A STUDY OF MAKING CONCENTRATED STARTER CULTURE FOR MALA PRODUCTION IN KENYA WITH SPECIAL EMPHASIS ON SMALL SCALE MANUFACTURERS.

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I affectionately dedicate this work to my wife Cecilia Kanyi, daughter, Mbui and two Sons, Manu, and `Nga'u, for their love and care which has given me the will to carry it on to completion.

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ABSTRACT.

The overall objective of this study was to produce a bulk concentrated mala culture for use directly in mala production by small scale mala producers. `Mala´ is a sourmilk product obtained by culturing pausterised milk with a liquid culture composed of *Streptococcus cremoris, S. diacetilactis* and *Leuconostoc citrovorum*. It is an improved version of a Kenyan traditional sourmilk product called `maziwa lala´. The concentrated mala culture should keep well under available preservation conditions in order to give the small scale manufacturers with adequate stocks to last them a convenient time period before replenishing. The growth of the culture was investigated in three media, namely skimmilk, whey and tryptone, both when buffered with calcium carbonate or not. It was then harvested by centrifugation and resuspended in the concentrated form in 10% sterile milk. The keeping quality of the concentrated culture was evaluated under frozen and freeze-dried conditions. Lactose, yeast extract, glycerol and glycerine were used as cryoprotective agents during the keeping quality studies. During the storage studies the culture was tested for its viability, species balance, activity and its functionality in mala

production.

It was found that the three media were equally good in supporting growth of the culture during preservation period. However, buffered whey was preferred because it is a by-product in cheese-making and was more appropriate in terms of ease of centrifugation and cell yield. The cryoprotective agents improved the keeping quality of the deep frozen culture. Glycerol and Glycerine were however more effective and could help maintain the quality of the culture upto 4 months. Temperatures at -32^{o}C were better than -20°C in this regard. The concentrated mala culture produced mala of acceptable quality. Quantities of 500g were sufficient to process upto 100 litres of milk into acceptable mala,compared to 3 litres of mala culture prepared conventionally. The processing of mala was accomplished by direct inoculation of 100litres of milk without sub-culturing or pre-incubation. Freeze drying of the culture lowered its viability and its ability to produce lactic acid. It also changed the species balance by lowering the proportion of *S. diacetylactics* and *L. citrovorum* responsible for aroma production. Deep freezing also reduced the viability and acidulating property of the culture, as well as lowering the proportion of the *S.*

diacetylactics and *L. citrovorum* but to a lesser extent. However, both freeze dried and deep frozen cultures managed to produce acceptable mala.

It is concluded that mala culture can be concentrated and preserved under the conditions of this study. The most economical method would be to grow the mala culture in whey buffered with calcium carbonate, concentrate by centrifugation and preserve by deep freezing at -32°C using glycerine as the cryoprotective agent.

1; INTRODUCTION.

Concentrated starter cultures (CSC) are defined as starter cultures that are grown under closely controlled conditions, concentrated into a small volume and frozen or dried for storage or transportation (25). They can be used for direct inoculation into the product vat (known as Direct-vat-starters or Superstarters), or for preparation of the bulk culture that can be used in the product vat. The CSC are advantageous as compared to conventional starter culture in their simplicity, reproducibility and security (10).

CSC have been used in the developed countries for the last twenty five years (91). This tended to solve the problems of inefficiency and culture failures that hitherto were manifested in the dairy industry of propagating their own starter cultures. According to Charmeymon, 1986, use of CSC accounts for 10-15% of all starters used in the developed countries and the proportion is increasing.

Concentrated starter cultures have been used successfully for preparation of various dairy products and especially cheese. This has worked particularly well with single strain streptococci cultures. Not all cultures can be concentrated however. It is necessary that research be done into concentration of every starter culture, and especially the mixed species types for making

various products (18,25,64,72,95).

The dairy industry in the developing countries depends on imported lyophilised cultures. These have to be propagated several times before use as a bulk starter culture. The propagation process is not only tedious but also expensive, requiring qualified labour, special equipment and transfer rooms. It is a risky process that calls for specially trained technicians to avoid possibilities of contamination with bacteriophage and other micro-organisms (25,102). To minimise these risks cultures are used for a short time (2-3 weeks only), before they are replaced (47). This, in effect means that the dairy industry in developing countries is compelled to import lyophilised cultures continuously, which is cumbersome and at times impossible due to non-availability of foreign exchange.

'Mala' is a cultured milk product that was introduced to the Kenyan market by the Kenya Co-operative Creameries (KCC) in the early seventies. It was later withdrawn due to problems of production. It is an improved version of the traditional consumed sourmilk product called 'maziwa lala'. The latter is obtained by spontaneous lactic fermentation of unpasteurised milk. Kurwijila, 1980, recommended the use of a mixed species mesophilic type culture to make a

product similar to mala, that was acceptable to the Kenyan consumers. KCC adopted this method and re-introduced the product in the market. Other small scale manufacturers joined in to make similar products with different brand names like 'Moori' in Nyeri. Wangoh (101) conducted research into flavoring of mala with locally available fruit pulps. This is yet to be commercialized but experience shows already that there is a fast increasing demand in consumption of mala and other cultured products in Kenya.

Some manufacturers of mala and similar products in Kenya rely on frozen liquid cultures prepared in the Department of Food Technology and Nutrition of the University of Nairobi. Others still use the traditional spontaneous fermentation method. The spontaneous fermentation method cannot guarantee quality reproduction. The frozen liquid cultures are liable to changes or even failure during their propagation through contamination. Furthermore the small-scale manufacturers may not posses or even afford the basic facilities necessary for culture handling. The production of concentrated starter cultures in Kenya that can be added directly to the product vat or to make bulk starter would therefore be a very welcome development to these manufacturers.

The aim of this work, therefore is to investigate possibilities of producing a

concentrated starter culture for mala production in Kenya. The underlying objectives are:

1. To develop and evaluate a suitable growth medium and growth conditions for mala culture to ensure high cell yields.
2. To concentrate the cells of mala culture and asses their viability and functionality in mala production, when used directly for mala fermentation.
3. To evaluate the keeping quality and viability of Mala CSC under storage conditions available to and affordable by the small scale dairy manufacturers.

2. LITERATURE REVIEW

2.1. Starter cultures

Starter cultures are bacterial cells available in liquid, lyophilised or concentrated forms (46,53,83). They may be present as single strain, multiple strain or mixed species (46). The strains are selected on rapidity of growth and lactic acid production, aroma and carbon dioxide production, viscosity of final product, lipolytic and protelytic activities and for resistance to bacteriophage attack (21, 46, 83).

There are two types of starter cultures (25).
1) The Thermophilic starters, also known as rodcoccus cultures are made of rod-shaped bacterium, usually *Lactobacillus bulgaricus, L. lactis* and/or *L. helveticus,* and include the coccus-shaped bacterium *Streptococcus thermophilus.* They are highly acidifying and have an optimum temperature at around 40^{o}C. Yoghurt is a typical product made by this type of culture (i.e. *L. bulgaricus* and *S.thermophilus*). The acidity of yoghurt can go up to 2.1% with a pH well below 4 and therefore this product has to be refrigerated in order to maintain a pH of 4.2-4.6 (46).
2) Mesophilic starter cultures made of bacteria in Group 14 of Bergy's Classification (7) which comprises of gram positive cocci, family

Streptococcaceae and genus Streptococcus and Leuconostoc. The term Lactic Streptococci includes three gram positive, homofermentative, catalase negative bacteria that occur in pairs or chains. They are *Streptococcus lactis, S. cremoris* and *S. diacetilactis*. The main species used from the genus leuconostoc is *Leuconostoc cremoris* (formerly *Betacoccus cremoris* syn. *L. citrovorum*, which is a heterofermentative, and utilizes lactose to produce lactic acid, acetic acid and carbon dioxide (figure 1) (7,58). *L. cremoris* together with *Streptococcus diadetilactis* utilize excess pyruvate to produce carbon dioxide, acetoin and diacetyl in the presence of citrate (figure 2) (7,58).

Mesophilic cultures are mild in acidification, stopping fermentation at 0.8-1.0% lactic acid (25). They have an optimum temperature between 20 and 30°C (25). Advantages over thermophilic cultures are that they do not require external heating during incubation, nor refrigeration at the end of fermentation to prevent overacidification. They have been used to produce buttermilk, sourmilk, sourcream and other mildly acidified dairy products such as mala (46). They can be classified according to aroma production as follows;

(a) B-type; Those containing only *L. cremoris* as the aroma producer.

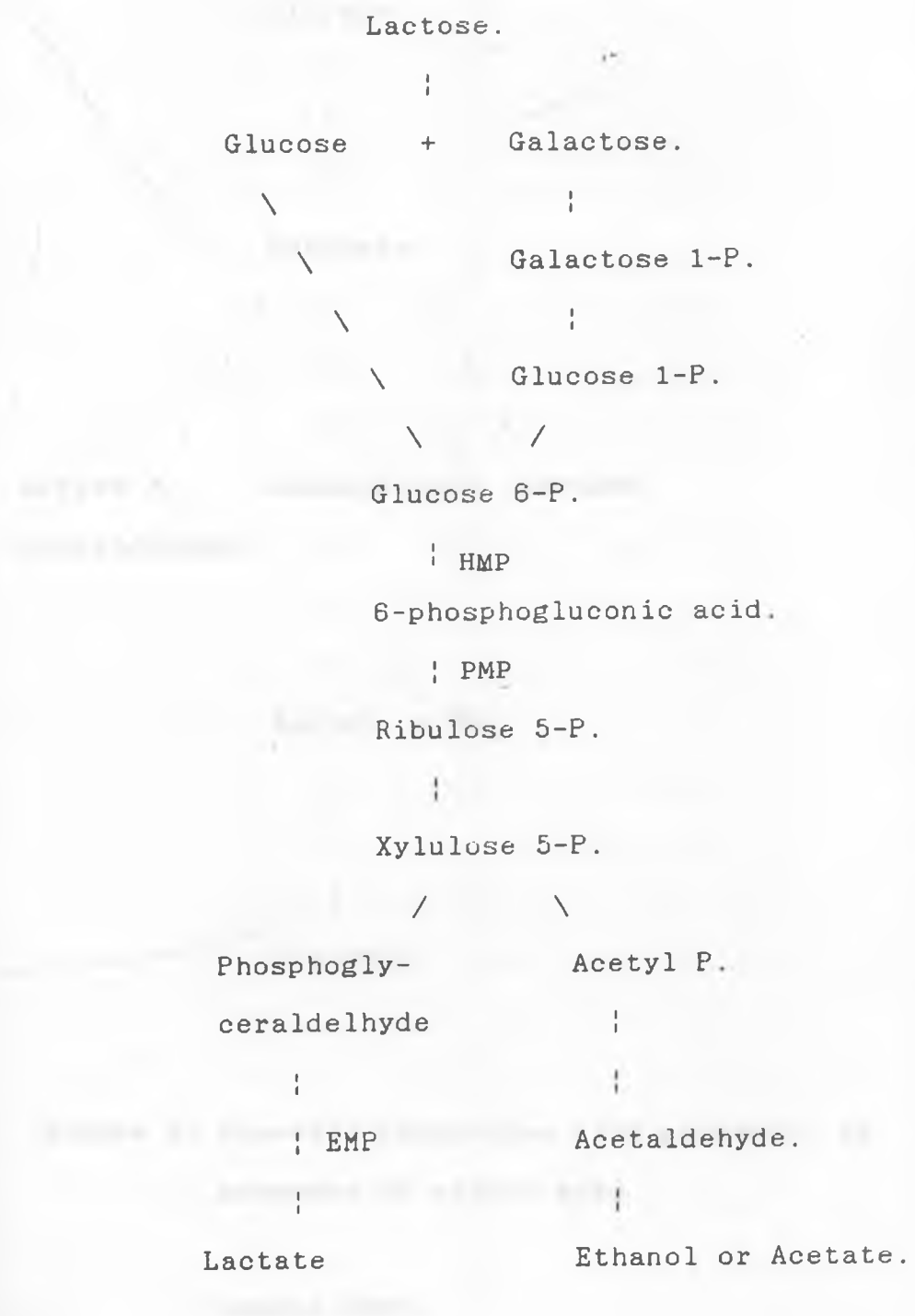


Figure 1: Heterofermentative transformation of lactose.

Source (58).

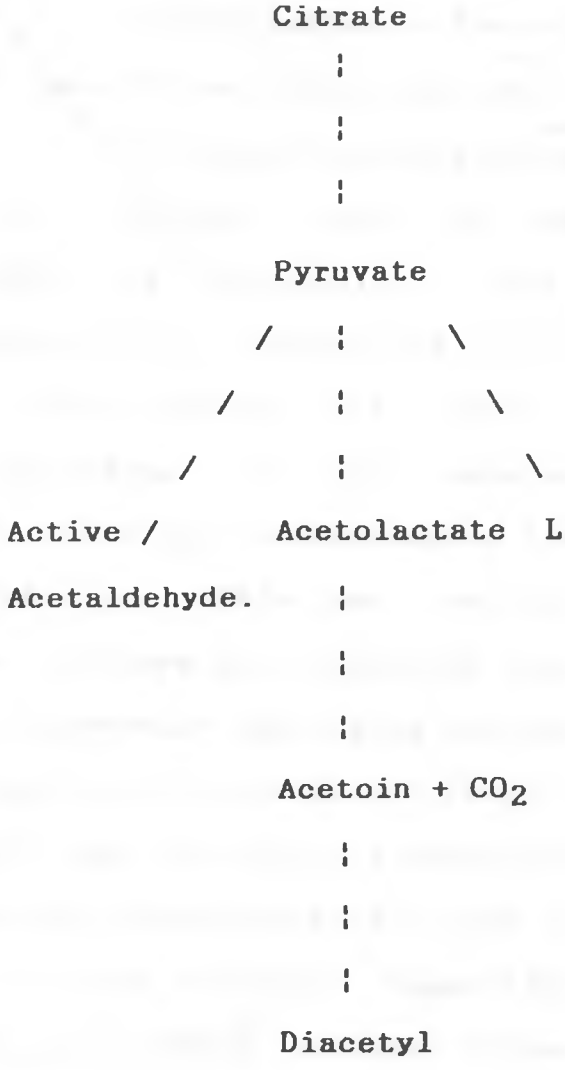


Figure 2: Diacetyl production from pyruvate, in presence of citric acid.

Source (58).

(b) D-type; Those containing only *S. diacetilactis* as the aroma producer.

(c) BD-type; Those containing both *L. cremoris* and *S. diacetilactis* as aroma producers.

The culture used to make mala is classified as mesophilic BD-type culture consisting of *S. lactis* 1-5%, *S. cremoris* 85-95 %, *S. diacetilactis* <5 % and *L. cremoris* 5-10% according to the manufacturers (Chr. Hansen's Laboratorium Copenhagen) (107).

Robinson, 1981a, has reviewed the dairy starter cultures and classified them according to their preservation methods as follows; (a) liquid, (b) frozen (short term at -20 to-40°C and long term at -196°C) and (c) dried (freeze-dried standard and freeze-dried concentrates for both bulk starter or direct to vat starter). Roger,1981, lists four classes i.e. liquid culture, frozen concentrated cultures, lyophilised cultures and direct to vat inoculation (DVI) cultures. A review by Russel,1983 gives seven classes namely, liquid, frozen liquid, freeze-dried, frozen concentrated, freeze-dried concentrated,frozen direct to vat and freeze-dried direct to vat cultures.

2.1.1. CONVENTIONAL STARTER CULTURES.

The consumption of fermented milk dates back to thousands of years. Fermentation was then caused

by contaminating organisms present in the milking shed and residing in cracks and crevices of milk containers (25). The initial step towards the development of the culturing process, that we know today, was the use of a portion of the cultured product to add to fresh milk. This led to the discovery and characterization of the fermenting bacteria and to the practice of inoculation with pure cultures of starter bacteria in the early part of this century (25). Further improvement came with the technology of lyophilising culture bacteria for long storage and ease of shipment.

2.1.1.1. LIQUID STARTER CULTURES.

Liquid starter cultures are bacterial suspensions in liquid form, used to make the bulk starter through serial transfers or propagation to increase the activity and volume of the culture. Russell, 1983, has shown the method of propagation as illustrated in Figure 3.

Liquid starter cultures are maintained by daily transfers in a suitable medium (25,47,82). However, these transfers can only maintain the culture in good performance for 2-3 weeks. During this time changes in the culture occur which result in slow or non-starters, faulty flavours, over- or under-acidification, undesired viscosity etc. These changes are attributed to improper

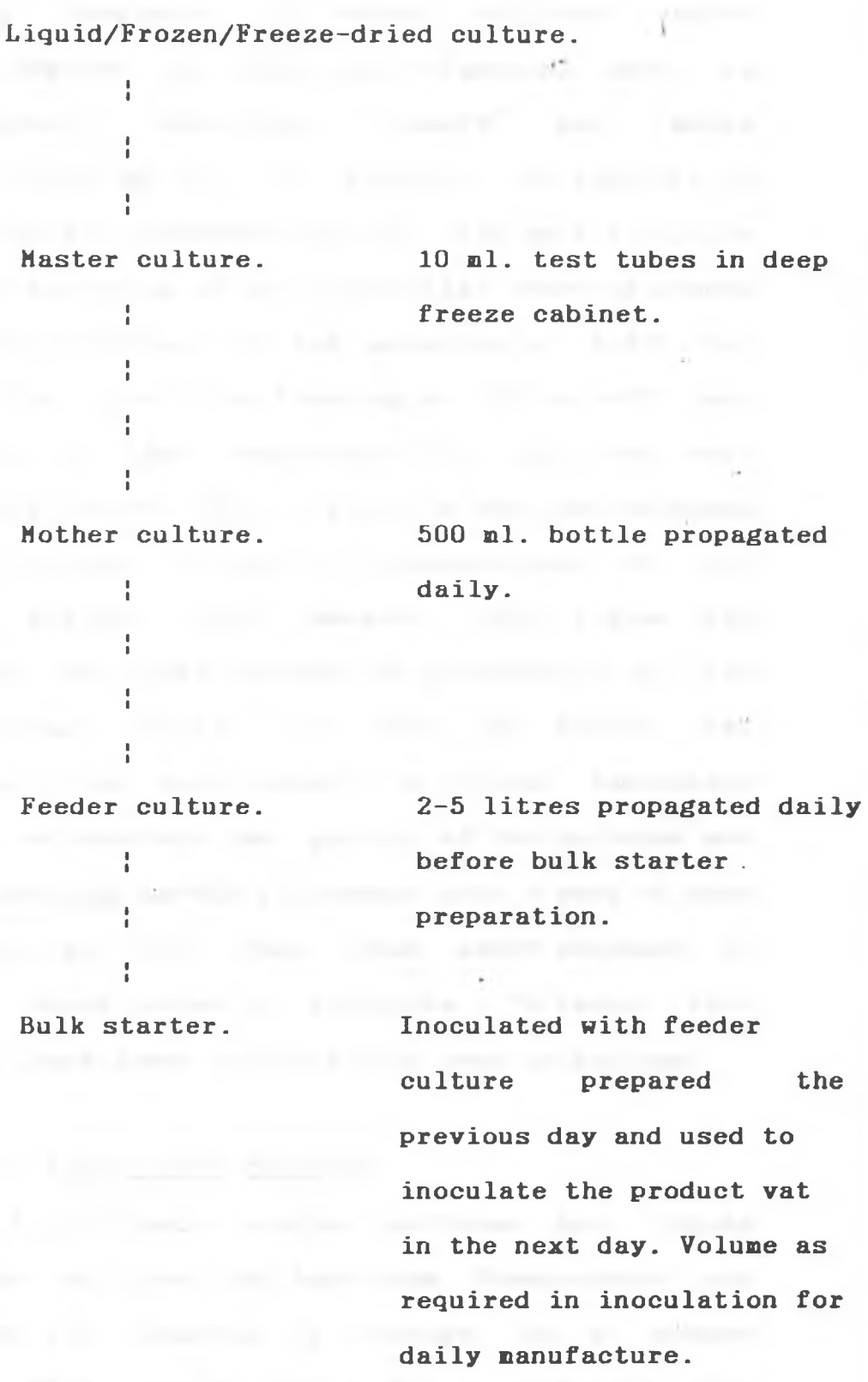


Figure 3: Propagation of starter cultures.
Source (82).

propagation, bacteriophage attack, species or strain imbalance in mixed cultures and/or contamination by other micro-organisms such as coliforms, Baccillus, yeasts and molds (11,21,23,80,83,92). To maintain the activity of cultures for prolonged periods, the active culture at the beginning of the exponential phase of growth can be preserved by refrigeration at 5-8°C for upto ten days or by freezing at -20 to -40°C and storing at that temperature for upto one year (21,46,47,66,96). The culture is then sub-cultured for further storage or preparation of the bulk starter. This, however, still leaves the tedious and risky business of propagation to the processing plants. To ease the burden, the manufacturers have turned to a central laboratory which can maintain the quality of the cultures and send the same to the processors once a week or once a fortnight (80). This often means shipment of bulk loads which is expensive. To reduce this cost, lyophilised cultures have been introduced.

