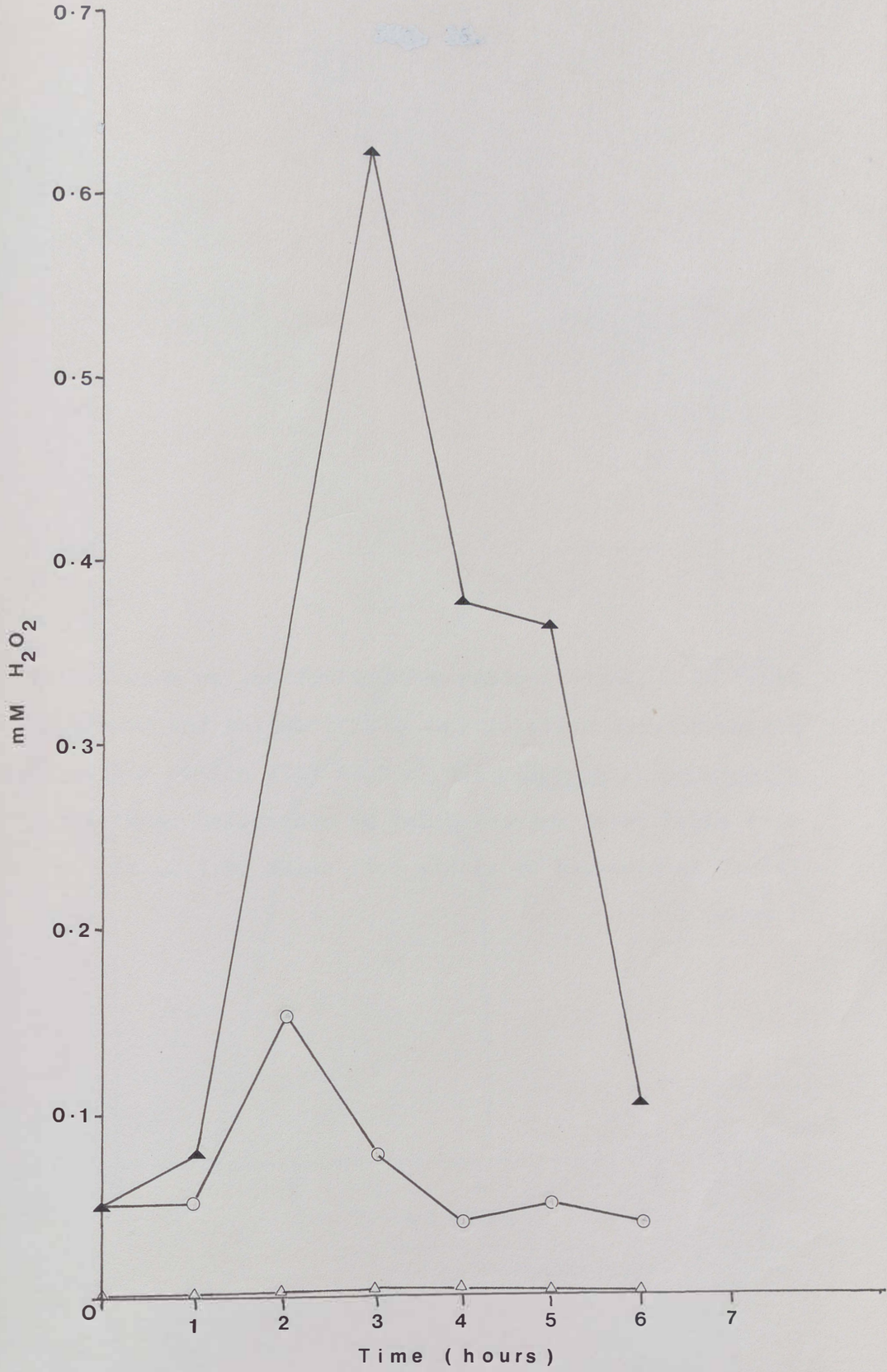


Fig. 26. Production of hydrogen peroxide in a Streptococcus cremoris 320 culture, pH controlled at 6.5, under aerobic (▲——▲) and anaerobic conditions (△——△). Levels for a non pH controlled culture are also illustrated (○——○). Growth medium consisted of 2.5% milk solids with 0.4% added Yeast Extract.

Fig. 27 . Bacteriophage multiplication, as measured by plaque forming units/ml (▲——▲), during the growth of Streptococcus cremoris 320 in 2.5% Milk solids Medium with 0.4% added Yeast Extract under pH controlled conditions. Growth is measured by viable cell count (c.f.u./ml) (○—○).

Fig. 27.

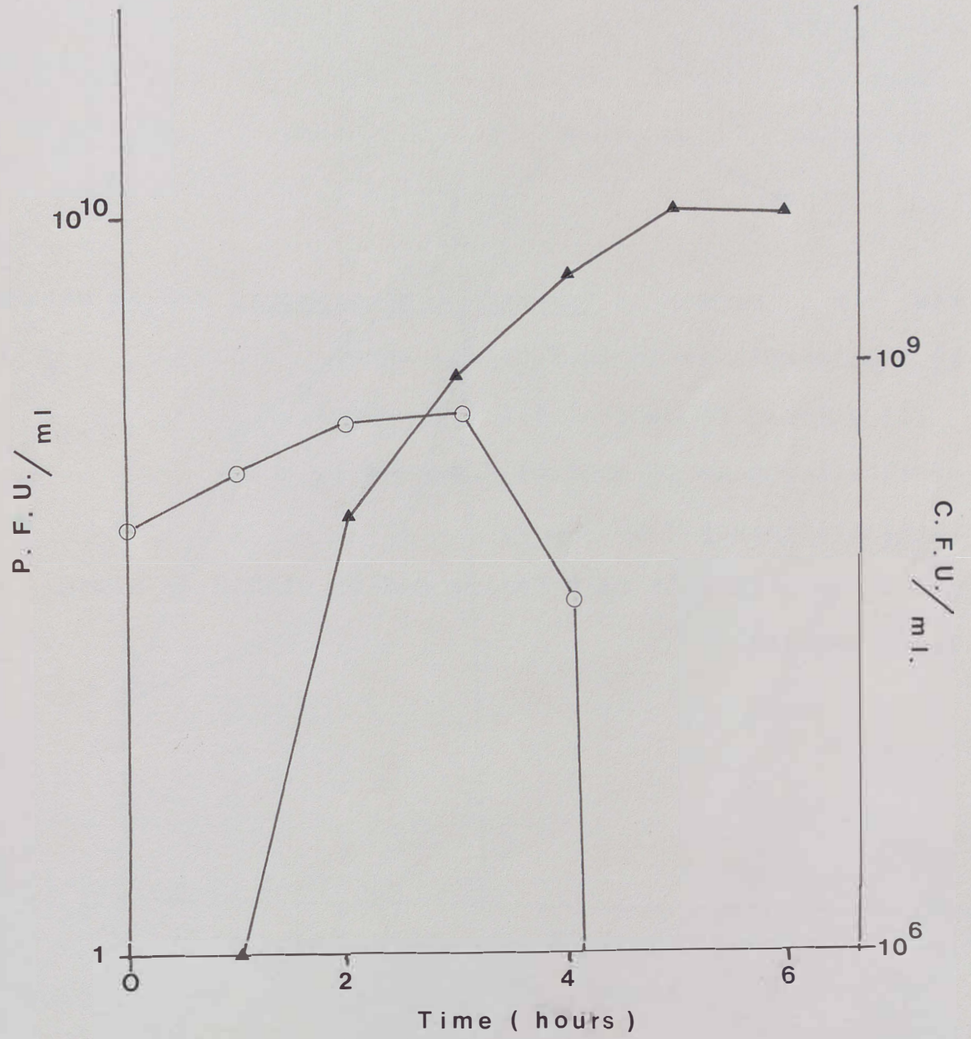


Fig. 28 . Growth of Streptococcus cremoris 320 as measured by viable cell count (c.f.u./ml) under