2.1.1.2.LYOPHILIZED CULTURES.

Lyophilized starter cultures are liquid starter cultures that have been freeze-dried and packed for shipping or storage in a powder form. They can be stored for a long time when in this form. But they have low initial activity

and have to be propagated before use. This means that, although they are easy to transport, they still have to be rejuvenated and propagated by the processors. They are also expensive when compared to liquid starter cultures.

2.1.2. CONCENTRATED STARTER CULTURES.

Concentrated starter cultures are produced by specialized laboratories and shipped to the processing plants in frozen or dried form (25). At the factories, they are used for preparation of bulk starter or for direct vat inoculation. In this way, the problems of propagation in the processing plant are eliminated. Concentrated starter cultures must at least have four times as many bacteria per unit of volume as conventional cultures (53). Martins, 1977, reported that it is feasible to concentrate starters cultures upto 180-240 times. He, however, experimented with cultures concentrated 60 times.

According to a recent review on dairy starters by Robinson, 1981 (78), there are four types of concentrated starter cultures namely;

a) Frozen concentrated starter cultures. In the liquid cultures the number of the viable bacteria is increased by use of neutralizing agents to maintain the pH in the growth medium at 6.0-6.3, thereby achieving a viable count of 10^9/ml.

The culture is then frozen and stored.

b) Freeze-dried concentrates; These are concentrated cultures that have been lyophilized for use in direct inoculation or for bulk starter production.

c) Direct to vat cultures (DVS) are liquid concentrated cultures as in (a), that have been centrifuged to get 10^9-10^{11} viable cells/ml (25,70, 71,74) and then frozen. They can be used for direct inoculation of the product vat.

d) Freeze-dried direct to vat cultures are DVS as in (c) which have been lyophilised and stored at refrigeration temperature, for direct inoculation into the product vat.

Starter cultures of many species of bacteria have been successfully concentrated. These include the thermophilic types (81,94). Others include the mixed species mesophilic type (6,8,18,35,50,59,71, 72,95), the lactic streptococci (4,14,26,27,30,36, 38,70) and leuconostoc (28,65). Similarly, many cultured dairy products have been made using concentrated starter cultures. Such products include mildly sour products (69) and cheeses such as cheddar (79,84,91), cottage (91), kostroma (2), Stilton (8), Gruyere (81), pickled (31) and manchego (64).

2.1.2.1 Advantages of concentrated cultures over conventional cultures.

A lot of research has been conducted on the production and preservation of concentrated starter cultures. Their performance has been evaluated and compared with that of conventional cultures. Gilliland, 1985, summarized the advantages resulting from use of concentrated cultures as follows: (a) The activity of the cultures can be carefully monitored and controlled prior to shipping to the processing plants. (b) The maintenance of stock cultures in the processing plants is not necessary. (c) Less labour is required in the processing plants in dealing with cultures. (d) Starter culture rotations are easier to establish in order to maintain control over the bacteriophage problem. (e) Quality of the culture can be well maintained to avoid contamination with yeasts, coliforms and other bacteria. (f) The manufacturing procedures of making cultured dairy products can be improved. (g) Chances of culture failure and subsequent economic losses are minimized. (h) The quality of the cultured product can be controlled with regard to acidity, flavour and consistency. Additional advantages include simplicity in use and low storage and transport cost (11,80). Finally the processing plants need not have highly skilled

microbiologists, since the mode of use and handling starter cultures is simplified.

2.1.2.2. Disadvantages of concentrated starter cultures.

Gilliland, 1985, summarized the disadvantages of concentrated starters as follows; (a) High cost of producing the concentrated starter cultures. (b) Requirement of adequate storage space at proper low temperature both for shipping and maintenance of cultures (c) Not all starter cultures can be concentrated successfully. Other additional disadvantages include changes in schedules in the processing plants, higher inoculation rates, low initial rate of acid production, and inadequate number of workable culture strains (79,80).

There is no doubt that concentrated starter cultures are the ideal inoculum for the bulk starters. But the main problem is to concentrate cultures, whose concentration has so far not been achieved, to make the process cheaper and to convince the processing plants to adopt CSC.

2.2. PRODUCTION OF CONCENTRATED STARTER CULTURES.

The production of concentrated starter cultures involves the growth of the cells under strictly controlled conditions in a medium from which the cells can be easily harvested and

concentrated into a small volume (25,26,78). The objective is to produce the maximum number of cells per unit volume,which will contain all the necessary enzymes to perform properly in fermentation processes. Gilliland, 1985, summarized the general stages involved in the production of concentrated cultures as shown in Figure 4. The stages include; 1) Inoculation of the suitable medium, 2) Incubation until culture reaches maximum population, 3) Concentrating the cells into a small volume, 4) Suspending the concentrated culture in a menstrum, 5) Freezing or drying for storage and, 6) Shipping to the processing plants where it is used or stored.

2.2.1.Inoculation

It is necessary that the culture used for inoculation have high activity for the resulting culture to function properly on concentration. This is ensured by transferring the culture 2-3 times in the same medium as growth medium (26,70,72,91). The inoculation rate used is 1 or 2% (12,14,27,45,59, 72). 5% has also been used (87). Kurwijila, 1978, used an inoculation rate of 3% for the propagation and use of mala culture. During incubation the culture should be exposed to the optimum conditions that lead to fast growth and high activity.

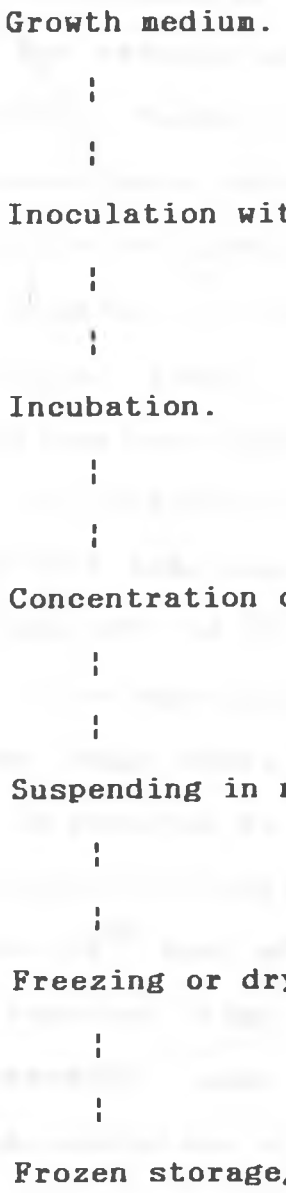


Figure 4: Generalized steps of preparing concentrated starter cultures.

Source (25).

2.2.2.Harvesting of the concentrated cells.

Two methods have been utilized in harvesting the cells. Faigh *et.al,* 1987, used microfiltration to concentrate dairy cultures to high population and activity levels. Other scholars have used centrifugation at different speeds for harvesting the cells. Lloyd, 1971, used a speed of 3000 revolutions per minute (RPM) and acquired a culture with 60-75% moisture content in skimmilk. Other speeds that have been used are in the range of 8000 to 50,000 RPM (34,56,60,70,72).

It is important that the culture be harvested at the stage where it has the highest activity. This is reported to be before the stationery phase (3,30,52,53,73,77,96), although it is shown to go upto the 36^{th} hour of growth (77). Peebles *et.al.*, 1976, reported that cultures grown at pH 6 could be harvested even six hours after reaching the maximum population with no loss in activity.

2.2.3. Storage And Shipment.

The storage and shipment of concentrated cultures require conditions that will lead to minimum loss in viability and activity so that the performance of the culture is preserved. Frozen storage is preferred. Frozen concentrated cultures can be packaged in plastic bottles (18) while freeze-dried cultures are distributed in aluminium sachets or vials (18).

2.3. FACTORS AFFECTING CELL YIELDS.

2.3.1. Growth Medium.

The growth medium should provide all the nutrients for maximum growth of the cells and ensure that they have the necessary enzymes to enhance fermentation (25). The cells should have optimum activity and remain stable during extended storage (25). Lloyd and Port, 1973, recommended that the growth medium for continuous production of the concentrated culture should be nutritionally adequate, economical, and should not precipitate during heat sterilization.It should contain lactose as the carbon source. Lactose is split by the homofermentative streptococci and lactobacilli into glucose and galactose. Glucose is fermented to pyruvic acid through the Hexose Diphosphate Pathway (HMP) (15). Pyruvic acid is then converted to lactic acid with trace amounts of acetic acid and carbon dioxide. If the concentration of lactose is low and especially under anaerobic conditions, more acetic acid is produced. This in terms of energy is justified since there is an extra mole of ATP by producing acetate instead of lactate. A high concentration of glucose stimulates the enzyme lactate dehydrogenase through fructose 1,6-di-phosphate (see figure 5). Although acetate is a flavour constituent, and stimulates

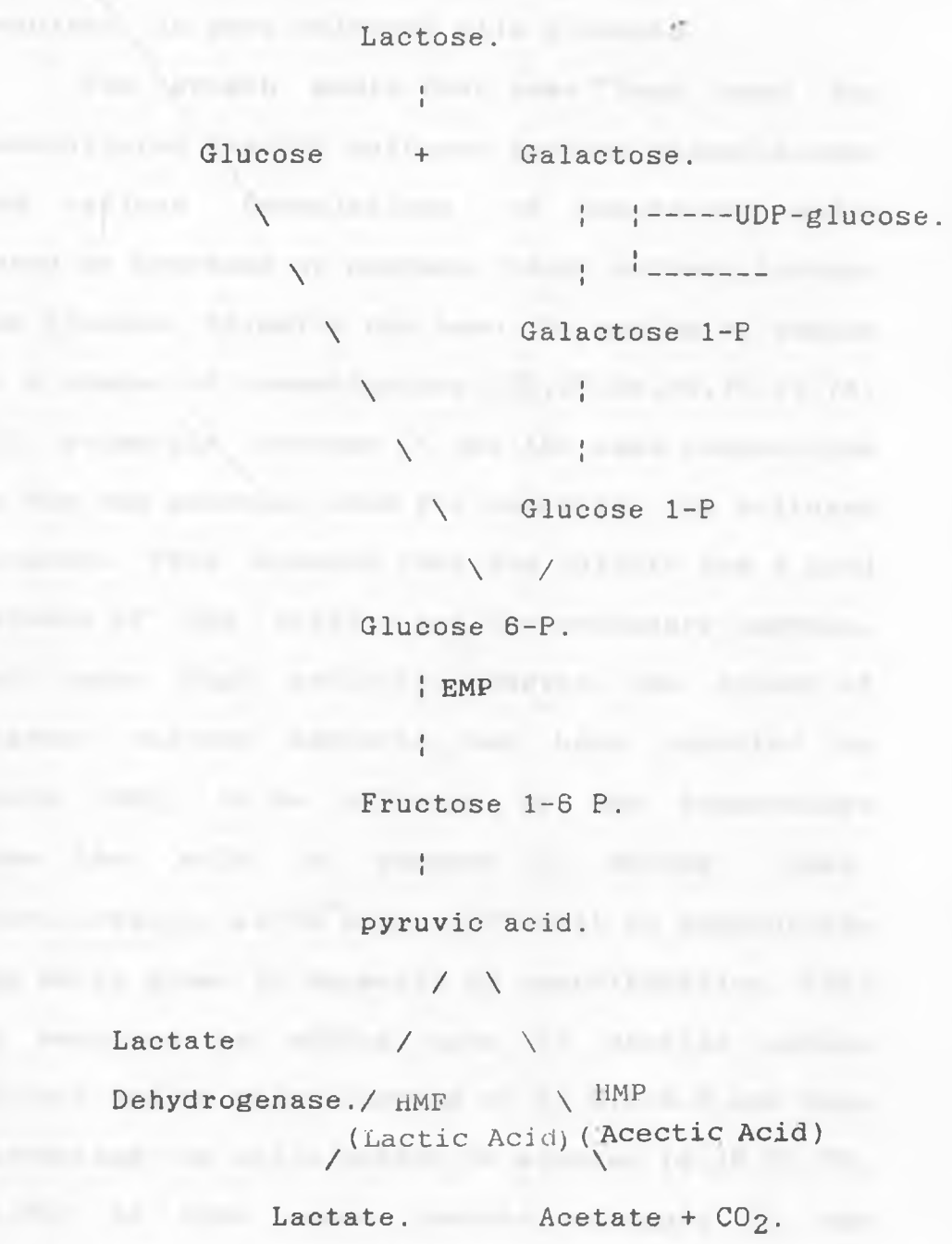


Figure 5: Homofermentative metabolism of lactose to lactate, acetate and CO_2. Source (15).

some useful bacteria while inhibiting pathogens such as salmonella, it is not required in most cultured milk products.

The growth media that have been used for concentrated starter cultures include skimmilk,whey and various formulations of laboratory media based on tryptone or peptone, yeast extract,lactose and glucose. Skimmilk has been the medium of choice to a number of investigators (25,27,54,56,70,72,74, 91), primarily because it has the same composition as the raw material used for preparing the cultured products. This ensures that the culture has a good balance of the strains and the necessary enzymes, and hence high activity. However, the growth of starter culture bacteria has been reported by Speck, 1962, to be affected by the temperature zone the milk is exposed to during heat sterilization. It is also difficult to concentrate the cells grown in skimmilk by centrifugation. This is overcome by adding upto 2% sterile sodium citrate and/or adjusting the pH to 6.8-6.9 and then harvesting the cells within 30 minutes (4,18,25,70, 74,95). In some cases, however, attempts to use sodium citrate to clarify the milk medium have not been very successful (25). Lloyd, 1975, used skimmilk supplemented with yeast extract, and added trypsin to digest the casein before centrifugation.

Whey has been used as a medium for producing

cell crops of starter culture, mainly because it is a by-product in cheese manufacture, and therefore inexpensive and it contains nutrients that can be utilized by bacteria (25,74). But it is prone to bacteriophage attack and lacks some nutrients that support maximum growth of starter culture bacteria. It can also precipitate during heat sterilization (25,30). To correct for the missing nutrients, much of the research using whey has been concerned with supplementation, using various proportions of concentrated maize extract, peptone, yeast autolysate, enzyme hydrolyzed casein, yeast extract and tryptone, either singly or in combination (48,65,74). Proteolytic enzymes for partial hydrolysis of whey proteins have been used to enrich the medium for growing bacteria and to prevent precipitation (56). But even with this enzymatic treatment, the medium has not been ideal for maximum growth of starter culture bacteria (56). Deproteination of whey has been used to prevent precipitation on heating but it renders it poorer as a medium (52). Acidic whey is neutralized before inoculation (8,72).

The formulated medium is chosen because it is easier to harvest the cells, and adjust the concentration of nutrients in the medium to the level which supports maximum growth of culture bacteria and produce high performance culture. The

medium consists of various concentrations of tryptone or peptone, yeast extract or yeast autolysate,and sugar,especially lactose and glucose (3,12,31,36,42,51,70). Sodium citrate is added for diacetyl production, and agents like Tween R80 to stabilize the media (28,30). The most common formulation used is the tryptone broth consisting of 2% tryptone, 1% yeast extract, 2.5% lactose and 2.5% glucose (26,27,30,42,70). Libudzisz *et.al.*, 1986, reported the optimum composition of ingredients as 0.6-0.8% lactose, 0.2-0.5% yeast autolysate, 0.6-0.7% peptone and 0.7-0.8 % sodium citrate.

The growth of starter culture bacteria in the three media has been compared. Petterson, 1976, compared the growth of a mixed species lactic starter in three different media namely, 9% reconstituted skimmilk (RSM), papain treated whey and tryptone medium. He found that, there was higher growth rate and yield in whey and tryptone medium than in RSM. Port and Holloway, 1968, reported higher maximum count and productivity (maximum cell count at the end of the logarithmic phase X the specific growth rate) of *Streptococcus lactis* when grown in skimmilk than in cheese whey, with 1% tryptone added to both media. But the specific growth rate was higher and the generation rate shorter when the culture was

grown in the whey medium than in the skimmilk medium.

2.3.2. Growth Conditions.

The environment the cells are exposed to during growth is bound to affect the yield of cell crops. Conditions that have been found to affect the cell growth include pH, temperature, growth factors and cultivation method.

2.3.2.1. pH.

A maximum population of stable cells is only obtained if the pH of the growth medium is maintained at a constant level of 6.0-6.5, near to that of milk (12,25,45,54,72,74). Lower concentrations of cells that are unstable during storage result when the pH is not controlled. Gilliland, 1977, found that the maximum population obtained if the culture is grown under controlled pH of 6.0 is about 15 times higher than when the pH is not controlled. He also found that the time to reach the maximum population was less when the pH was controlled at 6.0 than when it was controlled at 5.5, 6.5 or 7.0. Gilliland *et.al.*, 1970, reported that the maximum population was obtained when the pH of the growth medium was maintained at a constant pH between 6.0 to 7.0, while maximum diacetyl production was obtained when the pH was

maintained at 6.0. Peebles *et. al.*, 1969, found that maintaining the pH constant at 5.0 and 7.0 shortened the time to reach the maximum population considerably while the maximum population was only slighly lowered. Petterson, 1976, observed that the maximum cell yield was 4-20 times higher when the pH was maintained constant as compared to uncontrolled pH. There was, however, no significant difference in the growth rate and maximum cell yield, when the pH was maintained constant at 5.5, 6.0 and 6.5. The method of controlling pH also affects the growth of the bacteria. Two methods have been applied to control the pH: (a) Use of neutralizers and (b) Use of buffers.

(a) Neutralizers.

The neutralizers that have been most commonly used to control pH are sodium hydroxide and ammonium hydroxide. The use of ammonium hydroxide results in higher cell yield than that of sodium hydroxide, but the activity of these cells is lower (4,25,26,56,72). However, full activity is regained in the first subculture of the concentrated starter culture. This decreased activity is related to a decrease in the proteinase activity of the culture bacteria (25). Blaine *et.al.*, 1970, reported that the use of ammonium hydroxide or sodium carbonate

resulted in a higher specific growth rate and cell yield than the use of sodium hydroxide, calcium hydroxide or potassium hydroxide. The use of ammonium hydroxide by Gilliland, 1977, and Peebles *et.al.*, 1969, resulted in 1.5 to 2.5 times more cell yield when compared to the use of sodium hydroxide, but the cells had lower activity. However, Petterson, 1975, found no significant difference in the growth rate and maximum cell population due to the neutralizers used, although sodium hydroxide gave cells with decreased activity. It also affected the bacterial balance of mixed species lactic starters composed of *Streptococcus cremoris, S. lactis, S. diacetilactis* and *Leuconostoc citrovorum*. Sodium hydroxide decreased the proportion of lactic acid producing bacteria relative to the aroma bacteria. Concentrated cultures stored frozen at -30°C are reported to have poorer survival when the pH during growth is maintained by sodium hydroxide as compared to ammonium hydroxide (18, 36). Addition of these neutralizers results in a dilution effect on the culture bacteria (25,27). The use of ammonia gas and slightly soluble buffers such as calcium carbonate and calcium citrate have therefore been suggested (25).

(b) Buffers.

There is very little documented work on the use of buffers to control the pH of the growth medium for production of starter cultures. However, Bannikova and Lagoda, 1975, obtained stable concentrated cultures of mesophilic lactic acid bacteria grown in a whey/hydrolyzed milk/sodium citrate medium. They also found that buffering of a whey medium by sodium citrate for 6 to 8 hours followed by neutralization with sodium bicarbonate yielded the best results (48). A mixture of ammonium hydroxide and calcium carbonate has been found effective in producing cells of *Lactobaccilus acidophilus* (25). At pH 6.0 this neutralizer provides slow release of carbon dioxide which appears to benefit the growth of culture bacteria. Buffering by sodium citrate (66) and calcium carbonate (21,66) has been used as a means of preserving cultures when refrigerated or frozen.

2.3.2.2. Temperature.

The temperature of choice is the optimum temperature for growth of the bacteria culture or the temperature that gives the maximum cell yield if it does not have an adverse effect on the culture activity. Blaine *et. al.*, 1970, found that the specific growth rate and the maximum

population of a lactic streptococci culture were lowered at temperatures below and above 30°C. Petterson, 1975, reported that the maximum cell yield of mixed lactic starters composed of *Streptococcus cremoris, S. lactis, S. diacetilactis* and *Leuconostoc citrovorum* increased with temperatures between 20-30°C and especially in cultures grown in 9% RSM. Reif, 1971, found out that there was no advantage of culturing single and mixed strain cultures of the lactic streptococci in the temperature range of 15-21°C. Culturing at 21°C, however, took the shorter time to reach the maximum count and activity. The optimum conditions for a mixed species lactic streptococcus culture to give maximum cell yield and maintain species balance and activity were found by Efsthantiou *et. al.* 1975, to be 23-27°C and pH 6.1-6.5. The starter culture used for mala production has been cultured successfully at the ambient temperature of 23±1°C by Kurwijila, 1980, and Wangoh, 1988.

2.3.3. Growth factors.

Cell production often drops during the growth of the culture bacteria, before the nutrients are completely exhausted, while the acid production continues unhampered (25). This means that growth is stopped due to accumulation of growth inhibitors. Several inhibitors occurring in the

growth medium have been identified. These include oxygen,hydrogen peroxide, lack of carbon dioxide, accumulation of metabolites such as lactate and D-leucine, as well as excess nutrients such as lactose (3,25,30). Accumulation of lactate has been shown to have a negative effect on *Streptococcus cremoris* (3) and *S.lactis* (74). Excessive agitation has also an adverse effect on the growth of the bacteria cultures (12,40,41,74). Keen, 1972, explains that agitation decreases the growth rate of the organisms due to decreasing availability of carbon dioxide (carbon dioxide effect) and of the soluble nitrogen (soluble nitrogen effect). The carbon dioxide effect was observed only when carbon dioxide concentration was depleted by heat sterilization, but not when the medium was purged with carbon dioxide for 30 minutes before inoculation. The nitrogen effect occurred when agitation was more than 80 rpm. An effect of direct oxygen toxicity was also observed and it was more apparent at 20°C than at 30°C (41). However, low amounts of oxygen stimulated growth at pH 5.05-5.45 while accumulation of hydrogen peroxide affected growth at pH 5.45-6.35. Cogan *et. al.*, 1971, found no effect on growth by stirring rates of 600-700 rpm., but air sparging at one litre per minute decreased growth rapidly. Addition of carbon dioxide at one litre per minute had little effect

on growth, but addition of nitrogen at the same rate rapidly decreased the specific growth rate. They explained that this was probably due to carbon dioxide being swept away by nitrogen, and deduced that certain strains of the lactic streptococci have a partial or absolute requirement for carbon dioxide during growth. Marth, 1962, explained that lactic acid bacteria need carbon dioxide so as to make oxaloacetic acid. Oxaloacetic acid cannot be formed through the citric acid cycle, and it is an important precursor of essential amino acids such as asparagine, threonine and leucine (58).

2.3.4. Cultivation Method.

Both types of cultivation methods,i.e. batch and continuous,have been used for production of starter culture bacteria. The method of choice is the batch system. It is simpler and more convenient than the continuous system, in line with the equipment used for harvesting the cells, and does not require complex process steps (25).Important considerations when using the batch system, include control of pH of the growth medium, agitation and physiological age of the cells at harvesting time with respect to the activity of the concentrated culture (25,26,53,54). The use of the continuous system is associated with failure of the culture in

performance due to bacteriophage attack, loss of enzymes, mutations and contamination by other micro-organisms (25,53,56,57). There is also the need for more complex equipment and change in the production schedules for preparation of concentrated starter cultures. Important considerations in a continuous system are pH, dilution and length of time the system is maintained. Wilkoskie and Fouts, 1958, proposed a simple system of growing starter in skimmilk, and found that the critical pH level below which the growth rate declined was 5.3. For a lactic streptococci culture consisting of *Streptococcus cremoris* and *S.lactis*, Lloyd and Port, 1973, (56) found that the maximum yield for both species occurred at a dilution rate (flowrate / volume of fermentation) of 0.6, and pH 6.3 while the growth rate and activity of the culture increased as the dilution rate approached 1.0. It was explained that this was due to the dilution of lactose which acts as an inhibitor when in excess. However, the size of the cells increased as the dilution rate approached 1.0, while the cell activity of *S. cremoris* was reduced when the pH was above 6.0. Continuous cultures of the lactic streptococci have only been maintained for upto two weeks (56).

2.4. PRESERVATION OF CONCENTRATED STARTER CULTURES.

The preservation of starter cultures is a well researched topic. Several methods have been utilized to preserve cultures including buffering, addition of glycerol, sugar or salt, combination of high solids concentration and addition of low solubility neutralizers (66). This is then followed by freeze-drying, spray-drying or freezing (21) and/or storage at room temperature, or low temperature (21,66). The most common methods of preserving concentrated cultures are freezing and freeze-drying. More details on preservation of starter cultures are available from Foster, 1962, Olson, 1966, Reif, 1971 and Waes, 1970.

2.4.1. Refrigeration.

The use of refrigeration as a means of preserving concentrated cultures has been tried by a few researchers. Lagoda and Bannikova, 1948, stored a concentrated culture of mesophilic lactic acid bacteria in a refrigerator at 5-8°C, and after 2-5 days they found that it contained only 26-46% of the original viable cells. Girginov and Velichkova, 1971, and Lloyd, 1971, managed to store concentrated cultures at 5-8°C for 60 days, without change in their morphological, biological and biochemical properties. Besides they were more

phage resistant than other cell suspensions. However, the authors did not report on their survival rate. Refrigeration has been utilized successfully as a means of long-term storage of freeze-dried and spray-dried concentrated cultures (78).

2.4.2. Freezing and frozen storage.

Three temperature zones have been utilized for the preservation of concentrated starter cultures by freezing i.e. cryogenic freezing at -196°C, freezing at -110°C (the vapour phase of nitrogen) and deep-freezing at -20 to 40°C. It is fully agreed that freezing in liquid nitrogen at -196°C and storage in the same can be used to preserve concentrated cultures for a long time with no change in viability, activity, species balance, proteolytic activity and production of carbon dioxide (1,10,16,18,25,26,27,28,42,48,53,55, 56,57,59,70,71,72,76,77,78,100). Lloyd, 1975, Lloyd, 1978, and Lloyd and Port, 1973, have shown that freezing and storage of concentrated cultures at the vapour phase of nitrogen at -110°C is equivalent to freezing and storage in liquid nitrogen for periods upto one year. The above two freezing temperatures are difficult and expensive to maintain and research into freezing by means of commercially available deep freezers have

been suggested (18,52). Lloyd and Port, 1973, reported that the keeping quality of a concentrated culture of lactic streptococci was 42% at -20°C, 93-94% at -40°C and 100% at -110°C for four weeks. They attributed the low survival rate at -20°C and -40°C to low pH (less than 6.0) during storage. They found that the keeping quality was improved by adding cryoprotective agents such as glycerol. Lloyd, 1975, found that there is a decrease in viability, acid production and activity for cells stored at -30°C but not at -40°C for three months. He recommended that the temperature of storage should be less than -37°C. This was slightly lower than Bergere's, 1969, suggestion that the temperature of storage should be less than -30°C. Gilliland, 1977, reported that storage of a concentrated culture of the lactic streptococci at -37°C with 7.5% lactose for 60 days was the same as storage at -196°C but when stored at -20°C the culture reduced its viability and its proteolytic and acid producing ability. Storage of a mixed species concentrated culture at -30°C was found better than storage at -10°C (18). Bannikova and Lagoda, 1975, reported that cells of a mesophilic lactic acid bacteria concentrate lost 8-18% and 16-28% viability, after freezing in liquid nitrogen and then storage at -18°C and -6 to -8°C respectively for six months.

Lloyd,1978, suggested that concentrated cultures can be stored in a deep freezer for three months only. Concentrated cultures have been successfully maintained at -30°C for periods of upto four months (36,37,38,53,71,73).

Cryoprotective agents have been used to improve the shelf life of concentrated cultures during frozen storage. Glycerol has been reported to be most effective in maintaining the viability, activity and species balance of the culture (18,25,26,48). This has been attributed to the OH-groups which stabilize the conformation of the cellular constituents (18). Other agents that have been used include lactose, sucrose, yeast extract and the supernatant from the concentrated starter culture (1,18,26,48,55,57).

2.4.3. Drying.

Concentrated cultures have also been preserved by spray-drying and freeze-drying. Jansen *et.al.*, 1969, has explained a method of spray-drying in which 50% of the bacteria survived as compared to less than 10% survival in the freeze-dried cultures. However, the survival of freeze-dried cultures was raised to 50% by adding yeast extract during culturing. Freeze-drying of concentrated cultures leads to reduced activity and viability

(6,25,45,71). Krasnikova *et.al.*, 1981, reported a survival rate of 40-45% in a culture concentrate composed of *Streptococcus lactis, S. cremoris, S. diacetilactis* and *Leuconostoc dextranicum* after freeze-drying and storage at 3-5°C for six months. They found that there was less decrease in biochemical activity. Diacetyl formation dropped from 7.6 mg/l to 5.3mg/l. Bouillane *et. al.*, 1981, found that the viability of a freeze-dried lactic streptococci culture was the same as that of deep frozen culture while the activity was four times lower. Freeze-dried concentrates are reported to be stable at 6-8°C for six months and for two months at 18-22°C when a protective medium was used (48). The use of 5% monosodium glutamate and 7% lactose has been found to protect the cultures during freeze-drying (25,71,88,91). Freeze-dried concentrated cultures of the lactic streptococci were found to be more active and stable at 25°C, when the protective medium contained skimmilk and 15% glycerophosphate, instead of skimmilk and 10% maltose or sucrose peptone solution (105,106). The loss in viability of freeze-dried concentrated cultures has been attributed to the presence of oxygen in the culture during storage (25,64,71,104). This has necessitated vacuum sealing of the freeze-dried culture or packaging in an inert gas. Reddy *et,al*,.

1974, reported that lyophilization of concentrated cultures is preferable to freezing at -20°C for maintaining the component balance of mixed lactic cultures. Freeze-dried concentrated cultures have been used directly to manufacture cheddar cheese, but were found to be more expensive than frozen concentrated cultures (25,37,78). However, a freeze -dried concentrated culture would be advantageous as it is easier to ship to the processing plant and store than frozen concentrated cultures.

2.5. PERFORMANCE OF CONCENTRATED CULTURES.

The performance of concentrated lactic cultures has mainly been expressed by two parameters namely viability and activity. Other important parameters include species or strain balance, viscosity, flavour or diacetyl formation, proteolytic activity, as well as carbon dioxide formation and resistance to bacteriophage attack. In evaluation of the performance of concentrated cultures more practical data is obtained from the first sub-culture (76).

2.5.1. Viability.

The main method used to express the number of viable cells is the plate count method (3,18,26,57,64,70,72,76,91). Elliker *et.al.*, 1956, described an agar culture medium for the lactic

streptococci and most other lactic acid bacteria. The medium proved to be superior to all others with regard to ease of preparation, clarity, and ease of counting and colony development. This lactic agar has been used to express the total viable count of concentrated cultures by several workers (17,18,91). Other media that have been used include Oxoid-plate count agar (57), calcium citrate agar (72) and Reddy's differential medium for enumeration of the lactic streptococci (26).

Considering the need to know the viability of the cells of concentrated cultures within a short time more rapid methods than the pour plate technique have been used. The direct microscopic count has been used by Port and Holloway, 1968, and Keen, 1972. Karaski *et.al.*, 1976, have described a rapid method for estimation of bacterial population in milk. The dry weight determination has also been used (14,65).

2.5.2. Activity.

The activity of a culture is expressed as the quantity of lactic acid formed in milk inoculated at a given rate and incubated for a fixed time at a given temperature. There are three methods of expressing activity namely: (a) Determining the acidity at the end of coagulation of milk (18 -24 hrs.) at room or higher temperature. (b)

Determining the acidity at the onset or during the exponential phase of growth and, (c) By simulating the production of the cultured product i.e. checking the time of curdling or the time to reach a certain pH. The most common method is (b) where the acidity of a substrate containing 10-11 % RSM inoculated with a culture at the rate of 1-3 % and incubated at 30-37°C for 1.5-6.0 hrs. is determined by titrating with sodium hydroxide (0.1N) upto pH 8.2-8.4. (18,33,46,56,59,60,64,70,77,91). This method is preferred to the other two because it is quicker and more accurate since the activity of cultures decreases after the exponential phase of growth (3).

2.5.3. Flavour and Viscosity.

The main flavour constituents of cultured dairy products are diacetyl and acetaldehyde. But the determination of diacetyl alone has been used in expressing the performance of concentrated mesophilic cultures. Oberman *et.al*,. 1986, reported that the diacetyl and acetoin production of a deep frozen culture of *Leuconostoc cremoris* decreased by 25-50% after frozen storage for one month. The decrease in diacetyl production may however be due to decrease in aroma bacteria, reduction to acetoin and 2,3, butylene glycol by

means of diacetyl reductase enzyme, and\or volatilization (46). Pack *et.al.*,1964, have described a method of determining diacetyl in mixed strain starters which is a modification of the Owades and Jakovac method for diacetyl determination in beer. This method has been improved by Walsh and Cogan, 1974. Acetaldehyde is important as a flavour constituent in products made by using thermophilic starters. The diacetyl; acetaldehyde balance is important in some products such as buttermilk.

The consistency of a coagulum made from the concentrated culture is an important criterion for judging the performance of the culture. The inoculum should be as such to produce a coagulum similar to that made by the conventional culture in a milk substrate with the same solids-not-fat and butterfat, when subjected to the same heat treatment.

2.5.4. Species Balance.

The species balance is important in the concentration of mixed species cultures because it affects the performance of the culture in flavour formation. The most important factor in species balance is the ratio of aroma bacteria to the total bacterial population. Reddy's agar medium for differential enumeration of the lactic

streptococci (76) has been used to differentiate between the different species of the lactic streptococci on the basis of acid, ammonia and carbon dioxide development (18,26,76). Mullan and Walker, 1979 have described an agar medium and a simple streaking technique for the differentiation of the lactic streptococci on the basis of the colour of the colonies. These two media cannot be used to differentiate a mixed species culture that contains *L. cremoris* as one of the component species. The agar medium of Galesloot *et.al.* 1961, has been used for the isolation and enumeration of the aromabacter in starters (18,24). As the method of Galesloot *et.al.* is tedious and time consuming, the method of Nickels and Leesment, 1964, has been adopted (71,72). The differentiation of the aromabacter species in the last two methods is based on the size of clear zones around the colonies, which result from the fermentation of citrate. The difference in the size of the clear zones reflects the difference in the growth rate of the two species of the aromabacter.

2.5.5. Bacteriophage Resistance.

The performance of concentrated cultures is affected by bacteriophage attack and contamination by other micro-organisms. The

presence of bacteriophage can be determined by the change in colour of milk inoculated with the culture in the presence of bromocresol purple indicator (35,95), while the number of bacteriophages can be estimated by the plaque forming around the bacteria colonies (61).

2.5.6. Sensory Evaluation.

The final criterion for determining the performance of the concentrated starters is the acceptability of the cultured products made with the culture by the consumers. This is tested through sensory evaluation of the products by taste panels. Preference and ranking tests have been used (46).

3.0. MATERIALS AND METHODS

3.1. MATERIALS AND APPARATUS

3.1.1. Culture

The culture used was a frozen liquid type maintained in the Department of Food Technology and Nutrition (DFTN). It had been made from a lyophilised culture obtained from Chr' Hausen's laboratorium, Denmark. According to the manufacturers, the lyophilised culture is composed of "*Streptococcous lactis* 1-5%., *S. Cremoris* 89-95%, *S. diacetilactis* <5% and *Leuconostoc cremoris* 5-10%. The frozen liquid culture was thawed at room temperature, subcultured in skimmilk three times when maximum activity was regained and used to inoculate sterile skimmilk at a rate of 3% (v/v). The inoculated skimmilk was then asceptically filled into 100ml plastic bottles with screwcaps. These were immediately frozen in a deep-freezer to preserve the culture (46). Whenever needed a bottle of culture was removed from the deep freezer, allowed to thaw for two hours and incubated for 14-18 hours at room temperature. Such a culture was used for study after two serial transfers in skimmilk (45, 59, 72). It was only used for a maximum period of two weeks. The inoculation rate of 3% (v/v) was adopted in all transfers and experimental work (46,91).

3.1.2. Fresh milk

Fresh milk was obtained from the University farm and skimmed at the pilot plant of DFTN. The butterfat was standardised to 3.3%. The milk was heated to 90-92^{o}C and held at this temperature for 30 minutes. It was used to make products for sensory evaluation.

3.1.3. Reconstituted skimmilk

Non-fat milk powder was obtained from Kenya Co-operative creameries (KCC). It was reconstituted with water at 11% (W/V) (4,18) whenever needed. Calcium carbonate was added where applicable and the reconstituted skimmilk (RSM) sterilised by heating to 95-100^{o}C and holding it at this temperatures for 45 minutes (2). It was used for the growth of the mala culture.

3.1.4. Whey

Sweet-whey was obtained from the DFTN pilot plant as a by product of cheese making. It was pasteurised at 63^{o}C for 30 minutes, cooled, packed into plastic bags and preserved by deep-freezing at -32^{o}C (74). Whenever needed, the whey was thawed at room temperatures and treated in three ways:- 1) The pH was adjusted to 6.5 by 1 N sodium hydroxide (74), $CaCO_3$ added where applicable and the medium sterilized at 90^{o}C for 45 minutes (2). Such whey was used within 24 hrs. 2) The whey was heated to

92°C and held at this temperature for 3 minutes. 2% citric was added to pH 4.6 and the whey filtered. The pH of the clear whey was adjusted to 6.5, $CaCO_3$ added where applicable and the medium sterilised at 121°C for 15 minutes (74). 3) The whey was heated to 35°C, pH adjusted to 4.5 and papain added for partial hydrolysis of the whey proteins. The treated whey was boiled for 5 minutes to denature the enzyme and then filtered. The pH of the clear whey was adjusted to 6.5 and sterilised at 121°C for 15 minutes (74). The three whey preparations were compared for support of the growth of the mala culture. The most appropriate preparation was adopted for further study with the other two media.