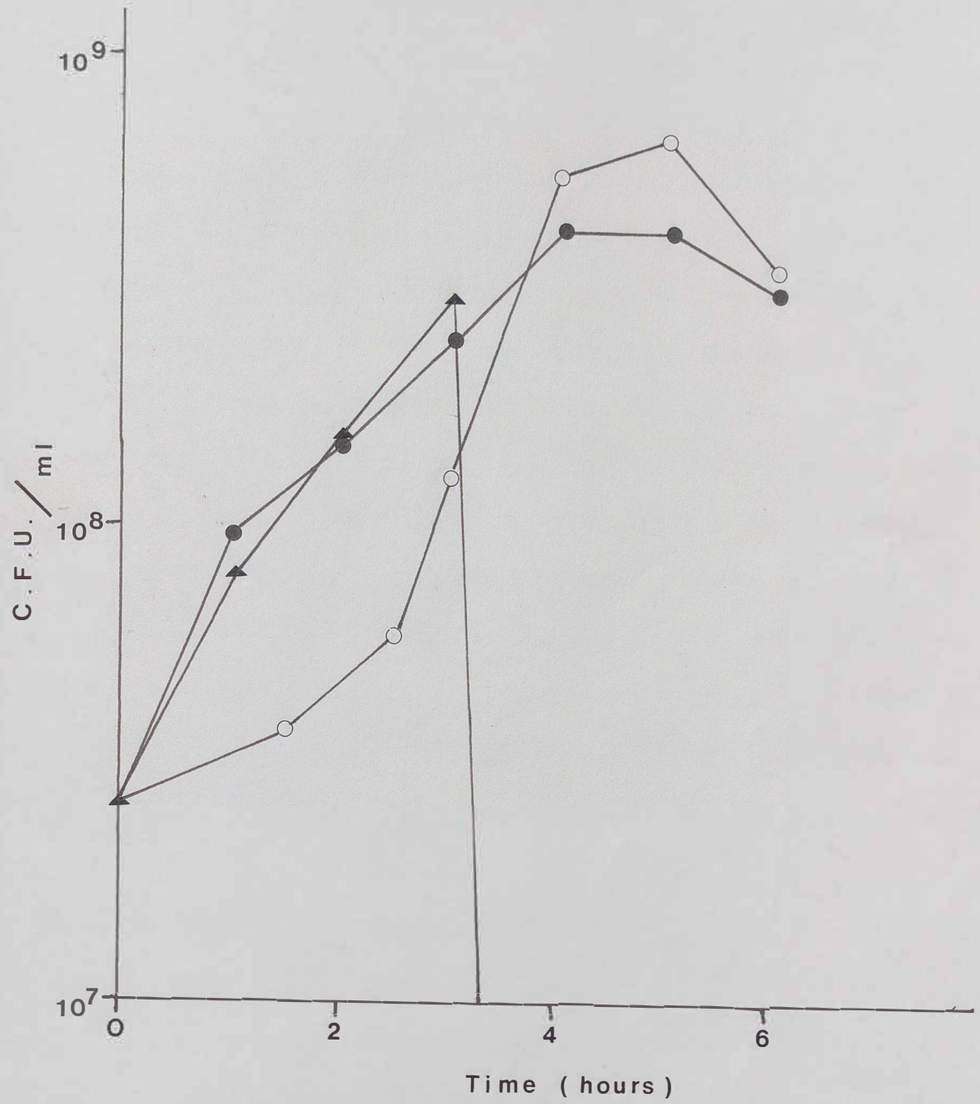
(i) stirred pH control 6.5 (▲——▲),

(ii) stirred no pH control (●——●),

(iii) stationary (○——○),

conditions, in 2.5% Milk Solids Medium with 0.4% added Yeast Extract.

Fig. 28.



to be the critical factor involved in the release of phage lytic for S. cremoris 320. At pH 6.0, as well as 6.5, phage release occurred under pH control. The pattern of phage release at pH 6.0 was similar to that found at pH 6.5, in that, after 1-1¼ hours incubation, lytic phage could be detected in cell free culture supernatants. Rapid phage multiplication occurred, leading to lysis of the culture and cessation of growth. However there was a 10-fold decrease in final phage numbers, due to a lower specific growth rate by the culture at pH 6.0. At pH 5.5, under controlled conditions, S. cremoris 320 grew normally, following a typical lactic growth pattern in low milk solids media, giving a developed acidity of 1.4% lactic acid compared with a final acidity of 0.4% lactic acid at pH 6.5 (Fig. 29). No phage could be detected at any stage of the growth cycle. Phage released at pH 6.5 will propagate, if inoculated at levels of less than two viable particles per ml of fermenter volume after 1 hour, in an S. cremoris 320 culture pH controlled at 5.5, totally lysing the culture in 4½-5 hours at 30°C.

The phenomenon of phage release as shown by S. cremoris 320 seemed to be confined to milk based media whether externally or internally pH controlled. Milk based media of high (10%) or low (2.5%) solids concentration, with or without added yeast extract, when pH controlled, all exhibited the phenomenon of phage release, with the exception of 2.5% milk solids, pH controlled at pH 5.5 (Table 21). Final phage levels, on culture lysis, depended on the growth rate of the culture. In 10% Skim Milk Solids media, while

Fig. 29 . Growth of Streptococcus cremoris 320 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract, as measured by developed acidity (% Developed Lactic Acid) under pH control at 6.5 (□——□) and 5.5 (■—■).

Developed acidity was determined from the amount of 5N NaOH required to neutralise the lactic acid produced by the culture.

Fig. 29.