3.1.5. Tryptone broth

The tryptone broth was composed of 2% tryptone 0.25% yeast extract, 0.5% glucose, 0.5% lactose and 0.1% sodium citrate dissolved in distilled water. $CaCO_3$ was added where applicable and the medium sterilised at 121°C for 15 minutes. The broth medium was used for the growth of the culture.

3.1.6. Agar media

The lactic agar was prepared from the ingredients according to Speck, 1984. It was sterilised at 121°C for 15 minutes. It was used for

enumeration of the total lactic acid bacteria count in the culture.

The potato dextrose agar and violet red bile agar was prepared from the ingredients according to Speck, 1984. They were sterilised at 121^{o}C for 15 minutes and used to check the contamination of the culture by yeasts and molds and by coliforms respectively.

The calcium citrate agar substrate was prepared from the ingredients and sterilised according to Nickels and Leesment, 1964. It was used as a constituent of the calcium citrate agar.

A fresh filtrate from the cultured milk was used as a constituent of the calcium citrate agar instead of the cultured cream filtrate recommended by Nickels and Leasment, 1964. It was sterilised at 121^{o}C for 15 minutes.

Calcium citrate was not available in the local market. It was prepared by the stoichiometric reaction between citric acid and calcium carbonate. 7.36g of citric acid ($C_6H_8O_7.H_2O$) was dissolved in warm distilled water to give 100ml of citric acid solution at 60^{o}C. 5.258g of calcium carbonate was reacted completely with all the 100ml citric acid solution. The reaction proceeded as follows:

$$3\ CaCO_3 + 2C_6H_8O_7.H_2 - Ca_3C_{12}H_{10}O_{14} + 3CO_2 + 3H_2O$$

The above quantities of citric acid and $CaCO_3$

are calculated from this stoichiometric equation so as to give a concentration of 10g calcium citrate per 100 ml in the final solution. The calcium citrate solution was used to make a suspension by mixing the 100 ml solution at 60^{o}C with 1.5g of carboxymethyl-Cellulose and blending the mixer with a kitchen blender for 5 minutes to get a stable suspension. The suspension was transferred into 100 ml dilution bottles and sterilised at 121^{o}C for 15 minutes. It was preserved in a refrigerator at 5-8^{o}C until when required. It was used as a constituent of the calcium citrate agar.

The calcium citrate agar was made by asceptically adding 10ml of the suspension and 5 ml of the cultured milk filtrate into 85ml of the calcium citrate agar substrate just before pouring into the plates (63). The calcium citrate agar was used to enumerate the aromabacteria in the culture (63).

3.1.7. Diluent solution

Peptone water was prepared according to Speck, 1984, sterilised at 121^{o}C for 15 minutes and used for dilution purposes.

3.2. CHEMICALS

The following chemicals were obtained from Howse and Mc.George Ltd, Nairobi. Acetone, Agar,

Ammonium hydroxide, Bromocresol purple, Calcium carboante, Calcium lactate, Citric acid, Gelatine, Glycerine, Glucose, Hydroxylamine,. Lactose, Posassium phosphate, Potassium sodium tartarate, Sodium Acetate, Sodium chloride, Sodium hydroxide, Sucrose, Tri-sodium citrate, Tryptone and Yeast extract. Other chemicals namely Casamino acids and Carboxymethyl cellulose were obtained from Difco Laboratories, Michigan, USA.

3.3. Apparatus

3.3.1. Laboratory Apparatus

The pH meter used was Tistriskop model E516, Switzerland.

Centrifugation in the laboratory was carried out in a Beckman, United Kingdom, model TJ-6, centrifuge using 100ml polyproplene tubes fitted with screwcaps.

Growth of the culture in large quantities was carried out in a Chemap, Switzerland, model AG, PC. fermenter with a capacity of six litres.

The freeze-drier used was Christ, Bremen, West Germany, model Beta I.

The viscosity was determined in the Torsion viscometer, model, Rheameter STV, from Contravenes A.G. Switzerland.

3.3.2. Pilot plant equipment

The fresh milk was skimmed in a manual

skimming machine.

Centrifugation of large quantities was achieved by using the Laboratory separator, Model LAPX 202, Switzerland.

3.4. EXPERIMENTATION METHODS

3.4.1. Growth characterization.

The growth of the culture in the three media namely reconstituted skimmilk (RSM), whey and tryptone broth, unbuffered or buffered with $CaCO_3$ was carried out in 200 ml of the medium in 300 ml glass jars with screw-caps. After inoculation the media was incubated at room temperature ($24\pm1^{o}C$). 15 ml samples were withdrawn at two hourly intervals for 24 hours. The samples were used for the determination of total viable count (TVC), activity, pH and acidity developed. The data obtained was used to plot the growth curves from which the characteristics of the culture were determined. The harvesting time of the culture was determined as the time when both the maximum TVC and activity are obtained.

3.4.2. Growth optimisation

The culture was grown as in 3.4.1. but samples were withdrawn immediately after inoculation and at the harvesting time (as determined from 3.4.1.). Five replicates were

conducted in each of the three media when unbuffered and buffered with $CaCO_3$. The samples were used for determination of TVC activity, pH, acidity and percentage aromabacter. The results were used to compare the growth in the three media and how buffering affects the growth of the culture.

3.4.3. Concentration

Cultures at harvest time were neutrilized by 1N NaOH to pH 6.9 (4,18). Cultures grown in RSM were further treated with 2% sodium citrate to break casein particles (4,37,91). The cultures were harvested by centrifugation in the Beckman centrifuge. The centrifugation conditions adapted were 4,000 r.p.m. for 30 minutes, the maximum speed possible in this centrifuge. The concentrated culture was analysed for TVC, activity, % aromabacter, as well as contamination by coliforms and yeasts and molds. The concentrated culture was suspended in RSM at 30% of original volume and re-incubated for four hours to determine the change in the activity (72).

3.4.4. Preservation of concentrated culture

Cultures for preservation studies were needed in large quantities. They could not be grown in the 300 ml glass jar nor concentrated in the 100 ml tubes of the Beckman centrifuge. Instead four

litres of the culture were grown in the six litre Chemap glass fermenter as shown in plate 1. Such culture was concentrated by the laboratory separation running at 10,000 r.p.m. and ejecting the concentrated culture every two minutes.

The concentrated cultures were resuspended in 3% RSM and mixed with cryoprotective agents where applicable. The following cryoprotective agents were investigated and compared to preservation with no agent; 10% glycerol, 10% glycerine, 7% lactose and 5% yeast extract (18,72). The cultures were divided into 100ml portions and filled into 250ml plastic bottles with screwcaps. They were deep frozen at -32 and -20^{o}C (5).

Concentrated cultures were also freeze-dried. The conditions used were 0.05 at pressure and -35^{o}C for 24 hours. The freeze-dried concentrated culture was packed in plastic bags and stored in a refrigerator.

3.4.5. Performance of concentrated mala culture during preservation.

Tests for thawing conditions of the culture were conducted on the frozen concentrated culture by holding the same at different temperatures for different time intervals as suggested by several workers (2,42,49,53,76,84,91). The optimum thawing conditions were determined and adapted for further

Plate 1. Fermentation equipment used to produce culture bacterial cell on masses.

1. Fermentation vessel 2. Sampling point 3. Thermometer 4. Gas outlet 5. Baffle 6. Gas inlet 7. Inoculation point 8. pH probe 9. Stirrer 10. Timber 11. pH meter 12. Water outlet 13. Water jacket 14. Water inlet 15. Pulsating pump 16. Chilled water reservoir 17. Stirrer speed control 18. Stirrer mortar 19. Stands 20. Gas source 21. Burret with 0.1 N NaOH 22. Samples

studies.

The culture bottles were removed from the deep freezer after one day and thereafter at three weeks intervals for four months. They were allowed to thaw as required and used to evaluate their performance during frozen storage. The freeze-dried culture was removed from the refrigerator and used immediately. The cultures were tested for TVC, species balance, activity and contamination. They were used to make mala which was tested for pH, aciditity, viscosity and diacetyl formation. The products were subjected to sensory evaluation and compared to mala made by the conventional culture.

3.4.6. Mala production

Mala was produced according to the method recommended by Kurwijila, 1980 and adopted by DFTN. The inoculation rate of 3% (V/V) was used for the conventional culture and an inoculaiton rate to give an equivalent number of cells of the concentrated culture. Fresh milk with 3.3% bufferfat was used.

3.5. MICROBIOLOGICAL DETERMINATIONS

3.5.1. Total viable count (TVC)

The TVC was determined by the pour plate colony count method in lactic agar. After pouring and overlying, the plates were incubated at $30^{o}C$

for 48 hours (19,22,91). The enumeration, recording and reporting of the colony count was done according to Standard Methods for Examination of Dairy Products (100).

3.5.2. Species Balance

Species Balance was expressed as the percentage aromabacter of the TVC. The aromabacteria count was determined in the calcium citrate agar according to the method of Nickels and Leesment, 1964.

3.5.3. Contamination

The culture was tested for contamination by yeasts and molds and by coliforms from the microbial count of the same in acidified potato dextrose agar and in violet red bile agar respectively (89). The enumeration, recording and reporting of the count was done according to the Standard Methods for Examination of Dairy Products (100).

3.5.4. Bacteriophage

Following the observation of rapid loss of viability in some cultures during preservation it was felt necessary to test the same for the presence of bacteriophage. The method by Hong, 1982, and Suzzane and Sandine, 1981, was adopted for the determination of bacteriophage presence in

the cultures.

3.5.5. Activity

The activity of the culture was expressed as the acidity developed in RSM by the culture inoculated at a specified rate and incubated in a specific temperature for a given time period. Two tests were used for determination of culture activity namely:-

1. 10% conventional culture or concentrated culture to give equivalent inoculation rate, incubated at 35°C for exactly five hours.
2. 3% conventional culture or concentrated culture giving an equivalent inoculation rate, incubated at room temperature for 18 hours.

The first test is more accurate than the second (33,95) and was used during the first part of the study. However, it was inconsistent with experimentation procedures of the later part of the study and the second test was used.

3.6. CHEMICAL ANALYSIS

3.6.1. Acidity

Acidity was expressed as the percentage lactic acid in the culture product. It was determined by titrating a 10 ml sample with 0.1 N NaOH to pH 8.4 (46). The percentage lactic acid was calculated as follows:-

% Lactic acid = Titre x 0.09 (89).

3.6.2. Diacetyl Determination

Diacetyl was determined according to the method of Owades and Jacovac as described by Pack *et. al.* 1964. The reagents were prepared according to the improved method by Walsh and Cogan, 1974. The standard curve is shown in figure 6 and was arrived at with standard solution of dimethyl glyoxine. The standard solution was prepared according to the A.O.A.C. method 10.47 (103) but treated according to the Owades and Jakovac method (67). The apparatus were arranged as shown in plate II.

3.6.3. pH

The pH was determined by the pH meter, E516, Titriskop, Switzerland.

3.6.4. Viscosity

The viscosity of the stirred cultured product was measured by Torsion viscometer, at 25^{o}C using system A and speed II (46). The viscometer was calibrated with glycerol.

3.7. SENSORY EVALUATION

Sensory evaluation tests were conducted to assess the product made by the concentrated culture and compare the same with products made by the conventional culture. Two tests were conducted

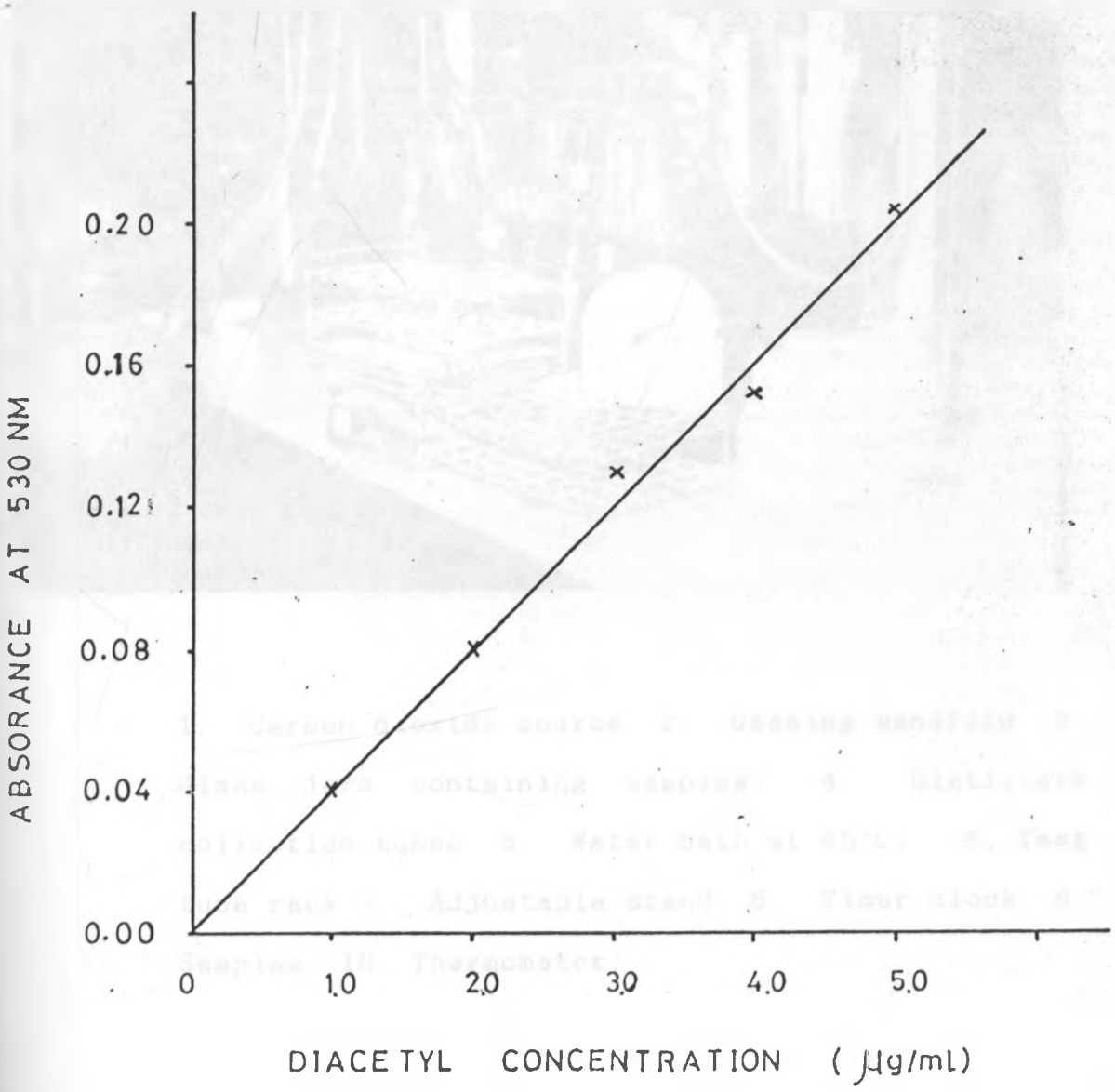


Fig. 6. Standard curve for diacetyl determination

Plate II: Apparatus used for distillation and collection of diacetyl.

1. Carbon dioxide source 2. Gassing manifold 3. Glass jars containing samples 4. Distillate collection tubes 5. Water bath at 65°C. 6. Test tube rack 7. Adjustable stand 8. Timer clock 9. Samples 10. Thermometer.

namely, Triangle test and Ranking Preference test. Samples were presented in coded cups together with the instruction forms. The procedure and analysis was conducted according to Hearthenbell, 1970. The instruction forms and analysis charts for the two tests are shown in appendices I to Iv.

4. RESULTS

4.1. GROWTH STUDIES

4.1.1. Growth of the mala starter culture

Data obtained from the growth of the mala culture in three different media, namely, skimmilk, whey and tryptone (broth) when unbuffered and when buffered with calcium carbonate were used to plot the growth curves shown in figure 7 From these curves, important parameters to characterise growth of the culture were determined and expressed in table 1. The growth of the culture as expressed by the total viable count (TVC) was different in the three media and also under the different conditions of buffering. When grown in the unbuffered media, the lag phase of the culture was about 33% shorter in skimmilk and tryptone than in whey. Similarly the stationery phase, the specific growth rate and maximum count were about 1.2 to 1.5 times higher when the culture was grown in milk than in the other two media. Buffering the media with calcium carbonate did not change the lag phase but the other characteristics were improved. The stationery phase was increased by 50% in whey and tryptone and 25% in milk. The specific growth rate was increased by 7-9% in milk and whey and 2% in tryptone. The maximum count was increased by 65% in tryptone, 20% in whey and 5% in milk.

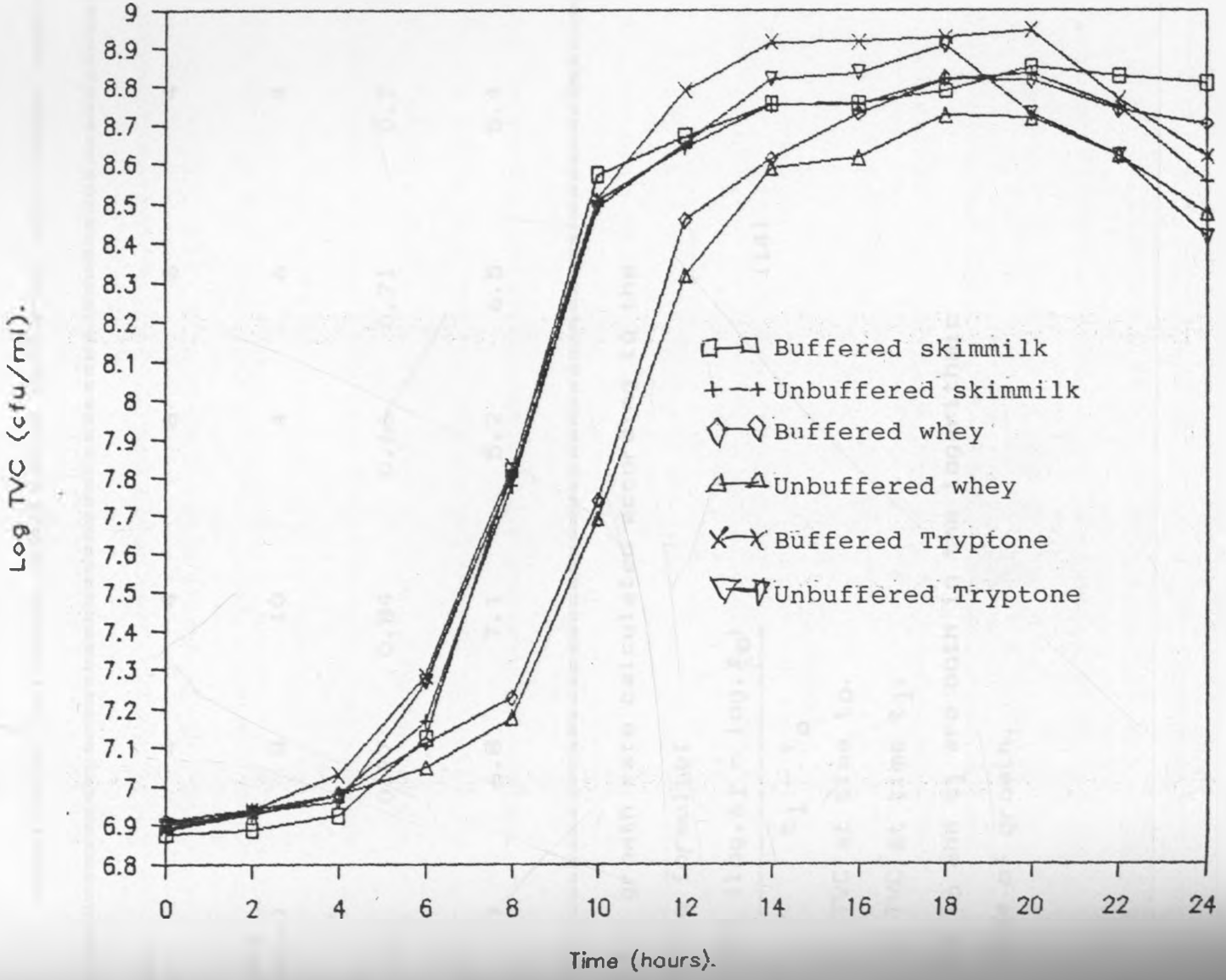


Figure 7: Growth Curves of Mala Culture

e 1; The growth characteristics of the mala culture.