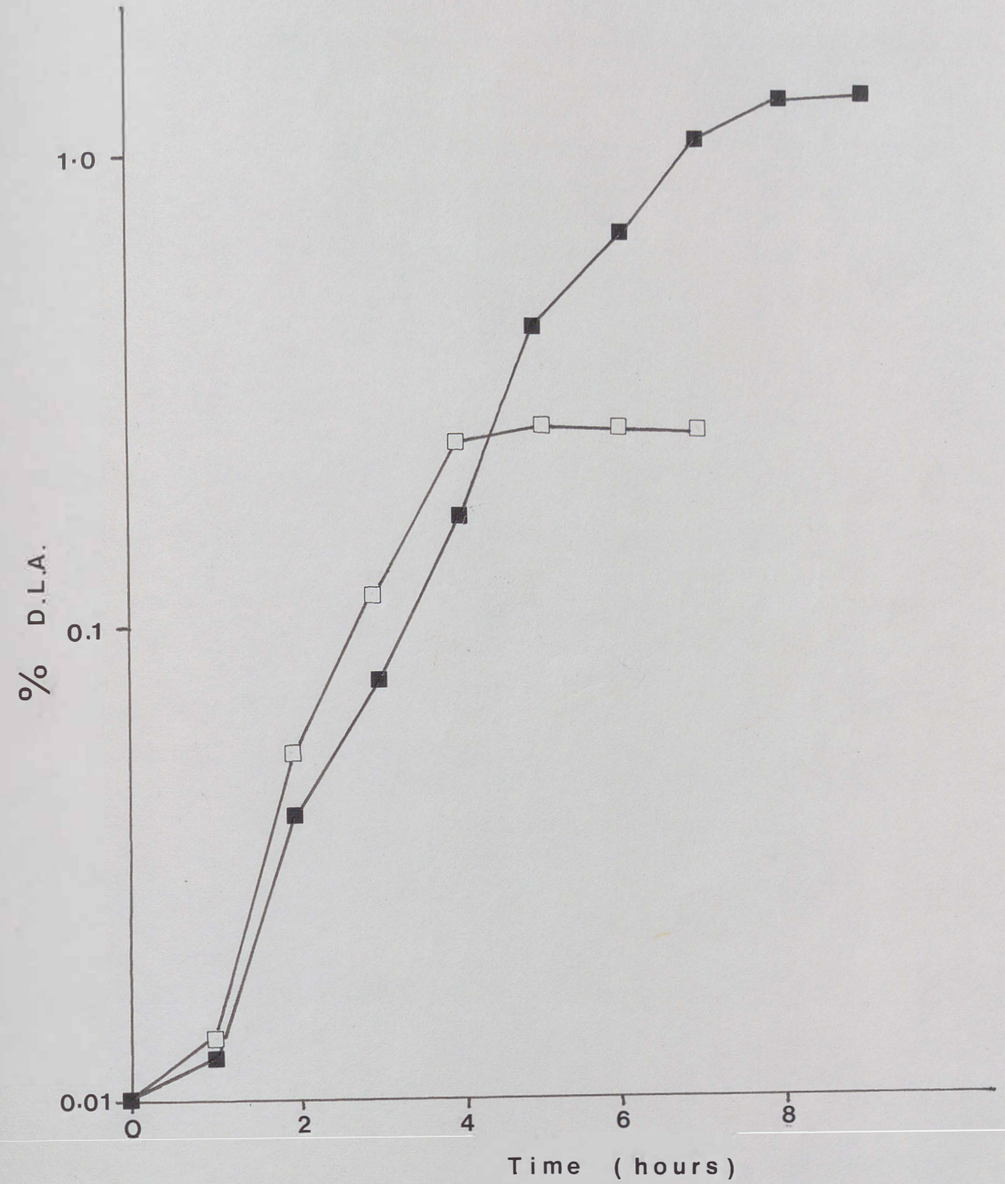


Table 21. The effect of different growth media on bacteriophage release by Streptococcus cremoris 320.

Medium	Controlled pH	Final Phage Titre (p.f.u./ml)
2.5% S.M. + 0.4% Y.E.	6.5	$3.5\text{-}5.0 \times 10^{10}$
"	6.0	2.0×10^{9}
"	5.5	None released
2.5% S.M. + 0.4% Y.E. + B-glycerophosphate	7.3-6.5	5.0×10^{10}
10% S.M. (aerobic)	6.5	2.0×10^{9}
10% S.M. + 0.4% Y.E. (aerobic)	6.5	1.0×10^{10}
10% S.M. + 0.4% Y.E. (anaerobic)	6.5	3.0×10^{10}
L.S.B.	6.5	None released
Phase 4	6.5-5.4	None released

the phage release pattern was similar in all cases, final phage levels depended on whether the medium was supplemented with yeast extract and whether the culture was incubated aerobically or anaerobically.

B-glycerophosphate was also used as a buffering agent from pH 7.3 to 6.5, where 5N NaOH was used to neutralise any acid produced and keep the pH at 6.5. The pH of the medium dropped from 7.3 to 7.16 after 2 hours. At this stage of the growth cycle, low levels of phage could be detected in the fermenter vessel. Before the pH of the culture had reached 6.5, there were greater than 1×10^6 p.f.u./ml present,final levels reaching 5.0×10^{10} p.f.u./ml after 6 hours incubation (Fig. 30).

No phage release could be detected in Lactic Streptococcal Broth (L.S.B.). A slightly longer lag phase and lower cell yield resulted from growth of S. cremoris 320 under pH control in L.S.B. under aerobic conditions at 30°C, compared with a statically grown culture, though both cultures had parallel growth rates (Fig. 31).

In the internally buffered "Phase 4" medium, no viable phage could be detected. Following an initial lag of one hour, the culture grew exponentially, reaching a developed acidity of 1.4% lactic acid (Fig. 32). Inoculation of S. cremoris 320 phage, released in milk under pH control and subsequently plaque purified, into a culture growing in "Phase 4" at low levels (1-2 viable particles per ml of fermenter volume), resulted in failure of the phage to propagate as would be expected in a phage inhibitory medium. However,the phage remain viable and will propagate once the

Fig. 30 . Growth of Streptococcus cremoris 320 in 2.5% Skim Milk solids Medium, with added B-glycerophosphate, as measured by viable cell count (c.f.u./ml) (■——■). Bacteriophage release and multiplication as measured by plaque forming units/ml (p.f.u./ml) (○——○) are also shown.

Note: B-glycerophosphate was autoclaved separately (15 psi x 15 min) and then added to the milk to give a final concentration of 18.9% (w/v).

Fig. 30.

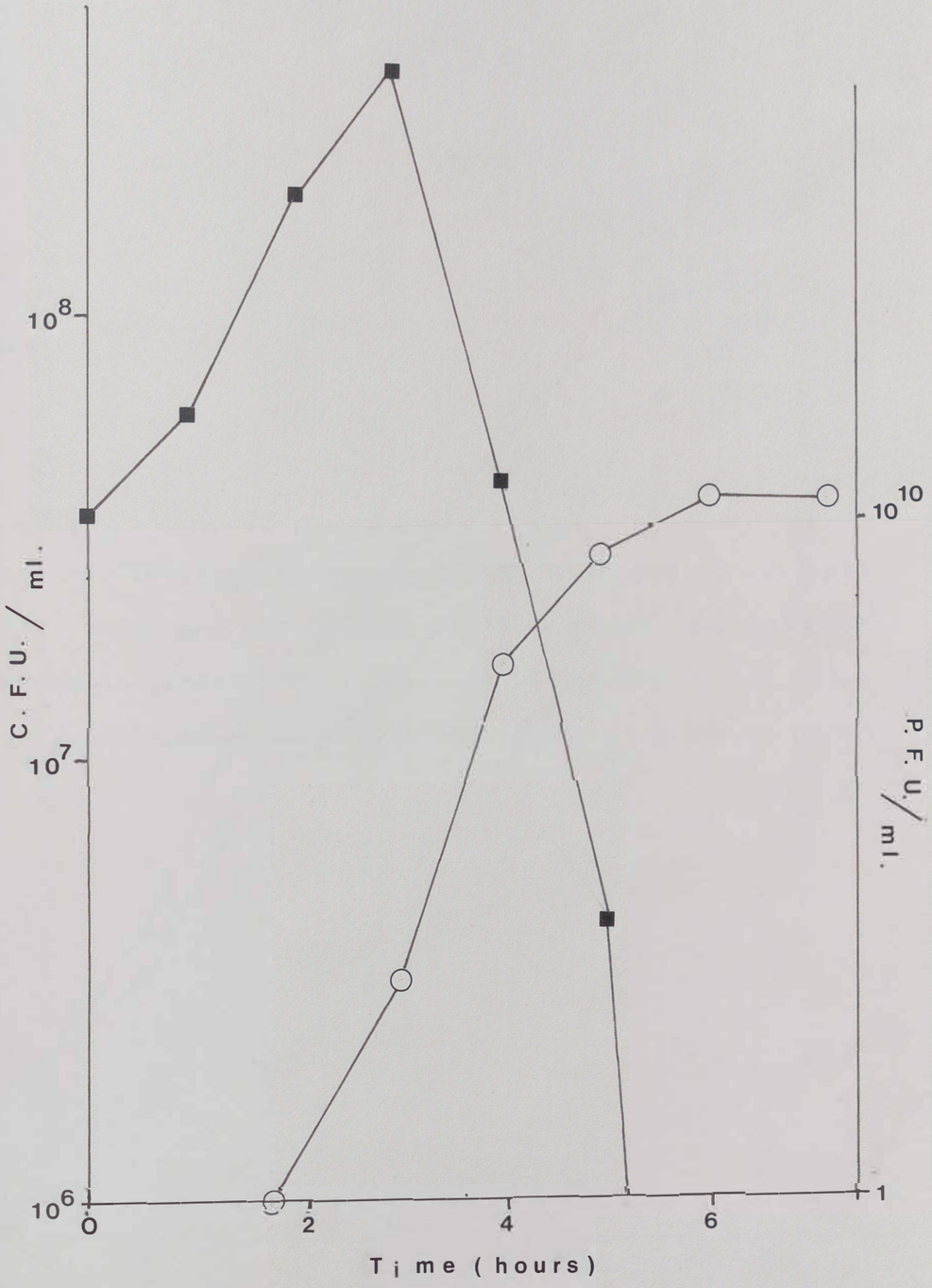


Fig. 31. Growth of Streptococcus cremoris 320 in Lactic Streptococcal Broth under pH control 6.5 (▲——▲) and under non pH controlled (△——△) conditions, as measured by an increase in absorbance at 600 nanometers (OD_{600}).