	Skim milk		Whey		Tryptone broth	
	unbuffered	buffered	unbuffered	buffered	unbuffered	buffered
phase ours)	4	4	6	6	4	4
tionery se (hrs.)	8	10	4	6	4	6
cific wth rate)	0.77	0.84	0.66	0.71	0.7	0.71
imum rt ( ) fu/ml)	6.8	7.1	5.2	6.5	5.4	8.9

Specific growth rate calculated according to the following formulae;

$$k = \frac{2.303\ (\log X_1 - \log X_o)}{t_1 - t_o} \qquad (14)$$

re X_o= TVC at time t_o.

X_1= TVC at time t_1.

and t_o and t_1 are both in the logarithmic phase of growth.

4.1.2. Buffering capacity of calcium carbonate

Skimmilk was used to evaluate the buffering effectiveness of calcium carbonate in the media during growth of the mala culture. The pH change and lactic acid development were followed during growth. A plot of the pH values against the corresponding lactic acid values is expressed in figure 7. The buffering effectiveness can be expressed by the slope of the curve, which is the rate of pH decline as compared to the lactic acid development. The slope of the buffered medium was 3.3 as compared to 3.8 for unbuffered medium. This shows that the calcium carbonate has a buffering effectiveness of 15% over that of the medium. It can also be seen from figure 7 that the final pH is higher in the buffered than unbuffered medium by about 0.70 pH units. And, the final acidity (% Lactic acid) developed in the buffered medium is about 27% lower than in the unbuffered medium.

4.1.3 Change in Activity of Culture During Growth.

The activity of the mala culture was followed during the growth of the culture in the three media and under the two levels of buffering for 24 hours. Activity was expressed as the percent lactic acid formed by a 10% culture inoculated into milk and incubated at 35^{o}C for exactly five hours.

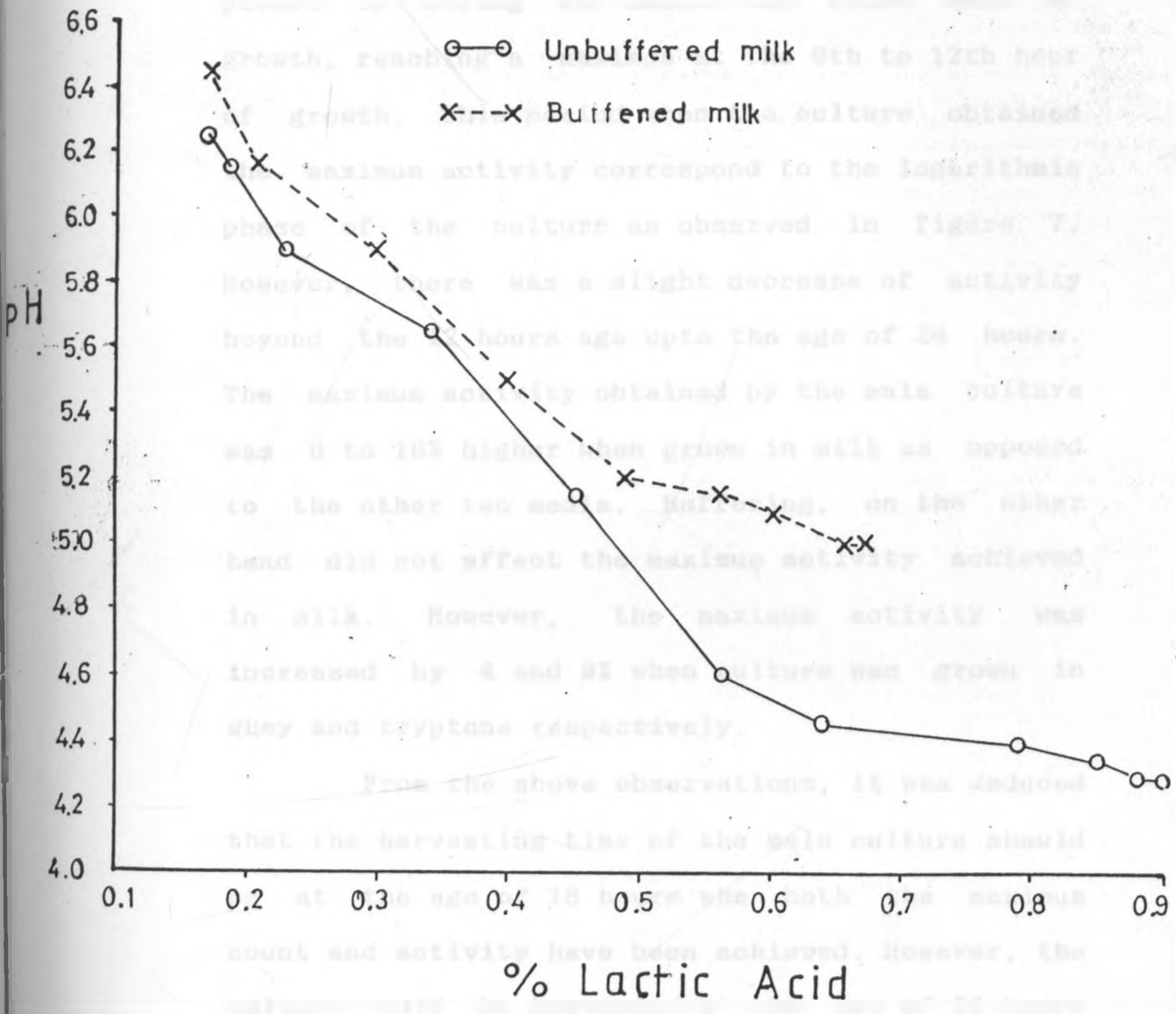


Fig. 8 The buffering effectiveness of calcium carbonate in skimmilk.

The change in the activity of the culture is shown in figure 9. The activity of the mala culture in whey and tryptone were initially very low, but picked up during the second and third hour of growth, reaching a maximum at the 8th to 12th hour of growth. This period when the culture obtained the maximum activity correspond to the logarithmic phase of the culture as observed in figure 7. However, there was a slight decrease of activity beyond the 12 hours age upto the age of 24 hours. The maximum activity obtained by the mala culture was 8 to 18% higher when grown in milk as opposed to the other two media. Buffering, on the other hand did not affect the maximum activity achieved in milk. However, the maximum activity was increased by 4 and 9% when culture was grown in whey and tryptone respectively.

From the above observations, it was deduced that the harvesting time of the mala culture should be at the age of 18 hours when both the maximum count and activity have been achieved. However, the culture could be harvested at the age of 24 hours with only a slight decrease in the maximum count and activity. For convenience of the working procedures, the harvesting age of 24 hours was adopted for the rest of the project.

4.2. GROWTH OPTIMISATION AND CONCENTRATION

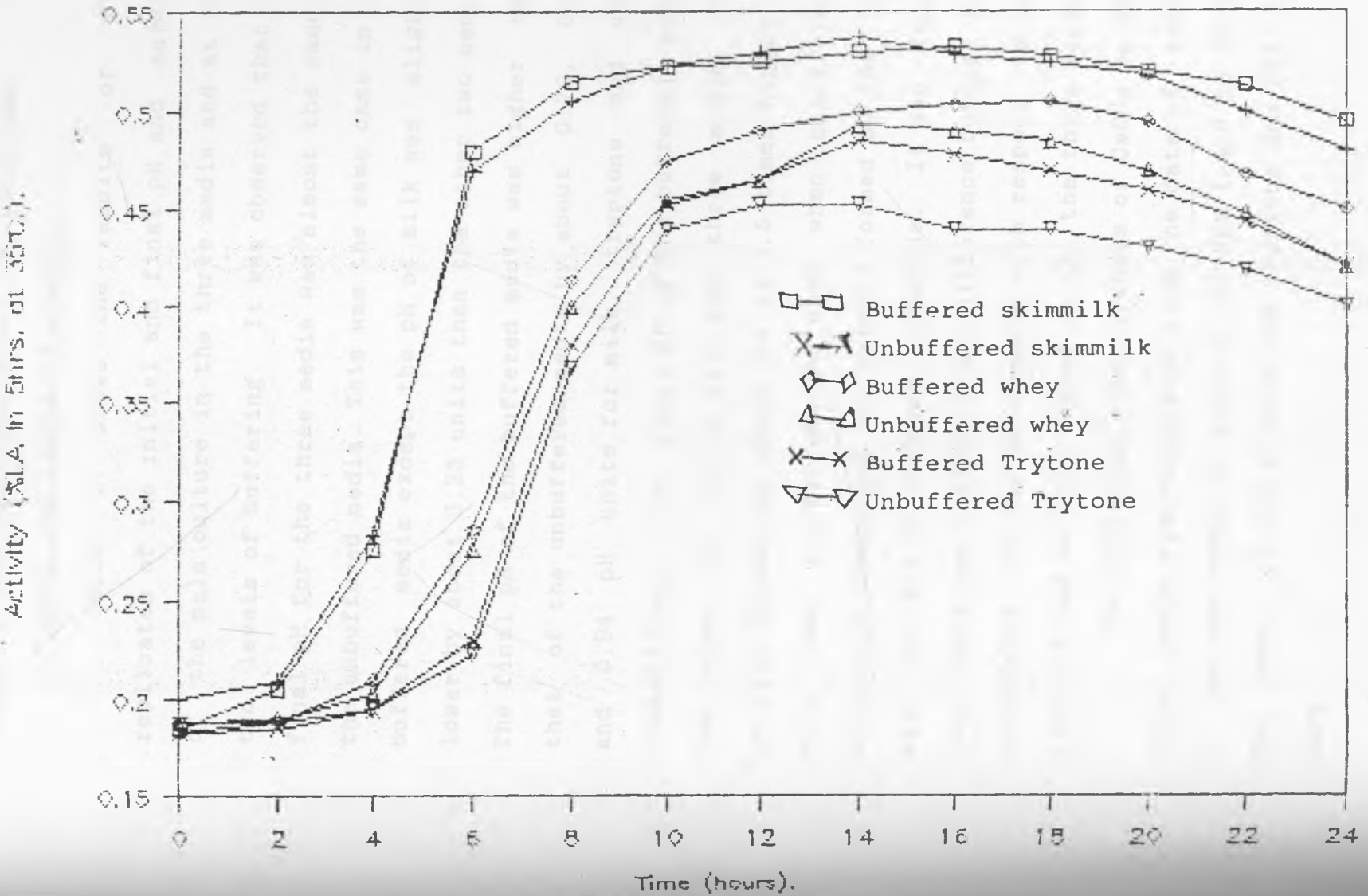


Figure 9: The Activity of Malt Culture

4.2.1. Effect of Growth Media and Buffering on pH and Acidity Formation.

Table 2 shows the results of five replicates of the initial and final pH and acidity of the mala culture in the three media and at the two levels of buffering. It was observed that the final pH for the three media was almost the same in the unbuffered media. This was the same case in the buffered media except the pH of milk was slightly lower by about 0.25 units than the other two media. The final pH of the buffered media was higher than that of the unbuffered media by about 0.70, 0.80 and 0.94 pH units for milk, tryptone and whey respectively. The final pH of the buffered media was above pH 5.0 in all the three media. The acidity formed was about 1.2 to 1.5 times higher in milk than in tryptone and whey when unbuffered. Buffering decreased the acidity formed by 16% in milk and 22% in whey and tryptone. It was found that there was a significant difference between the unbuffered and buffered media with respect to the final pH and acidity formed in all the three media.

The buffering effectiveness of $CaCO_3$ on the three media was calculated from the data of table 2, and are shown in table 3. It was found to be in the order of 15% in milk and tryptone and 22% in whey.

Table 2: Effect of growth media and buffering on the pH change and lactic acid development of the mala starter culture.

Growth media	buffering with $CaCO_3$	initial (0 hrs.) pH[a]	initial (0 hrs.) %LA[a].	final (24 hrs.) pH[a]	final (24 hrs.) %LA[a].
Skimmilk	unbuffered	6.52	0.17	4.40[b]	0.88[c]
	buffered	6.60	0.16	5.10	0.76
Whey	unbuffered	6.23	0.14	4.42[b]	0.60[c]
	buffered	6.53	0.11	5.36	0.49
Tryptone Broth	unbuffered	6.19	0.22	4.53[b]	0.72[c]
	buffered	6.41	0.21	5.33	0.59

a: Mean of five replicates.

b: Significant difference ($p<0.05$) in PH between the buffering and unbuffering in each medium .

c : Significant difference ($p<0.05$) in % lactic acid between buffering and unbuffering for each medium.

Table 3: The buffering effect of calcium carbonate on the media during the growth of mala culture.

Growth Media	Buffering effectiveness of $CaCO_3$[a]		
	growth media alone (pH/%LA.)	growth media with buffer (pH/%LA.)	buffer alone (% of growth media)
Skimmilk	2.96	2.50	15.50
Whey	3.93	3.08	21.60
Tryptone	3.32	2.84	14.50

a: Buffering effectiveness is expressed as

$$\frac{\text{Initial pH - Final pH.}}{\text{Final \% LA. - Initial \% LA.}}$$

4.2.2. Effect of Growth Media, and Buffering on the total viable count (TVC).

The effect of growth media and buffering with $CaCO_3$ on the TVC of the mala culture is shown in table 4. There was a net increase of 110-140 times in the TVC of the mala culture during the 24 hours of growth. But an extremely high increase (of 190) times was reported in buffered whey. The TVC at harvest was found to be within a range of 7.0 - 9.0 x 10^8 cfu/ml with low values being reported in tryptone. Buffering the media increased the TVC of the culture at harvest time and after centrifugation. The net increase in TVC during growth was by 1.7 times in tryptone but by 1.3 and 1.2 times in milk and whey respectively due to buffering . On the the other hand the TVC on centrifugation was increased by 1.4 times in whey and tryptone and decreased by 25% in milk. There were large standard errors in the values of TVC after centrifugation. This was due to inaccuracies resulting from errors associated with the centrifugation method. Due to the above factor, the cell yield was considered as a better way of evaluating the effect of media and buffering on the concentration of the mala culture.

4.2.3. Effect of Growth Media and Buffering on Cell Yield of the Mala Culture.

Table 4: Effect of growth media and buffering on the total viable count (TVC) of mala culture.

Growth Media[b]	Buffering with $CaCO_3$[c]	Total viable count (cfu/ml.)[a] ($*10^6$)			
		Initial (0hrs.)	Final (24 hrs.)	Net Increase	After harvest
Skimmilk	unbuffered	6.2	740	734	85000
	buffered	6.3	900	894	64000
Whey	unbuffered	6.6	870	863	51000
	buffered	6.3	1200	1194	70000
Tryptone Broth	unbuffered	7.1	440	433	56000
	buffered	6.8	750	743	78000

a: Mean of five replicates.

b: No significance ($p<0.05$) in TVC between the three media.

c: No significance ($p<0.05$) in TVC between buffering and unbuffering in each medium

The cell yield of the mala culture was expressed as the weight in grams of the concentrated cells recovered by centrifugation per 100 ml of the culture harvested. This value was corrected for solids-not-cells using a blank. The cell yield obtained from the three media at the two levels of buffering are shown in table 5. It is observed that in the unbuffered cultures, cell yields were highest in milk, 16% less in whey and 62% less in tryptone. But in the buffered cultures, the cell yields was highest in whey, 12% less in milk and 66% less in tryptone. Buffering, therefore, increased the cell yield by 47% in whey and only 9 and 10% in milk and tryptone respectively. There was a good relationship between the final pH of the media, the TVC at harvest and the cell yield of the culture (Table 2, 4 and 5.)

4.2.4. Effect of Growth Media and Buffering on the Activity of Mala Culture

Table 6 shows the activity of the mala culture after 24 hours growth in the three media, after centrifugation and after re-incubation in milk for 18 hours. It can be observed that pH was not sensitive in differentiating the activity as a result of various treatments of media and buffering. Percent lactic acid was found better in expressing the activity of culture. The activity

Table 5; Effect of growth media and buffering on the cell yield of the mala culture after concentration.

Buffering With $CaCO_3$	Growth Medium		
	Skimmilk[b]	Whey[b]	Tryptone Broth[c]
Unbuffered[d]	2.99	2.52	1.14
Buffered[e]	3.26	3.71	1.26

a; Mean of five replicates.

bc; Significant difference ($p<0.05\%$) in cell yield between the three growth media

de; Significant difference ($p<0.05\%$) in cell yield between Buffering and unbuffering in all the three media.

Table 6: Effect of growth media, buffering and re-incubation on the activity of the mala culture[a].

Growth Medium [b]	Buffering with $CaCO_3$ [c]	Activity after 24 hours		Activity after centrifugation		Actvity after re-incubation	
		pH	%LA.	pH	%LA.	pH	%LA.
Skimmilk	unbuffered	4.45	0.81	4.53	0.75	4.41	0.86
	buffered	4.45	0.82	4.56	0.77	4.20	0.81
Whey	unbuffered	4.38	0.79	4.58	0.75	4.30	0.89
	buffered	4.38	0.81	4.62	0.74	4.50	0.90
Tryptone Broth	unbuffered	4.54	0.76	4.65	0.71	4.50	0.83
	buffered	4.57	0.79	4.63	0.72	4.55	0.81

a: Mean of five replicates.

%LA. = Percent lactic acid.

b: No significant difference ($p<0.05\%$) in activity between the three media.

c: No significant difference ($p<0.05\%$) in activity between the buffering and unbuffering in each medium.

(as % lactic acid) of the culture at harvest was similar when the culture was grown in tryptone and whey but only slightly higher by about 3-7% in milk. However, the activity of the culture grown in all the three media decreased by about 5-10% on centrifugation. But on re-incubation this decrease in activity was regained by the culture grown in milk and increased to levels beyond those for cells at harvest in whey and tryptone. Buffering in all the three media had a very slight effect on the activity of the culture at harvest, after centrifugation or after re-incubation.

4.2.5. Applicability of Concentrated Mala Culture in Mala Production

Before the concentrated mala culture could be evaluated for storage, it was necessary to test for its viability and lactic acid production ability as shown in Tables 4 and 6 respectively. From the TVC of the concentrated mala culture ($5.0-9.0 \times 10^{10}$ Cfu/ml), it was calculated that an inoculation rate of 0.01-0.02% would give the required initial count of around 6.0×10^{6} cfu/ml (Table 4). Although the activity of the concentrated mala culture was lower than that of the conventional culture, such difference was quite low and of no significant since the acidity achieved was over 0.70%. Due to this, the culture

could be used directly to make products.