Fig. 31.

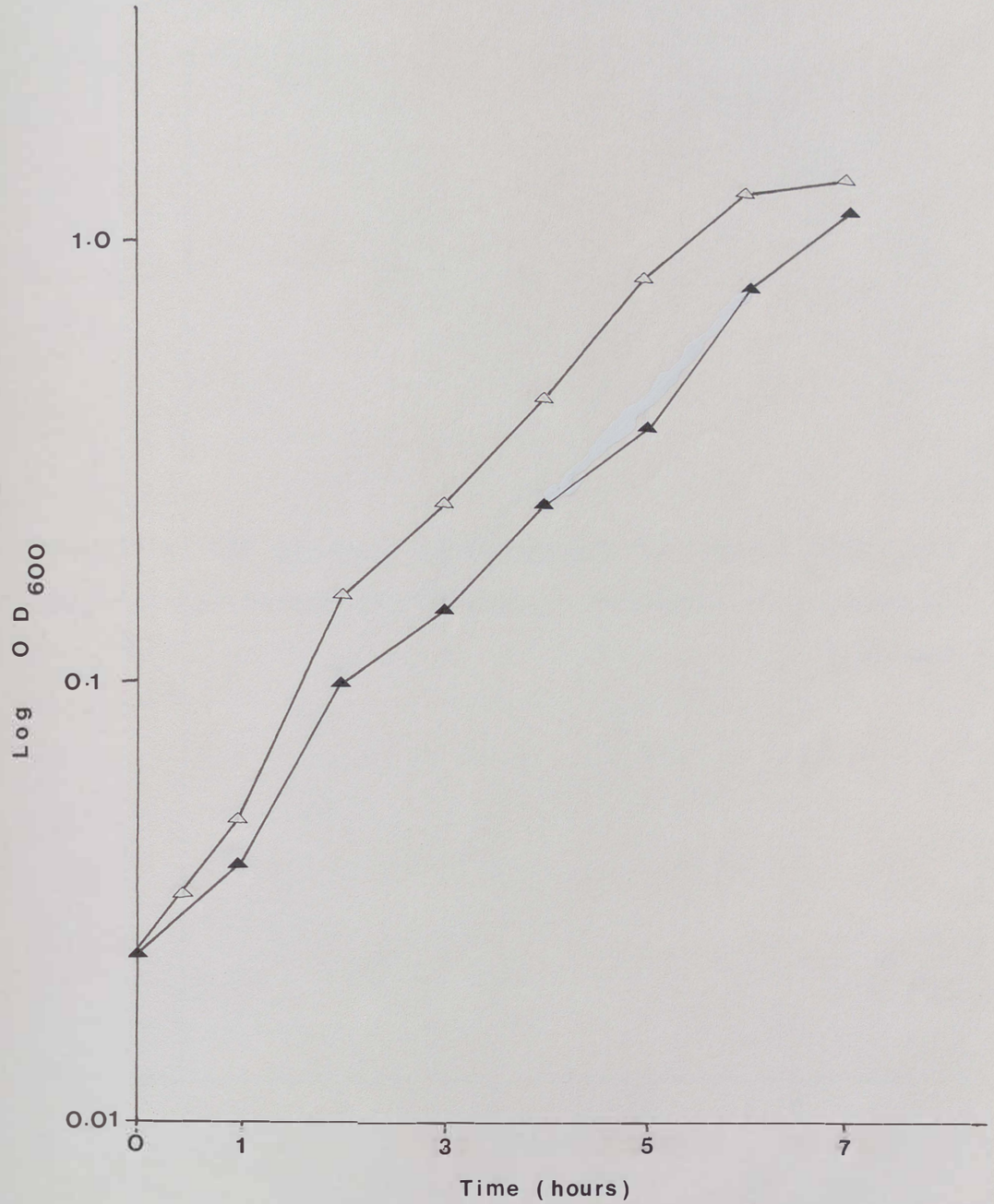
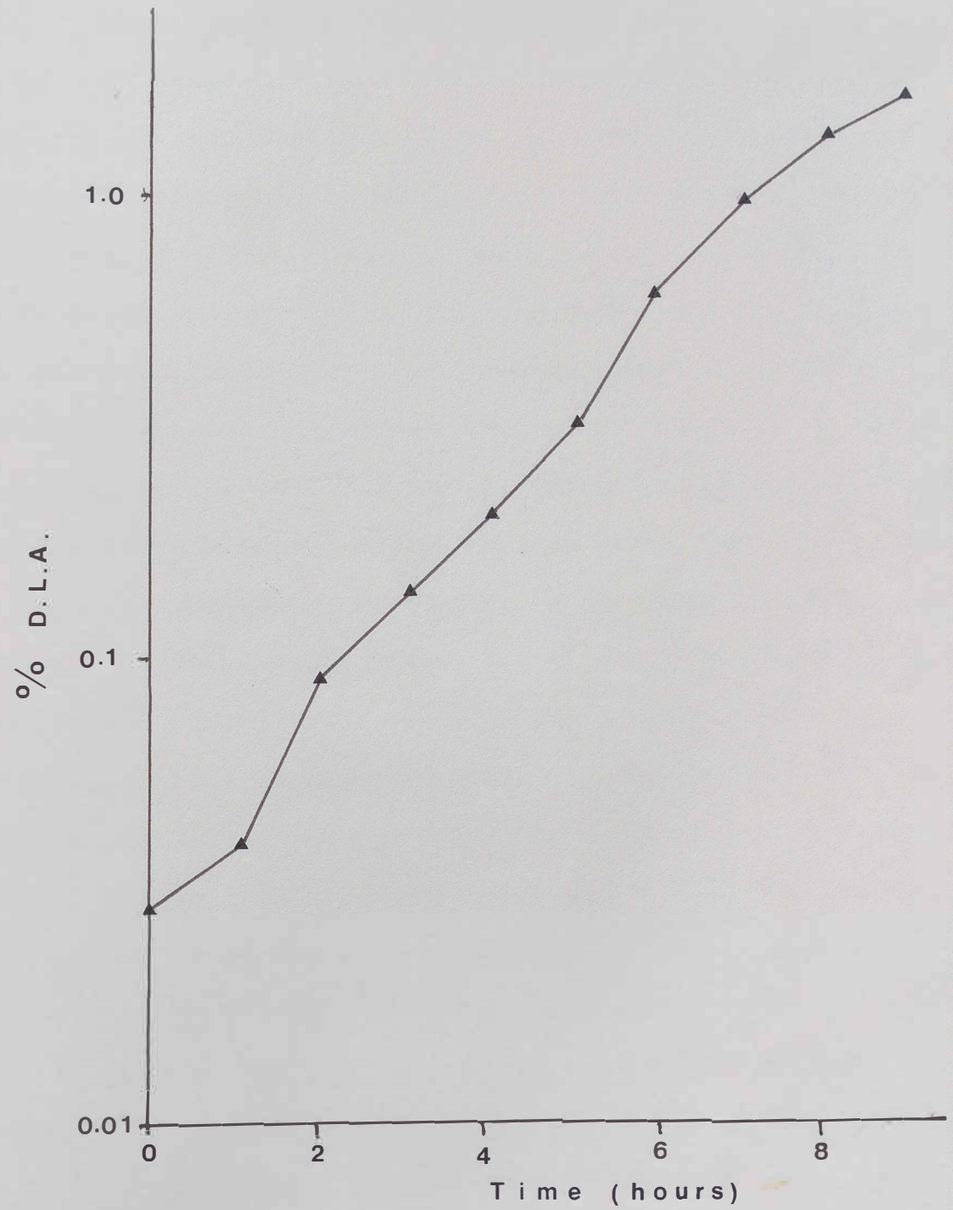


Fig. 32. Growth of Streptococcus cremoris 320 in "Phase 4" as measured by developed acidity (% Developted Lactic Acid) (▲——▲).