In addition to the above tests, the concentrated mala culture was tested for its ability to make mala of good acceptability as compared to that made by the conventional culture. Two products were made, one by the concentrated mala culture at 0.015% inoculation rate and other by the normal conventional mala culture at 3%. The products were evaluated for their acceptability by triangle test involving twelve panelists. Only three panelists could correctly identify the odd sample. The panelists ability to identify the samples was, therefore, statistically insignificant ($p<0.05$). All the three panelists who could correctly identify the sample, showed a preference for the product made by the concentrated mala culture

4.3. VIABILITY OF CONCENTRATED MALA CULTURE UNDER FROZEN CONDITIONS

The concentrated mala culture was treated to various cryoprotective agents and frozen at -32 and $-20^{o}C$. Samples were taken after freezing and thereafter at three weeks intervals and tested for TVC, % aromabacter, activity as well as viscosity and diacetyl formation. The data obtained was used to plot the graphs shown in figures 10, 11, 12 and 13. It was also used for statistical analysis. The

Table 7. The Statistical coefficients (F-values) for growth media and buffering on the storage of frozen concentrated mala culture.

	Growth media	Buffering		Buffered Milk vs Buffered Whey
	Milk vs Whey	Buffered vs Unbuffered		
		Milk	Whey	
Viabilty	131.60**	0.05	11.65*	0.18
% Aromabacter	7.45*	1.31	1.20	-
Acid producing activity	47.62**	0.82	7.22*	2.01
Viscosity	3.36	4.55*	6.70*	4.21*
Diacetyl formation	2.97	6.05*	4.63*	3.39

* ; Significant ($p<0.05$).

** : Highly significant ($p<0.01$).

Table 8. The Statistical coefficients (F-values) for cryoprotective agents used for storage of frozen concentrated mala culture.

	Agents vs Control			Grycerol vs (lact.[a] + Y.E.[b])			Lact.[a] vs Y.E.[b]		
	milk	buffered		milk	buffered		milk	buffered	
	control	milk	whey	control	milk	whey	control	milk	whey
Viability	35.90*	10.80*	9.23*	9.80*	1.32	1.04	0.12	-	-
% Aroma-bacter	4.95*	6.98*	3.07*	3.21*	0.77	3.10*	3.70*	-	2.99
Acid producing activity	2.59	8.75*	4.60*	-	7.43*	12.00*	-	0.00	1.41
Viscosity	26.33*	2.50	7.67*	2.16	-	5.35*	-	-	0.08
Diacetyl formation	3.50*	14.00*	3.33*	4.92*	6.10*	1.47	2.00	5.69	-

* ; Significant ($p < 0.05$).

a: Lact. means lactose.

b: Y.E. means yeast extract.

statistical coefficients are shown in tables 7 and 8.

4.3.1. Change in Viability of the Culture.

The change in the viability of the concentrated mala culture during freezing and under frozen storage is expressed in figure 10. It was observed that, there is a general decline in the viability of the culture during frozen storage, with a sharp decrease in the first three weeks. This sharp decrease was due to a major drop in the viability of the culture during the freezing process in the first one or two days of putting the culture in the deep freezer (see table 9). There was a decrease in viability of 20-25% in the control samples and 5-15% where cryoprotective agents were used. Cultures grown in milk were found to have a significantly higher survival than those grown in whey. Buffering the media during growth, improved the survival of the culture at -32^{o}C significantly when grown in whey but not in milk. It was apparent that the survival of the culture grown in unbuffered whey declined rather rapidly after the sixth week of storage. This was attributed to bacteriophage attack whose presence was detected after the three months storage. The survival was also higher when cryoprotective agents were used during frozen storage at -32^{o}C. Glycerol

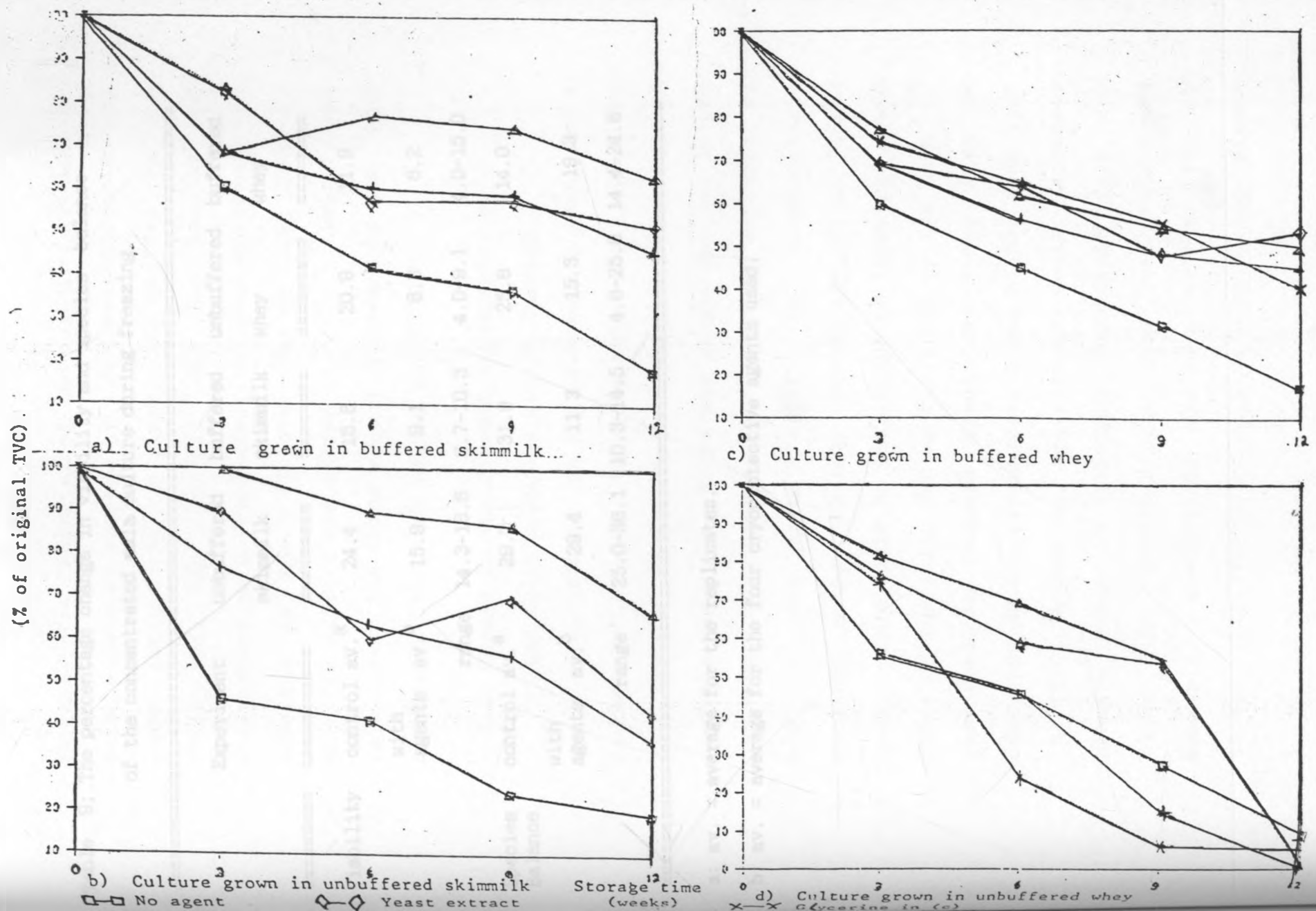

Viability
(% of original TVC)
a) Culture grown in buffered skimmilk
c) Culture grown in buffered whey
b) Culture grown in unbuffered skimmilk
d) Culture grown in unbuffered whey
Storage time (weeks)
No agent
Yeast extract
Glycerine in (c)

Table 9; The percentage change in viability and species balance of the concentrated mala culture during freezing.

	Experiment		unbuffered skimmilk	buffered skimmilk	unbuffered whey	buffered whey
Viability	control	av.[a]	24.4	18.8	20.9	11.9
	with agents	av.[b]	15.9	9.1	6.8	8.2
		range	14.3-16.6	6.7-10.3	4.0-9.1	4.0-15.0
Species Balance	control	av.[a]	29.2	31.9	25.8	14.0
	with agents	av.[b]	29.4	11.3	15.3	19.3
		range	25.0-36.1	10.3-14.5	4.6-25.8	14.4-24.8

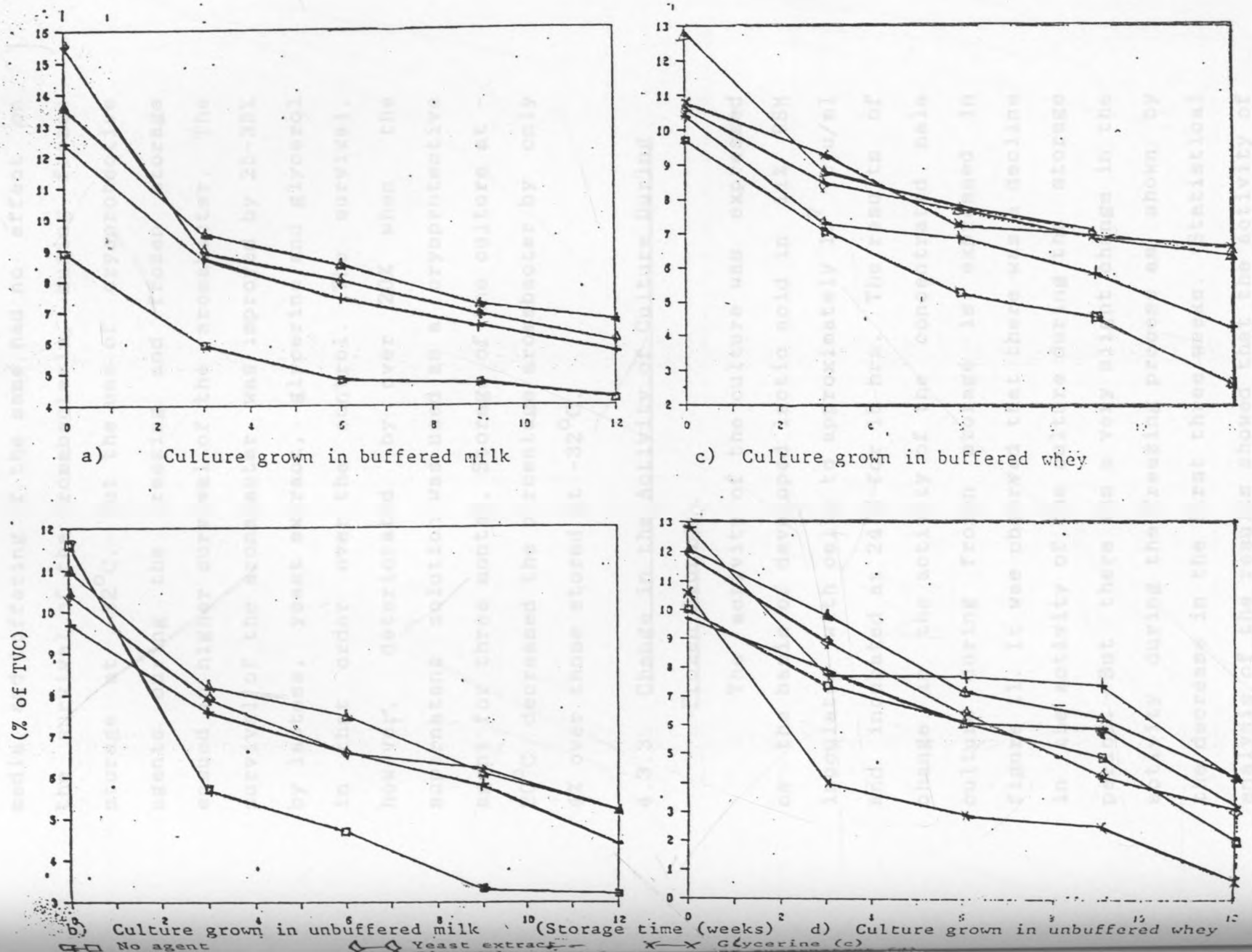
a: av. = average for the replicates.

b: av. = average for the four cryoprotective agents used.

improved the survival of the culture during the three months of storage by 1.8 times, glycerine and yeast extract by 1.6 times, and lactose by 1.2 times over the control. But the use of the supernatant as a cryoprotective agent decreased the survival by about 10% over that of the control. When the culture was stored at -20°C, the survival rate over the three months decreased by 10% over storage for the same period at -32°C.

4.3.2. Change in the Percent Aromabacter of the Culture During Frozen Storage.

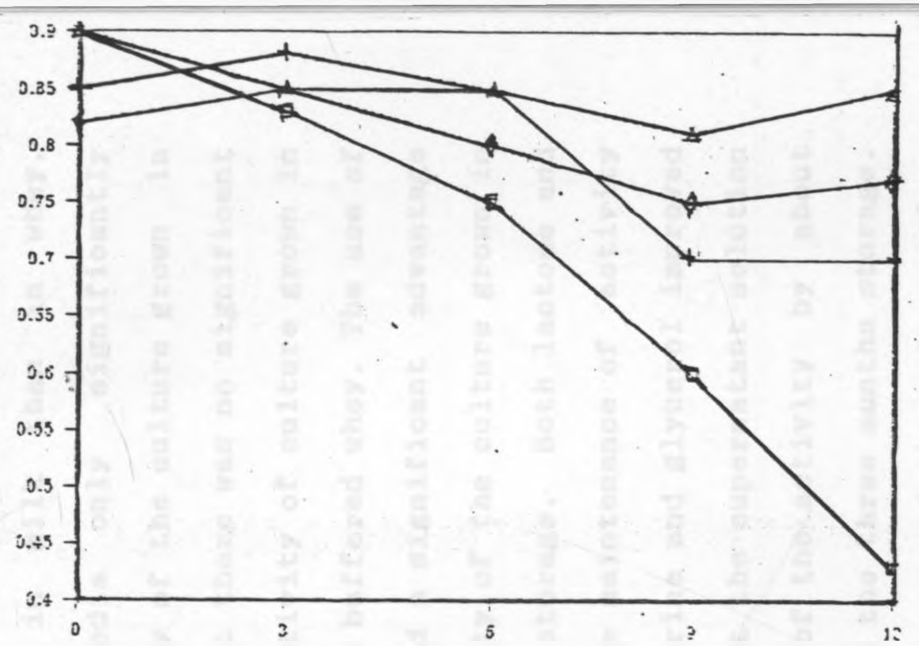
The change in the aromabacter count as a percentage of the total viable count of the concentrated mala culture during frozen storage is shown in figure 11. There was slight decline in the percentage aromabacteria during frozen storage. As was the case with the TVC the aromabacteria declined rather sharply during the first three weeks. This could be attributed to increased death rate of the cells during first one or two days of the freezing process (table 9). The rate of declining of the aromabacteria was higher than that the TVC suggesting accerelated death kinetics brought about by freezing. Such death rate was higher in aromabacteria than in acidulating bacteria. Statistical analysis of the results showed that the use of milk or whey as the growth


Aroma bacter count
(% of TVC)
(Storage time (weeks)
a) Culture grown in buffered milk
c) Culture grown in buffered whey
b) Culture grown in unbuffered milk
d) Culture grown in unbuffered whey
No agent
Yeast extract
Glycerine (c)

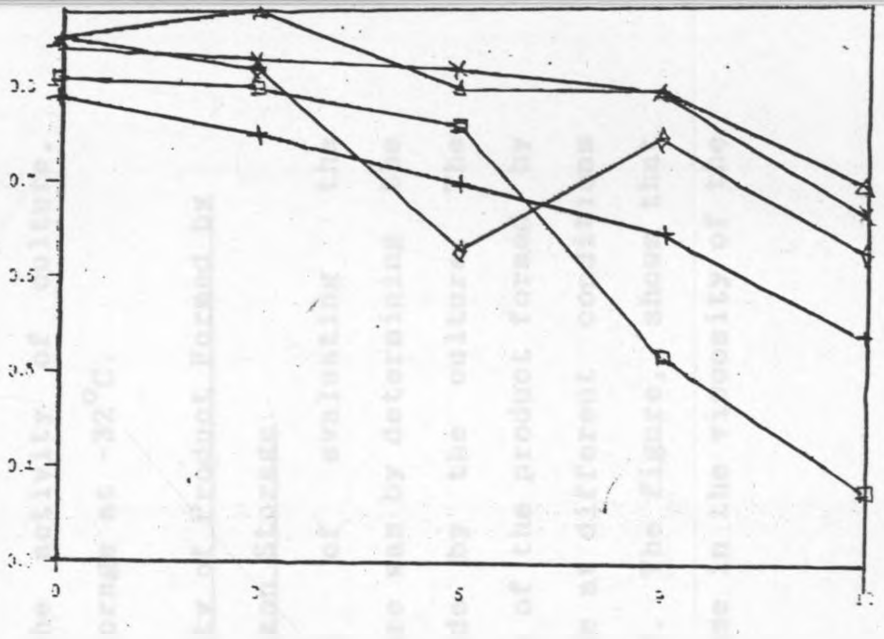
media and buffering of the same had no effect on the survival of the aromabacteria, during frozen storage at -32°C. But the use of cryoprotective agents during the freezing and frozen storage ensued in higher survival of the aromabacter. The survival of the aromabacter was improved by 25-35% by lactose, yeast extract, glycerine and glycerol in that order over the control. The survival, however, deteriorated by over 20% when the supernatant solution was used as a cryoprotective agent for three months. Storage of the culture at -20°C decreased the percentage aromabacter by only 4% over those stored at -32°C.