Fig. 32.



culture is transferred to milk.

To determine whether or not there were two or more cell types in the S. cremoris 320 culture population, single colony isolates were obtained by plating the culture on L.S.B. agar plates as well as on Fast Slow Differential Agar (F.S.D.A.). Sequential restreaking of single colony isolate was repeated ten times so as to reduce or eliminate the possibility of the culture carrying phage. Plating of S. cremoris 320 on F.S.D.A. showed that 62-80% of the culture comprised fast acid producers while the remainder of the culture was slow (proteinase negative). Slow isolates were also obtained following the restreaking of S. cremoris 320 on L.S.B. agar plates. Both types of single colony isolate were checked against phage obtained from three different sources, namely, a factory cheese vat whey, phage from laboratory stocks and from the S. cremoris 320 pH controlled culture. Results are shown in Table 22. All three bacteriophage isolates plaqued against all the single colony isolates tested. All single colony isolates except for isolate A showed reduced plaquing ability with isolate C having the most restictive plaquing ability.

Isolates A to F were then checked for ability to release phage under pH control in 2.5% Skim Milk Solids Media with 0.4% added yeast extract. All isolates, with the exception of F, were "fast" acid producers, F being proteinase negative. While F did not release phage under pH control, other slow variants did. In all cases, phage release occurred later and final culture lysis took 8-10 hours at 30 °C. This was due to a prolonged lag phase on

Table 22. Evaluation of single colony isolates of S. cremoris 320 using phage lytic for 320 from three different sources.

Phage used:

1. Ø 320 J Ø released under pH control
2. Ø 320 M Ø obtained from factory cheese vat whey
3. Ø 320 A Ø obtained from lab. stocks.

Culture isolate	Ø Titre		
	Ø 320 J	Ø 320 M	Ø 320 A
A	5.5×10^{10}	2.0×10^{9}	6.3×10^{9}
B	4.2×10^{10}	1.9×10^{9}	7.6×10^{9}
C	5.5×10^{9}	9.0×10^{7}	1.2×10^{8}
D	2.0×10^{10}	8.0×10^{8}	7.8×10^{9}
E	4.1×10^{10}	1.0×10^{9}	1.0×10^{10}
F	4.0×10^{9}	5.3×10^{9}	1.5×10^{10}
G	9.0×10^{9}	6.4×10^{9}	9.3×10^{9}
H	3.6×10^{9}	8.0×10^{9}	9.0×10^{9}
I	4.5×10^{9}	7.6×10^{9}	1.0×10^{10}
J	2.4×10^{9}	5.6×10^{9}	1.0×10^{10}

Plaque assay carried out at 30°C.

inoculation of the culture into the pH controlled media. Table 23 summarises the phage releasing ability of isolates A-F. Isolates A, B and C released a phage which gave three plaque types (large, >1mm, clear; small, <1mm clear and small, <1mm hazy) when plaqued on both a broth culture of the isolate and on the S. cremoris 320 from which it was derived. However isolates D and E gave only one plaque type when plaqued on themselves, but when plaqued on the parent S. cremoris 320 or on other single colony isolates again gave the three distinct plaque types outlined above.

Bacteriophage insensitive mutants of S. cremoris 320, when grown under pH control fail to release any detectable phage particles. These mutants, derived from S. cremoris 320, cannot be attacked by phage isolated from the factory environment or by phage from the pH controlled cultures. S. cremoris 320/1 a phage insensitive mutant, after an initial lag of one hour grew exponentially under pH control reaching a developed acidity of 1.4% lactic acid after 9 hours growth (Fig. 33). At no stage could viable phage particles be detected in cell free filtrates of the culture fermenter.

Table 23. Bacteriophage release as measured by plaque forming units/ml, of individual isolates of Streptococcus cremoris 320, grown under pH control 6.5 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract.

Isolate	Final Phage Titre (p.f.u./ml)	Plaque type*
A	4.6×10^{10}	LC, SC, SH
B	8.0×10^{9}	"
C	1.3×10^{10}	"
D	2.0×10^{9}	SH
E	2.1×10^{9}	LC
F	None released	--

*Plaque type

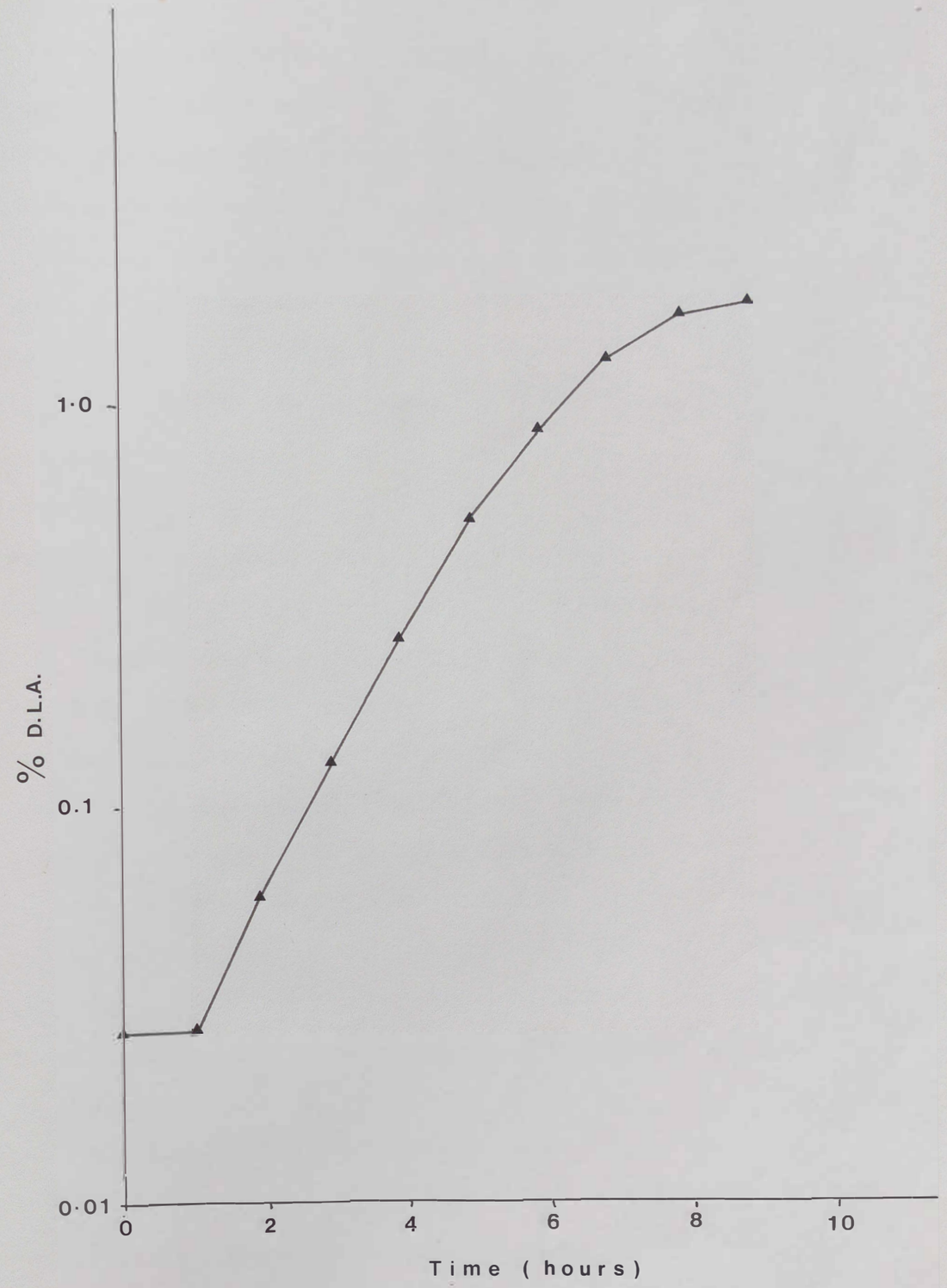
LC = Large Clear

SC = Small Clear

SH = Small Hazy

Fig. 33. Growth of Streptococcus cremoris 320/1 under pH control 6.5 in 2.5% Milk Solids Medium with 0.4% added Yeast Extract as measured by % Developed Lactic Acid (% D.L.A.)
(▲——▲)

Fig. 33.



In the last twenty years the Irish Dairy Industry has been rationalised with the evolution of large scale production units which are highly mechanised and fully automated. Such production units have not been confined to the areas of butter and milk powder manufacture. The cheese industry which mainly produces Cheddar has been streamlined with fewer factories. These factories have, however, increased throughput which has put enormous pressure on the lactic acid bacteria, used as starters, to maintain acid production at a steady rate. This system has to ensure high quality products of desirable flavour, texture and composition without any interruption of manufacturing schedules.

Traditional starter preparation for the cheese industry involves three main concepts. Firstly, most starters used in Ireland were undefined in that they contained an unknown number of strains present in various combinations. Secondly, these starters were mostly phage sensitive and had to be rotated in order to minimise losses due to "dead" vats caused by the build up of bacteriophage in the factory. Thirdly, the starter had to be subcultured in the factory before inoculation into the cheese vat maximising the possibility of phage infection.

The above problems had been tackled as early as the 1940's in New Zealand by Whitehead and coworkers and later in Australia and the United States where Dairy Research Institutions in collaberation with the cheese industry selected the best strains on the basis of flavour, acid

production and resistance to phage. This strain system allowed multifilling of vats with a consistent end product quality as well as simplifying bulk culture propagation and culture supply.

Up the late 1970's undefined strain systems were normal in Irish cheese factories with large, complex rotations being necessary to maintain production in larger factories. Cheesemakers were reluctant to adopt the defined strain system as they had been led to believe that starters always had to be rotated. They also believed that Cheddar flavour was associated only with undefined cultures. Introduction of the New Zealand single strains led to their phaging out due to the differences in the phage pools present in the new environments. As problems increased with increasing production, the Irish Dairy Industry in collaberation with the Dept. of Dairy Microbiology,University College, Cork and An Foras Taluntais Moorepark, attempted to analyse the problem and developed a strain system best suited to the Irish cheesemaking environment.

Selection of suitable cheesemaking strains was carried out using criteria described in New Zealand (Limsowtin et al., 1977; Lawrence et al., 1976) U.S.A. (Daniell and Sandine, 1981; Richardson et al., 1980, 1981) and Ireland (Hurley et al., 1982). Of the two hundred and ten strains screened approximately 35 survived detailed evaluation, including all those strains outlined in Table 1.

As this defined culture system involved the use of individual strains which would be produced in one location, it was therefore logical to understand how the cultures

differed from each other as regards growth characteristics under pH control, as well as survival and maintenance of activity when stored under adverse conditions.

Parameters for the production of starters have already been established. Even though concentration of culture was not employed batch fermentation was considered to be most suitable to prevent loss of activity (Lloyd and Pont, 1973a) or the appearance of undesirable strain variation. NaOH was selected as a neutraliser since cells produced using NaOH have been found to have better activity when compared with equal numbers of NH_4OH neutralised cells, even though the latter gives higher final cell numbers (Gilliland, 1977; Sandine, 1977; Lloyd and Pont, 1973a). Temperature and pH values of 30°C and 6.5 respectively are considered normal for the production of batch concentrates of cells. The advantages of low lactose concentration,in the growth medium, to reduce acid build up and prevent cell injury has resulted in several types of media being used in concentrate production. These media have been both milk based (Lingren et al., 1974; Valles and Mocquot,1968; Lloyd and Pont, 1973a) or artificial (Condon and Cogan, 1973; Peebles et al., 1969; Lamprech and Foster, 1963). No attempt has been made to use skim milk powder diluted to give a low lactose content with nitrogen and mineral complement loss being compensated for by the use of yeast extract.

As can be seen from the results presented, the cultures studied can be divided into three broad categories on the basis of their growth characteristics in the reduced lactose medium.

DISCUSSION

In the first category are the majority of cultures examined, which grew well both aerobically and anaerobically in the 2.5% milk solids medium. While only S. cremoris 310 and S. lactos 021 were examined for growth in media containing differing amounts of yeast extract, results indicate no major difference in the growth patterns or in final cell numbers. This would suggest, that for most cultures a low concentration of yeast extract (0.4%) is sufficient to ensure optimum culture growth in low milk solids medium. Milk contains enough nutrients to allow starter cells to grow under pH control to levels equal to those found in conventional, non pH controlled cultures. Similar results were obtained by Stadhouders and coworkers (1969). They concluded that the addition of 3% yeast extract to skim milk was of little or no advantage in ensuring survival of bacteria or retention of activity.

While colony counts are dependent on the procedure used (Martley, 1972) it has been found that conventional milk cultures contain 1-2 x 10^9 c.f.u./ml compared with pH controlled cultures containing 1 x 10^{10} c.f.u./ml before concentration. These cultures can be used in bulk tanks without concentration (Turner et al., 1979). In Table 3 it can be seen that cell numbers of pH controlled cultures are, with the exception of S. lactis 021, two to ten fold greater than milk cultures grown at 21°C. For S. lactis 021 cell numbers were approximately the same in both instances. This would allow a low lactose medium to be used to produce bulk tank inoculants without concentration.

Following an initial lag period of 1 - 1.5 h these

strains grew exponentially for 4-5 h before entering stationary phase. Maximum cell numbers were reached approximately two hours prior to complete lactose utilization,as measured by continuing acid production. This indicated a lack of nitrogen or growth factors in the medium. As shown previously additional yeast extract did not prevent the uncoupling of acid production from growth indicating that some other factors were involved. These may include, inhibition of protein synthesis (Breheny et al., 1975) or autolysis of the culture (Vegarud et al., 1983) due to the absence of salts such as sodium chloride. Continued incubation during this period did not have an adverse effect on cell numbers for most of the strains listed. However, S. cremoris strains 091, 6 and 310 showed a decline in cell numbers on entering stationary phase of the growth cycle indicating the importance of determining for each strain the optimum harvesting time in a given culture medium.