4.3.3. Change in the Activity of Culture During Frozen Storage.

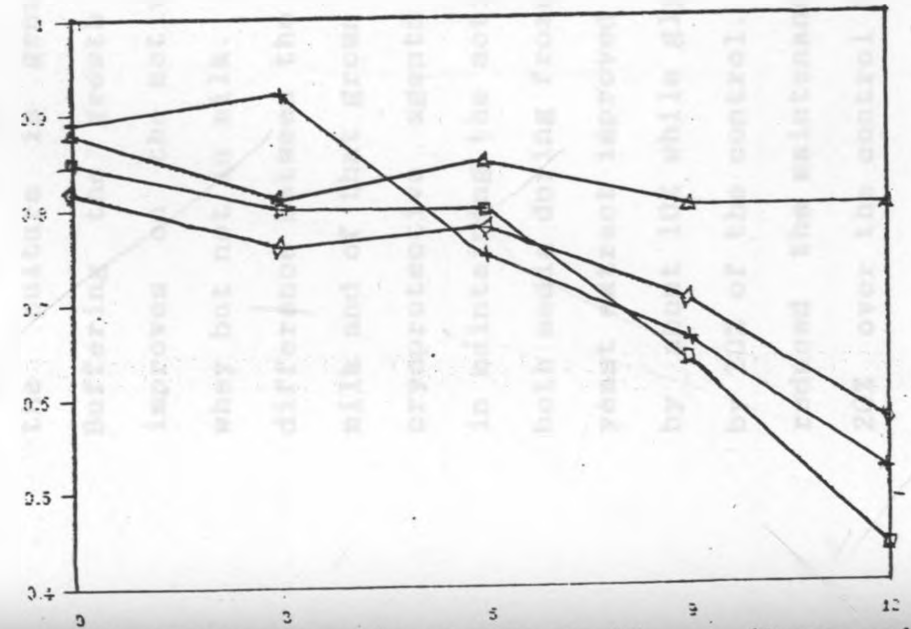
The activity of the culture was expressed on the basis of developed lactic acid in 11% RSM inoculated with cells to approximately 10^7 cfu/ml and incubated at 24°C for 18 hrs. The results of change in the activity of the concentrated mala culture during frozen storage is expressed in figure 11. It was observed that there was a decline in the activity of the culture during the storage period. But there was a very slight change in the activity during the freezing process as shown by the decrease in the first three weeks. Statistical analysis of the results showed that the activity of



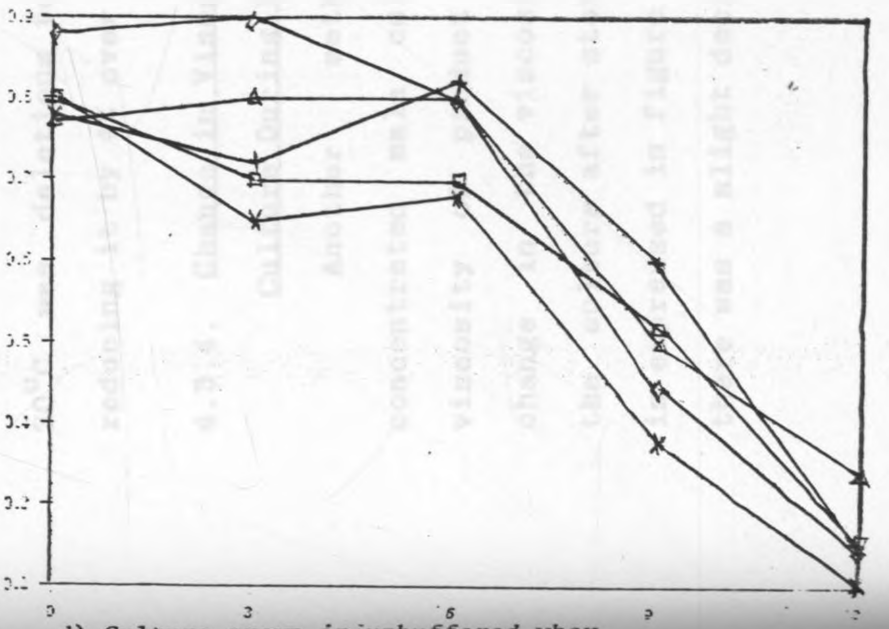
a) Culture grown in buffered milk



c) Culture grown in buffered whey



b) Culture grown in unbuffered milk



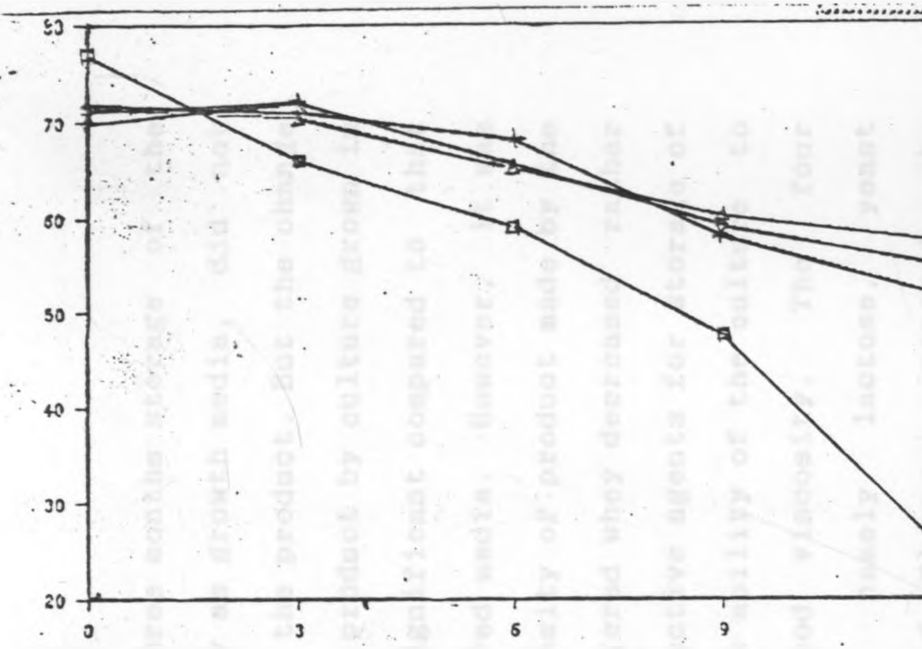
d) Culture grown in unbuffered whey

(Storage time
weeks)

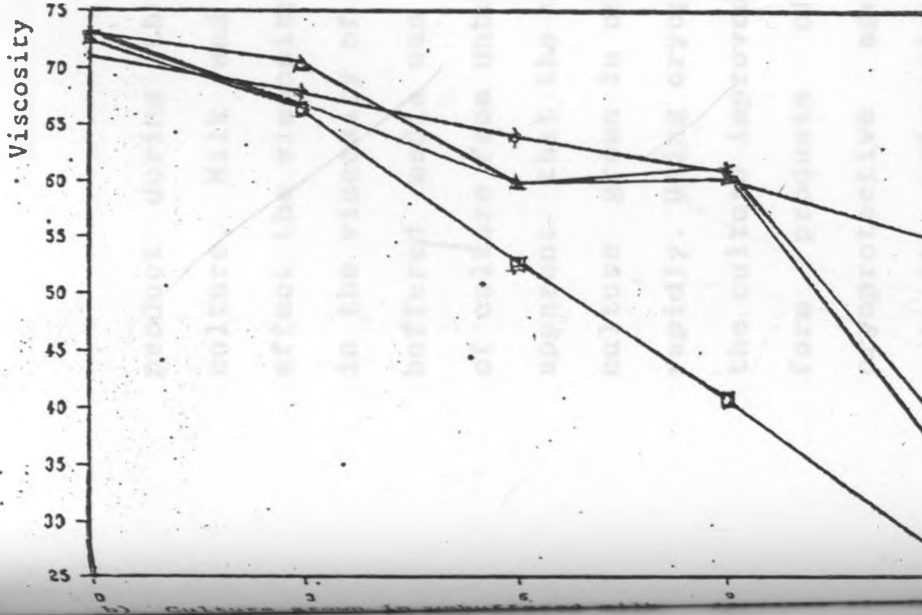
the culture is significantly maintained higher, if the culture is grown in milk than in whey. Buffering the growth media only significantly improves on the activity of the culture grown in whey but not in milk. But there was no significant difference between the activity of culture grown in milk and of that grown in buffered whey. The use of cryoprotective agents had a significant advantage in maintaining the activity of the culture grown in both media during frozen storage. Both lactose and yeast extract improved the maintenance of activity by about 10% while glycerine and glycerol improved by 20% of the control. But the supernatant solution reduced the maintenance of the activity by about 20% over the control for the three months storage. On the other hand the storage of the culture of $-20^{o}C$ was deletious to the activity of culture, reducing it by 4% over storage at $-32^{o}C$.

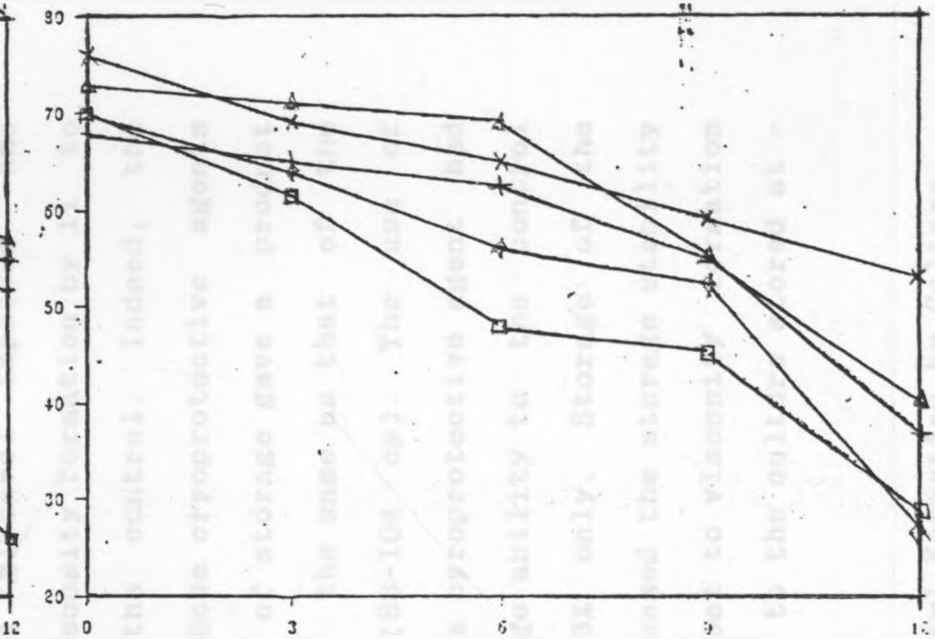
4.3.4. Change in Viscosity of Product Formed by Culture During Frozen Storage.

Another method of evaluating the concentrated mala culture was by determining the viscosity of product made by the culture. The change in the viscosity of the product formed by the culture after storage at different conditions is expressed in figure 13. The figure, shows that there was a slight decrease in the viscosity of the

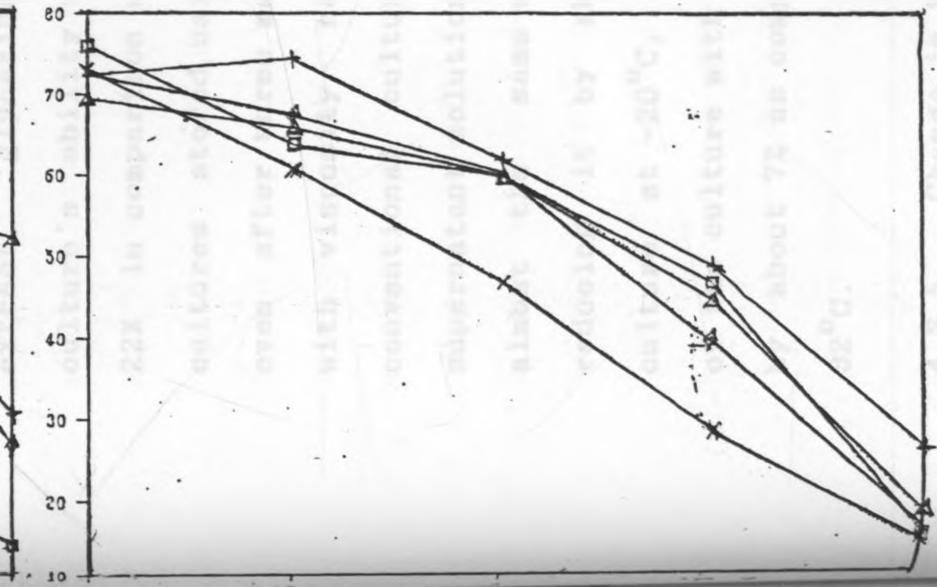


a) Culture grown in buffered milk





c) Cu'ture grown in buffered whey

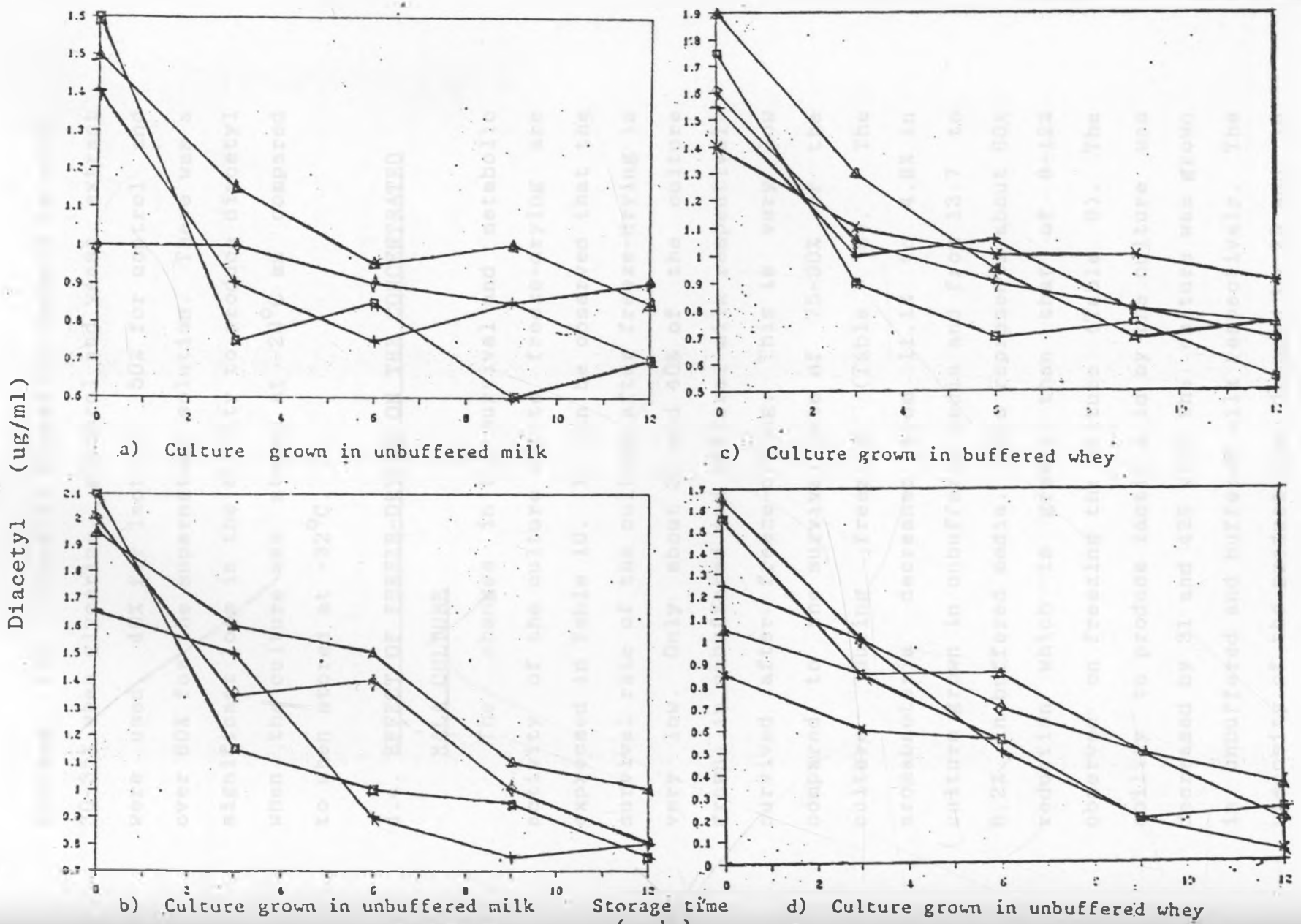


product during the three months storage of the culture. Milk and whey as growth media, did not affect the viscosity of the product. But the change in the viscosity of the product by culture grown in buffered media was insignificant compared to that of culture from unbuffered media. However, it was apparent that the viscosity of product made by the culture grown in unbuffered whey decreased rather rapidly. Using cryoprotective agents for storage of the culture improved the ability of the culture to form products of good viscosity. The four cryoprotective agents namely lactose, yeast extract, glycerine and glycerol improved the culture's ability to viscosity formation by 11 to 22% in comparison with the control. Indeed, the cultures stored using these cryoprotective agents even after three months of storage gave a product with viscosity nearly the same as that of the conventional cultures (68-104 cp). The use of supernatant solution as a cyroprotective agent had almost the same storage ability to the control reducing it by about 3% only. Storage of the culture at -20^{o}C, decreased the storage stability of the culture with respect to viscosity formation by about 7% as compared to the culture stored at -32^{o}C.

4.3.5. Change in Diacetyl Formation by Culture

During Frozen Storage.

The ability to produce diacetyl by culture stored under different conditions was evaluated. The results of changes in diacetyl produced by the stored culture in shown in figure 14. There was a general decline in the diacetyl formed by the culture stored under different conditions. A higher rate of decrease was observed in the first three weeks of storage. This period coincided with the sharp decrease in viability and percent aromabacteria of the culture as shown in figures 10 and 11 respectively. Statistical analysis of the results showed that the ability of the culture to produce diacetyl was equally conserved during storage irrespective of whether it was grown in milk or whey. In the buffered media, the decline in diacetyl production tended to stabilise after three to six weeks, while in unbuffered media, diacetyl production declined throughout the three months storage. The use of cryoprotective agents during frozen storage at $32^{o}C$, significantly enhanced the ability of the culture to form diacetyl in general. Glycerol, glycerine and yeast extract were found more effective than lactose in conserving the ability of culture to produce diacetyl. However lactose was more effective than the control while the supernatant solution was less effective as compared to the control. During the three months


Diacetyl (ug/ml)
a) Culture grown in unbuffered milk
c) Culture grown in buffered whey
b) Culture grown in unbuffered milk
d) Culture grown in unbuffered whey
Storage time (weeks)
No agent
Yeast Extract
Glycerine in (c)

storage, the diacetyl formation dropped by about 30-35% when glycerine, glycerol and yeast extract were used, 40% for lactose, 50% for control and over 80% for the supernatant solution. There was a significant loss in the ability to produce diacetyl when the culture was stored at -20°C as compared to when stored at -32°C.

4.4. EFFECT OF FREEZE-DRYING ON THE CONCENTRATED MALA CULTURE

The changes in the survival and metabolic activity of the culture due to freeze-drying are expressed in Table 10. It can be observed that the survival rate of the culture after freeze-drying is very low. Only about 30 and 40% of the culture grown in unbuffered and buffered milk respectively survived after freeze-drying. This is very low compared to the survival rate of 75-90% of the culture during freezing (Table 9). The aromabacteria decreased from 11.1% to 4.6% in culture grown in unbuffered media and from 13.7 to 6.2% in buffered media. This represents about 60% reduction which is greater than that of 8-12% observed on freezing the culture (Table 9). The ability to produce lactic acid by the culture was decreased by 31 and 42% when the culture was grown in unbuffered and buffered milk respectively. The viscosity of the product also dropped by 25 and 18

able 10 The effect of freeze-drying on the survival and metabolic activity of concentrated mala culture.

	Non buffered media			Buffered meddia		
	Frozen	Freeze-dried	% change	Frozen	Freeze-dried	% change
ight (g).	280	61.4	78.1	280	60.1	78.5
C ( ) (cfu/ml	10	92		10	120	
urvival.		32.6			39.8	
Aromabacter.	11.1	4.6	58.6	13.7	6.2	54.7
of product.	4.55	5.1	12.8	4.45	4.95	11
LA developed.	0.81	0.57	29.6	0.84	0.64	23.8
scosity (cP).	70.5	54	23.4	70	58	17.1
cetyl (ug/ml)	1.35	0.55	59.3	1.15	0.7	29

cp when prepared by culture grown in unbuffered and buffered media respectively. The diacetyl formation by culture grown in unbuffered milk decreased from 1.35 to 0.55 ug/ml while by culture grown in buffered milk decreased from 1.55 to 0.70 ug/ml. These changes were more drastic than those in lactic acid producing ability, viscosity and diacetyl formation after freezing of the concentrated mala culture.

4.5. SENSORY EVALUATION

4.5.1. Triangle Test

The functionality of the concentrated mala culture in mala production after various storage periods was evaluated by sensory evaluation of the product, mala, made by the same, as compared to mala made by using normal conventional culture. The acceptability of the two such product was compared by triangle test. The results are shown in Table 11. It is observed that only about a third of the panelists could correctly identify the samples. This was compared to that shown in the chart of appendix I. This number was statistically insignificant to establish a difference in any of the two samples. Over 50% of the panelists who could identify the samples, preferred the product made by the concentrated mala culture.

le 11; Comparison of products made with concentrated mala culture and conventional mala culture by triangle test.