In the second category were S. lactis 007 and S. cremoris 108 both of which grew poorly in the low lactose medium under aerobic conditions. Developed acidities of 0.60 and 0.77 after 10 h and 8 h for S. cremoris 108 and S. lactis 007 respectively,compared poorly with developed acidities of greater than 1.60 for the other strains studied. Both strains, however, grew well in a similar medium but without pH control, under both aerobic and anaerobic conditions (Fig. 14). Both cultures were susceptible to aeration by stirring, in any growth medium, even 10% reconstituted skim milk. As indicated by Anders and coworkers (1970) agitation of milk or milk based media results in the formation

of hydrogen peroxide (H_2O_2) which inhibits the growth of lactic streptococci. However the presence of yeast extract was not sufficient to decompose any H_2O_2 produced and thereby stimulate growth as has been reported (Selby-Smith et al., 1975), probably due to the low concentration used in the 2.5% milk solids medium.

In the third category is S. cremoris 320, which has been discussed separately (see later), as it appears to be a novel system,demonstrating phage release under specific growth conditions.

Once the optimum growth conditions have been established, each culture has to be harvested quickly,without loss of activity or viability, and usually stored at subzero temperatures to maintain activity. Various differences in freezing survival and recovery have been established for both undefined mixed strain cultures and for defined single strains indicating that all bacteria survive freezing differently depending on conditions used during the pocess. Parameters established as being important include freezing rate, storage temperature and the type of cryoprotectant used. Among the cryoprotectants which have been examined are lactose and glycerol. The use of buffers in preventing freeze injury has not been assessed other than B-glycerophosphate which has been added to lyophilized cells (Speckman et al., 1974). The addition of physiological saline (ringers) has not been examined.

Even though our studies involved the use of a low lactose medium, rapid cooling to 4°C while still under pH control was considered essential. This is because the point of

optimum harvest and the absence of lactose do not occur at the same time (Bergere, 1968; Lloyd, 1973a). Cooling under pH control to 4°C even if lactose is used as cryoprotectant will result in samples being stored at sub zero temperatures, at pH values in the range 6.2 to 6.5. Freezing at these pH values results in better maintenance of activity (Stadhouders et al., 1971; Lamprech and Foster, 1963).

Storage of all strains examined in the spent ferementation medium at 4°C resulted in maintenance of activity for periods up to 14 days. In the case of S. lactis 021, activity was maintained for greater than 2 months at 4°C (Tables 4 and 5). This meant that the low milk solids medium was an ideal medium for the suspension of lactic streptococci at low temperatures without having any deleterious effects on the cells. Similar results were obtained by Lamprech and Foster (1963), the rate of survival at 4°C being dependent on the initial pH of the medium, with cells surviving better at pH 7.0 than at pH 5.0. The addition of lactose to the spent fermentation medium prior to storage at 4°C resulted in a rapid loss of activity, due mainly to the fermentation of the lactose causing a fall in pH from 6.5 to 4.4 - 4.6. Concentrated cell suspensions can be stored at 4°C in the absence of lactose and without any other cryoprotectants are essentially, cell for cell as active as fresh cultures.

This can be contrasted with storage at -20°C in the absence of any cryoprotectant. There was a rapid decline in activity on storage whether the culture was frozen previously at -80°C or not. Most strains differed in their

ability to survive at -20°C without cryoprotectant. For S. lactis 021, stored at -20°C without cryoprotectant a rapid loss of immediate activity occurred which was not mirrored by a significant decrease in cell numbers. Similarily S. cremoris 047 and S. cremoris 091 showed little or no decline in cell numbers for periods up to 150 days. The addition of cryoprotectants such as lactose and glycerol did not have a marked effect on cell viability but did improve immediate activity. Freezing and thawing injures cells lethally and non lethally according to strain and cell wall composition (Morichi and Irie, 1973; Gilliland and Speck, 1974b). Both fatty acid composition and the amount of capsular material present are important in minimising cell injury during freezing (Goldberg and Eschar, 1977). Non lethally injured cells can recover on complex media such as M17, much more quickly than they can in milk at 30°C,where they exhibit an extended lag phase. This time may be required for injury repair prior to cell multiplication (Ray and Speck, 1973). Compounds such as glycerol and lactose enhance retention of activity by protecting the cell surface rather than cell interiors as has been previously shown (Mazur, 1970). Protection of the cell surface is particularly important in the lactic streptococci as it has been shown that the decrease in activity on frozen storage is due to a change in the cell bound proteinase (Speck and Cowman, 1969) or its complete dissociation from the cell (Cowman and Speck, 1967).

Incubation of S. lactis 021 and S. cremoris strains 047 and 091 at 21°C prior to checking activity at 30°C

indicated that most of the cells stored at -20°C were sublethally injured. Incubation of these cells at the lower temperature allows cell repair before metabolic rates corresponding to higher temperatures are attained. This knowledge is particularly useful if the cells are to be used as a bulk tank inoculant or direct vat starter. In the former, time temperature combinations are more favourable to cell revovery than are the temperatures of 31° C to 39°C employed in the cheese vat.

For other strains stored at -20°C, including strains S. cremoris 310 and 6, a decline in immediate activity was mirrored by a corresponding decline in cell numbers. Samples stored with added ringers solution showed the quickest decline in both activity and viability, with glycerol and lactose protecting the cells, glycerol being the more effective. Quick frozen (-80°C in less than ten minutes) cultures stored at -20°C showed the same rate of decline in cell numbers and activity as cultures frozen and held at -20°C. However more cells survived the quick freezing, giving slightly higher activities than cultures frozen and held at -20°C.

The importance of cryoprotectants can be seen from Figs. 20 and 23 where their presence in the freezing medium protected the cells during the freezing process. The greatest initial loss of viability, in the absence of glycerol and lactose, was prior to the first sampling point during the frozen storage. This indicates most cell death occurred during the freezing process not during frozen storage. The absence of cryoprotectants allows the formation

of ice crystals which cause membrane damage and cell dehydration during slow freezing (Thunell et al., 1984). Cell death during rapid freezing,in the presence of excess water,results in small intracellular ice crystal formation (Ray and Speck, 1973). At low temperatures (-20°C) these crystals tend to be unstable, growing to a size sufficient to cause membrane damage. The presence of cryoprotectant binds water, inhibits intracellular ice crystal formation, forming instead more stable extracellular ice (Ray and Speck, 1973; Baumann and Reinbold, 1966). Subculturing of S. cremoris strains 310 and 6 at 21°C did not lead to full recovery of viability or activity as has been reported for the other strains studied. This was especially true when the cultures were stored with added ringers solution, which would seem to suggest that there are at least two mechanisms involved in freeze injury to strains of lactic streptococci, one being reversible under suitable conditions of medium and temperature, the other irreversible leading to cell death.

In the light of the results obtained at -20°C with the strains studed, it can be concluded that the advantages of such storage are outweighed by the disadvantages. This is true for all except short term storage in the presence of cryoprotectant. Indeed the results obtained if applied to mixed strains and defined multiple strains frozen together, would lead to strain dominance as has been previously reported (Cowman and Speck, 1965; Lamprech and Foster, 1963; Efstathiou et al., 1975). This is an important consideration in ensuring the success of a culture system by minimising bacteriophage attack.

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Of the two storage temperatures, 4°C and -20°C, results prove for most, if not all strains, that for time periods of up to two months refridgeration is better than freezing for retention of culture activity. This is substantiated by other workers (Thunnell et al., 1984; Gibson et al., 1965; Lamprech and Foster, 1963).

At -80°C, where freezing is rapid, the presence of cryoprotective agents as has been previously reported (Stadhouders et al., 1969, 1971) does not significantly affect cell survival or subsequent activity. Compared with lactose, glycerol only marginally improved viability for all strains studied, with the exception of S. cremoris 310, where cells with added lactose and ringers solution had lower viabilities than glycerol stored cells. This loss of viability and activity seemed to be irreversible as culturing at 21°C did not lead to full recovery of activity, more so for lactose and ringers than for glycerol protected cells. For S. cremoris strains X and 107, lactose enhanced protection of -80°C stored cells particularly if the storage period was greater than 3 months. This would indicate that some strains of lactic streptococci are susceptible to freezing independent of temperature, rate of freezing and the presence or absence of cryoprotectants.

The importance of cell wall composition to freezing at temperatures between -17°C and -23°C has been established (Goldberg and Eschar, 1977; Gilliland and Speck, 1974). At -80°C no correlation between cellular composition and decline in activity has been reported, except for Lactobacillus bulgaricus. Incorporation of Tween 80 in the

growth medium aided in the resistance to liquid nitrogen freezing (Smittle et al., 1972), by changing the cell wall fatty acid composition (Smittle et al., 1974). Tween 80 in the freezing medium did not have the same effect, indicating that cell wall composition was the important factor in preventing cell death in strains prone to freeze injury.

For some strains, notably S. cremoris 6, 091 and 310, cell numbers appeared to rise during storage particularly at -80°C. This rise is unexplainable but similar results with other strains of lactic streptococci have been reported (Thunnell et al., 1984; Baumann and Reinbold, 1966).

From this work it can be concluded that the low lactose medium studied is suitable for the growth of defined strains of lactic streptococci and the spent medium is as effective as "Phase 4" in maintaining activity on refrigeration as shown by Birlison and Stanley (1982). The addition of cryoprotectants allows the cells to be stored at -80°C for periods up to six months without loss of activity. In this condition, the cells could be used as a bulk tank inoculum, without the need for preincubation or mother culture handling. Concentration by centrifugation would be the next step in producing direct to the vat starter.

While the strains studied generally grew well in the low-lactose medium, one strain, S. cremoris 320, did not grow properly under pH control. Growth of this strain followed a normal pattern at the beginning of the fermentation but within $4\frac{1}{2}$ h growth, as measured by lactic acid production, had ceased. No further acid production could be detected on continued incubation even though batch non-pH-controlled

cultures grew to a pH of 4.1 before cell numbers finally declined from the maximum obtained - 7×10^8 c.f.u./ml. Measurement of cell numbers in the pH-controlled culture showed a peak of 3×10^8 c.f.u./ml with numbers dropping to between 10 and 50 c.f.u. per ml.

Possible causes of inhibition of lactic streptococci include the accumulation of H_2O_2 (Selby-Smith *et al.*, 1975; Anders *et al.*, 1970) or lactate (Peebles *et al.*, 1969; Blaine *et al.*, 1970) or the presence of bacteriophage. In the case of *S. cremoris* 320 lactate concentration was ruled out as the cause of culture inhibition since growth had ceased after only four hours incubation. In addition, the culture had "died", not entered stationary phase, as measured by viable cell counts. H_2O_2 was excluded as the cause of culture death by growing a pH-controlled culture under anaerobic conditions. No H_2O_2 was detectable under such conditions, yet the culture "died" at a rate comparable to an aerobically-grown culture containing six times as much H_2O_2.

Evidence for phage attack causing culture inhibition in the production of cell concentrates of lactic streptococci has been documented (Lloyd and Pont 1973c). Examination of cell-free filtrates from the fermentation of *S. cremoris* 320 under pH control revealed the appearance of phage after 1-1½ h. This phage then multiplied rapidly, lysing the culture almost completely in 4 h.

The origin of phage in the cheese factory environment has been the cause of considerable arguement over the past three decades. In the case of *S. cremoris* 320, the source of phage was either through external contamination or the

culture itself.

External contamination could possibly be due to the growth medium, the growth environment or from the fermentation equipment. In the case of S. cremoris 320, external contamination can be ruled out because of a number of factors. Firstly, the fermentation equipment was sterilised by autoclaving and the growth medium was heated at 110°C x 10 min prior to use. When duplicate samples, one under pH control, the other non-pH controlled were run phage was not detected in the latter even though greater than 10^{10} phage/ml were present in the adjacent vessel. The most conclusive proof that the source of phage was not external comes from the fact that S. cremoris 320 could be grown under pH control at pH 5.5 without inhibition by phage, even though the phage obtained from S. cremoris 320 filtrates was capable of replicating on S. cremoris 320 at pH 5.5 when added exogenously.

This suggests that the source of phage released under pH control was S. cremoris 320 itself. Apart from the lytic cycle of phage infection lactic streptococci and their bacteriophages can coexist in two other states, lysogeny and the phage carrier state.

While most lactic streptococci are considered lysogenic, the scarcity of indicator strains has greatly retarded research in this area. Classical lysogeny, as demonstrated for lactic streptococci by Gasson and Davies (1980), involves the inability of induced phage to plaque on their lysogenic hosts unless the hosts were prophage cured. The phage - host relationship as shown by S. cremoris 320 does not appear to be explained by the criteria established for classical

lysogeny. While the phage was released by single colony isolates, the culture was not immune to this released phage. If some of the S. cremoris 320 population were prophage cured only partial lysis of the culture would occur on release of the lytic phage. In addition, the recognised inducers of lysogenic phage such as U.V.-light and mitomycin-C, did not stimulate the release of phage particles lytic for S. cremoris 320.

The phage-carrier-state (pseudolysogeny) is established by a virus forming a persistent but incomplete, lytic association between itself and the host cell (Barksdale and Arden, 1974; Limsowtin and Terzaghi, 1977). While the phage-carrier-state can be eliminated by repeated single colony reisolation, the system described using S. cremoris 320 was stable for ten successive reisolations.

As all the cells in the population seem to have the latent ability to release lytic phage, failure to do so during routine propagation in milk suggests that cultivation under abnormal conditions such as pH control placed a stress on the bonding between phage and host, possibly at the cell wall level, leading to release of already formed intact phage particles, which under normal batch growth conditions were not be able to dissociate from the host cell. Phage detection was dependent on the pH at which the culture was grown. It has been reported (Terzaghi and Sandine, 1975) that a decline in pH results in restriction of streptococcal phage plaques, while the use of B-glycerophosphate in M17 medium is important in enhanced plaque formation, as well as better culture growth.

The phage-release phenomenon was observed in milk based media to which no Ca^{++} chelating agents had been added but not in the phosphated "Phase 4" medium. However, Huggins and Sandine (1977) have reported phage induction in commercial phage inhibitory media and also that such inducton is Ca^{++} independent. Spontaneous induction of phage under conditions differing from those required for U.V. and mitomycin-C induction has also been reported (Meister and Ledfored, 1979), while Lloyd and Pont (1973c) reported difficulties with phage in the continuous production of culture concentrates in milk-based media.

While work with other lactic streptococci has resulted in no phage-related problems in growing cultures under pH control, the findings with S. cremoris 320 show that care has to be taken when exposing lactic streptococci to conditions involving pH control for prolonged periods. The increasing acceptance of pH control by the dairy industry to increase bulk tank capacity (Heap and Richardson, 1984; Limsowtin et al., 1980) may lead to altered environmental conditions suitable for phage induction or phage release from a previously stable phage carrier state.

At present S. cremoris 320 represents a model situation where a defined strain releases phage that are lytic for the releasing culture. The extact nature of the phage-host relationship could possibly be established by the use of phage-antisera. This would determine whether or not the phage particles are bound to the cell wall in a manner that allows release if exposed to prolonged environmental stress. This, in addition to prophage curing, could either eliminate

areas where the virus may be associated with the cell or, alternatively, establish the mechanism whereby this phage-host relationship, normally stable, can be "shocked" into phage release and culture lysis.

The phage-associated inhibition of S. cremoris 320 when grown under pH control is an interesting finding. Further research is warranted to determine the nature of the phage-host relationship involved. In practice, this strain could not be used in a system which otherwise proved succesful for the growth of lactic streptococci.

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