	Storage time (weeks)				
	0	3	6	9	12
mber of nelists	11	12	10	15	12
mber of rrect identification	3[a]	3[a]	4[a]	5[a]	4[a]
mber of correct entification preferring ference sample	0	1	2	2	0
mber of correct dentification preferring st sample	3	2	2	3	4

[a] No significance at $p < 0.05$. between the two similar samples and the odd sample.

4.5.2. Preference Test

Panelists ranging from 10-15 were provided with four coded mala products. One product was made by the conventional culture while the other four were made by the concentrated mala culture. The panelists were required to taste the samples and rank them according to their preference. The panelists ranks were converted into scores according to the chart shown in appendix II. The total scores for each product is shown in Table 12. The total scores were checked for significance. It was found that there was no significance difference in the total scores of the four products during the first six weeks of storage for the concentrated mala culture. But at the ninth week of frozen storage, the product made by culture grown in unbuffered whey (4) was significantly inferior to the others. Otherwise all the others had no significant difference in scores. At the 12^{th} week of storage the above product was not tested. Instead a product made by the freeze-dried concentrated mala culture was tested with the others. It was found that there was no significant difference in the total scores of all the four products including that made by the freeze-dried concentrated mala culture.

Table 12; Comparison of products made with concentrated mala culture and conventional mala culture by preference test.

Storage time (weeks)	Number of Panelists	Total scores for product samples 1	2	3	4	5
0	11	0.6	-1.2	0.73	0.6	
3	12	1.03	0.43	-1.76	0.3	
6	10	1.33	0.73	-1.46	0.6	
9	15	2.03	1.73	2.92	-6.68*	
12	12	2.63	0.6	2.06	-	4.12

* Product significantly different from the others ($p < 0.05$)

\- Product not tested.

5. DISCUSSION

5.1. Growth Characterisation of the Mala Culture.

The lag phase for the mala culture was 4 hours when grown in milk and tryptone and 6 hours when grown in whey. The stationery period was between the 14^{th} and 20^{th} hours in milk, 16^{th} and 20^{th} in whey and 14^{th} and 18^{th} in tryptone. Thus the duration of the stationery phase was about 50% longer in milk than in the other two media. The specific growth rate was highest in milk at 0.77 cfu/ml per hr. and about 30% higher than in the other two media. On the other hand the maximum count was nearly the same for three media at 8×10^8 cfu/ml. This, however decreased by about 50% in milk and 60% in tryptone and whey by the 24th hour. These findings show that the mala culture grows at a faster rate in milk but the level of cells achieved is the same. However, the higher level of cells can be maintained for a longer time in milk. These observations are in contrast to that of other researchers. The lag phase of *Streptococcous lactis* grown in tryptone was reported as 2 hours by Port and Holloway, 1968. The maximum count of 9.5×10^9 per ml was achieved at the 12th hour. This however decreased by about 68% to 3×10^9 per ml at the 18th to 24th hour. This differences from the mala culture, can be expected, since *S. Lactis* as a

pure culture grows faster than the other species in the mala culture (7). The specific growth rate of mala culture was higher than that of 0.68 cfu/ml/hr reported by Blaine *et. al*, 1970, for a lactic streptococci culture grown in milk.

Buffering the media did not affect the lag phase of the mala culture, although the duration of the stationery phase was prolonged by 25% in milk and 50% in tryptone and whey. The specific growth rate was increased by 7 - 9% in milk and whey, and by 2% in tryptone. The maximum count was increased by 30, 20 and 70% in milk, whey and tryptone. These findings show that the $CaCO_3$ buffer improved the growth media better in whey and tryptone than in milk. These observations were comparable to those of other cultures elsewhere (4,26,72,74). Port and Holloway, 1968, reported that there was no decline in the maximum count of *S. lactis* for upto 36-48 hours when the pH was controlled at 6.3 (74). This represented an increase in the duration of stationery phase by about 200-250%. Blaine *et. al.* 1970, reported that the specific growth rate of a lactic streptococci culture grown in milk increased from 0.68 to 0.72, 0.74 and 0.94 cfu/ml per hour when pH was controlled at 6.3 by NaOH, KOH and $Ca(OH)_2$, and Na_2CO_3 and NH_4OH respectively. This represented increases of about 6, 9 and 40%

respectively. The maximum population of this culture was increased by 48-58% when NaOH, KOH and $Ca(OH)_2$ was used as the neutralizer and 74% when NH_4OH and Na_2CO_3 were used. The maximum cell counts for *Streptococcus cremoris* was increased by 9-14 times when the pH was maintained at 5.5, 6.0 and 6.5 compared to where pH was not controlled (70). Control of pH at 6.5 by neutrilisation using NaOH increased the maximum cell counts for lactic streptococci 15 fold in tryptone broth (26).

In the buffered media, the lag phase of mala culture was longer in whey by about 50% times over the other two media. The stationery phase was same in tryptone and whey, but about 20% higher in milk (table 1). The maximum count in whey was higher by 1.3 and 1.6 times than in milk and tryptone respectively (table 4). These findings show that in buffered media, the mala culture grows fast in milk but a higher population was achieved in whey. Therefore, the best media would be buffered whey. These observations are comparable to those of other workers (72, 74). The maximum cell population of *S. Lactis* was two times higher in milk than in whey when the pH of both media was maintained at a constant of 6.3. Petterson, 1975, has also reported that the growth rate and maximum cell yield of a culture containing *S. Lactis, S. Cremoris, S. diacetilatics* and Leuconostoc

bacteria, similar to mala culture were better by about three times, when grown in whey and tryptone as compared to milk when the pH was maintained at three levels of 5.5, 6.0 and 6.5.

The activity of the mala culture when grown in milk was 8 and 16% higher than when grown in whey and tryptone respectively. Buffering the media had no effect on the culture when grown in milk. The activity of the culture, however was slightly increased when grown in buffered whey and tryptone media. These findings are in agreement with others that the activity of a culture is higher when cultivated in milk than in other media (25,30). Lloyd and Pont , 1973b, reported that the activity of a culture of *S. cremoris* and *S. lactis* in trypsin - digested skimmilk with continuous pH control at 6.3 by NaOH was similar to that of control where the pH was not controlled. But there was a decrease in activity of several cultures when NH_4OH was used to control pH unlike when NaOH was used (26, 27, 70).

The above arguments show that, buffering the media with $CaCO_3$ improved the growth of the mala culture. Such improvement was higher in whey and tryptone than in milk. However, this improvement fell short of what would be expected from maintaining pH at a constant value of 5.0-6.5

by using neutralizers. But the buffering of whey medium with $CaCO_3$ to maintan the pH above 5.3 at end of growth was found as effective as control of pH at a constant by NaOH, KOH, or $Ca(OH)_2$.

5.2. Effect of Growth Media and Buffering on Cell Yield and Activity of the Mala Culture

The pH for the three media; milk, whey and tryptone at harvest time was the same. But the acidity was 1.2 and 1.5 times higher in milk than in tryptone and whey respectively. However, on buffering the media, the pH at harvest time was increased by 16% in milk, 22% in whey and 17% in tryptone. A corresponding decrease in acidity of 16%, 22 and 22% respectively was observed (table 2). But the TVC at harvest increased by 20, 30 and 70% respectively (table 4). The effectiveness of buffering by $CaCO_3$ was found to be 16, 21 and 15% over milk, whey and tryptone respectively (table 3). These findings show that the $CaCO_3$ buffer improved on the growth of the mala culture in the increasing order of whey, tryptone and milk. It is well established that the control of pH at constant level of 5.0-6.5 results in high counts (TVC) of cultures (4,26,27,70,72). The improvement on the TVC on using a $CaCO_3$ buffer was thought to be due to slowing the change of pH during growth

and maintaining the pH above the critical level of 5.0 (105). Cultures are also known to benefit from release of carbon dioxide, which meets a partial or absolute need of the culture bacteria (14,25,58). The use of $CaCO_3$ can lead to a release of carbon dioxide from its reaction with lactic acid formed during growth.

It was found that the TVC at harvest was 40% higher in buffered tryptone and whey while 25% lower in milk than when unbuffered. On the other hand the cell yield increased by 9, 11 and 47% in milk, tryptone and whey respectively. These findings suggest that the recovery of the cells by centrifugation was more effective in whey than in the other two media. This, however, could be attributed to the difficulties and errors accompanying the centrifugation and TVC determination. Such errors included transferring, sampling and weighing of the recovered cells and at the same time avoid contamination. It is well known that the recovery of cells by centrifugation is much difficult in cultures grown in milk (4,25,72).

There was a good relationship between the pH at harvest and the cell yield of the culture after harvest in milk and whey. The cell yield was much higher when the pH at harvest was higher than 5.0. This observation was in agreement with what Peebles *et. al.*, 1969, has reported that there is

little difference in the maximum cell yield and time to reach this yield if the pH is maintained constant at pH above 5.0. Petterson, 1975, also reported no significant difference in the growth rate, maximum cell yield and the acid production ability by cultures grown in media whose pH was maintained at 5.5, 6.0 and 6.5.

The activity of the culture decreased slightly during centrifugation. This decrease in activity was however regained on first subculture of the concentrated mala culture. These findings show that there is only little difference in the activity of the culture. Hence, this culture can be used directly into the product vat. The activity of *S. cremoris* grown in tryptone was found to decrease when the pH was controlled at a constant as compared to when pH is not controlled (70). Gilliland, 1977, has explained that the ability to produce lactic acid by culture can be lowered through concentration, due to alteration in cellular metabolic activity, cell injury and strain imbalance especially if the growth medium is not milk. The lowered activity is usually regained in the first subculture when the injured bacteria are repaired (26,70).

5.3. Media and Buffer Effect on Storage of

Frozen Concentrated Mala Culture Storage.

It is not well established if the growth media affect the stability of the concentrated culture during frozen storage (25). But it has been reported that the survival of *S. cremoris* during frozen storage was better when grown in milk than in tryptone (26). This stability under frozen conditions was attributed to the content of oleic fatty acid in the bacterial cells picked from the growth medium. It is also possible that some constituents of the media recovered together with the cells such as protein molecules can act as cryoprotective agents. Results in this study have shown that growing the mala culture in milk, improved significantly its stability in viability and metabolic activity during storage at $-32^{o}C$. But the stability and metabolic activity of mala culture grown in buffered whey was same to that grown in milk. From these findings it was therefore deduced that there was no difference in the stability of mala culture during frozen storage, when it is grown in whey and milk. The above discrepancy, being due to the unexpected apparent instability of the culture when grown in the unbuffered whey after the sixth week of storage.

Mala culture grown in buffered milk and whey media, concentrated and frozen at $-32^{o}C$ were

more stable in terms of acid production but not in viability during storage. The species balance was also affected by storage as expected due to instability in viability. It has been shown that the growth of lactic cultures in media whose pH are maintained constant around 6.0 using neutralizers, tend to improve on their storage with respect to viability and activity as compared to culture grown without pH control (18,25,30,72). The use of NH_4OH as the neutralizer has been reported to give better storage stability in viability and activity compared to other neutralizers (18). Oberman *et. al.* 1986, have however, reported that the ability of multiple leuconostoc species culture to produce acid and diacetyl was not affected by storage at -30°C for three months.

5.4. Effect of Cryoprotective Agents on storage of frozen concentrated mala culture.

The use of lactose, yeast extract and glycerol as cryoprotective agents during frozen storage of cultures is well known. Concentrated mala culture stored at -32°C and containing the above cryoprotective agents in addition to glycerine, was found to have improved viability, species balance and the metabolic activity. During the freezing process in the first one day the

viability dropped by 5-15% when the above agents were used as compared to 20-25% when frozen without cryoprotective agents giving survival rates of 85-95% and 75-80% respectively. This survival rate, was however, lower than that reported by Bannikova and Lagoda, 1975, of 91-98% for a mixed culture suspended in a protective solution composed of glycerine, sucrose and sodium citrate. During the storage of the concentrated mala culture, over 50% of the original viability remained in the three months storage period when the cryoprotective agents were used but much less when no agent was used. Glycerol was found to be the best protective agent in maintaining the viability, species balance and metabolic activity of culture. This appears to be in agreement with Efsthanthiou *et. al.* 1975, as reported for multiple starter concentrates stored frozen. Using yeast extract as a cryoprotective agent, the lactic acid bacteria and aroma bacteria were affected in a similar way. Lactose on the other hand, was equally good in sustaining the lactic acid producing ability of mala culture, but very poor with respect to maintaining the aromabacteria. This was contrary to what has been reported regarding lactose as a good cryoprotective agent. It is said to offer better protection to the aromabacter than to acidulating bacteria (18,76). The use of the supernatant from the concentrated culture as a cryoprotective agent was found to have an adverse effect of the viability, species

balance and metabolic activity of the concentrated mala culture. This observation was in contrast to that by Lloyd and Pont, 1973, who reported survival rate of 88% of *S. lactis* when stored at -10°C protected by the culture supernatant.

5.5. Effect of Storage Temperature on Concentrated Mala Culture

Mala culture grown in unbuffered whey and frozen using glycerol as the cyroprotective agent was maintained at two temperatures of -20 and -32°C. They were tested for viability. % aromabacteria and ability to produce lactic acid, form viscosity and diacetyl. It was found that the mala culture stored at -32°C, sustained the viability, % aromabacter and diacetyl formation better than that stored at -20°C. But there was no difference in the lactic acid production by the cultures and viscosity in mala produced by these cultures when stored under the two different temperatures. These findings showed that frozen storage of the mala culture at -32°C was better than at -20°C. Bergere, 1969, has recommended that the storage temperatures of concentrated cultures should be less than -30°C. Lyold 1975, recommended storage temperatures of less than -37°C.

5.6. Effect of Freeze-drying on the

Concentrated Mala Culture.

Freeze drying appears to have greater reduction effect on the survival rate, % aromabacter and metabolic activity of the concentrated mala culture as compared to deep freezing. The survival rate of the concentrated mala culture decreased by about 30 to 40% as a result of freeze drying. This is within the reported survival values of 10 to 100%, depending on the culture, and protective agents used (6,21,37,45,64). The lower survival rates are reported when no protective medium is used (21,37). Intermediate rates have been observed when the protective media are yeast extract, lactose, glycerol and monosodium glutamate used singly (27, 37, 45). The higher survival rates are registered when a combination of the above agents are used (6,21,64). The survival rate of the concentrated mala culture was, however, lower than that reported for a culture freeze dried with the same protective medium, yeast extract (37). The decrease in the % aromabacteria of the concentrated mala culture was similar to that reported by Zickrick *et. al*, 1982, who established that the proportion of the aromabacter, *S. diacetilactis* decreases in proportion to the acidulating bacteria during freeze drying.

The lactic acid forming activity of the concentrated mala culture was reduced two-fold by freeze drying as compared to deep freezing. The diacetyl and viscosity formation of the culture were also reduced by about the same margin. In spite of this, it was found that the freeze dried concentrated mala culture could still be used directly to make a good product. The reduction in the activity of the mala culture was less when compared to that reported by Bouillanne *et. al.*, 1978. They showed that the activities of 62 freeze dried concentrated mixed cultures, were reduced by four times as compared to when deep frozen. They did not, however, indicate whether any protective agents were used during freeze-drying.

5.7 Applicability of the Concentrated Mala Culture in Making Mala.

The concentrated mala culture were used directly to make mala. These were compared to similar products made by the conventional starter culture. It was found that there was no difference in the acceptability of the two products. Products made by the concentrated mala culture after three months storage were equally acceptable to that of conventional culture. Products made by using the freeze dried concentrated mala culture directly, were equally preferred to the other products. These

findings were significant in that both the deep-frozen and freeze-dried concentrated mala cultures can be applied directly into the manufacture of good quality mala. This was the purpose of the study.

6; SUMMARY AND CONCLUSIONS.

The purpose of this work was to produce a bulk concentrated mala culture that can be used directly to make good quality mala. Such a culture should be stored for a reasonable period of time under conditions that can be achieved by small scale dairy industries in Kenya.

This purpose was to be achieved by optimising the growth of the culture. Conditions considered to this end were the growth media and buffering the same. Three media, namely skimmilk, whey and tryptone broth plus buffering of the same with calcium carbonate were evaluated. The objective was to obtain the best medium in terms of growth, cell yields, viability, activity and the species balance of the culture. The culture was then concentrated by centrifugation in a laborotary centrifuge. The concentrated mala culture was preserved by deep freezing at -32 and -20°C plus use of cryoprotective agents and by freeze-drying. During preservation the concentrated mala culture was tested for viability, species balance and metabolic activity. The performance of the concentrated mala culture was evaluated by making mala using the same and comparing this with mala made by the conventional starter using sensory techniques.

It was found that although each media affected the growth parameters of the culture differently, the overall surpport of the media to the culture growth was similar in the three media. There was only slight difference in the TVC and activity of the culture grown in the three media. However, centrifugation of the culture was difficult in milk and tryptone. The cell yield of the culture was lower in tryptone than in milk and whey. The growth media did not affect the stability of the concentrated culture during frozen storage. Buffering of the media was advantageous into the growth, concentration and preservation of the culture. During growth, buffering produced less change in pH as compared to lactic acid formation. This resulted in a higher pH and lower lactic acid developed at harvest time as compared to unbuffered media. This led to an increase in the growth rate, cell population, cell yield and activity of the mala culture. Such increases were higher in whey than in the other two media. Buffered whey was thus deduced to be the most appopriate medium. Cultures that were grown in buffered media were found to be more stable in sustaining the viability, species balance and the metabolic activity of the concentrated mala culture during frozen storage. The stability of the concentrated mala culture during frozen storage for three months was greatly

improved by use of cryoprotective agents. Glycerol was particularly very effective as a cryoprotective agent. The culture preserved frozen using glycerol stayed for four months with slight change in its quality. Glycerine was found to have the same effect as glycerol. This was of great practical importance because the former is locally available unlike the latter. Storage of the culture at -20°C caused greater instability of the culture than when stored at -32°C. Freeze drying of the concentrated mala culture resulted in lower activity and viability than deep freezing.

From the results of the viability and activity of the concentrated mala culture, it was deduced that this culture, can be used directly to make mala. It was calculated that an inoculation rate of only 0.2% could achieve the same initial cell concntration as the 3.0% rate used for the conventional culture. This rate was used to make the products during the storage of the concentrated mala culture. By comparing such products with those made by the conventional culture, it was found that the products of the concentrated mala culture were equally acceptable. The practical application of these findings are enormous. The mala culture was grown, concentrated and packaged in 500g in plastic bottles. These cultures were stored successfully by deep freezing at -32°C for at least four months.

The culture-in-bottles was found enough to inoculate directly into 100 litres of milk in the vat. One such bottle could be used for two weeks. This volume of milk would otherwise require three litres of the conventional starter culture, prepared through propagation in five days and with a proportionate increase in the cost of production. The concentrated mala culture was successfully preserved in the long term by freeze-drying. The freeze-dried concentrated mala culture was found effective as a Direct Vat Starter (DVS). But due to the small amounts involved, it could well be utilised as the source of the deep-fozen concentrated mala culture. From the foregoing it is concluded that the purpose of this work has been achieved.

The production of the bulk concentrated mala culture, using whey buffered with calcium carbonate and concentration of the culture by centrifugation is low cost and practically feasible. Such a culture can be safely preserved by deep freezing at -32°C with the help of glycerine as a cryoprotective agent. There is need to study other dairy cultures used in Kenya such as those for yoghurt and Kenyan-made cheeses on similar lines with the aim of improving and encouraging the use of these cultures by small scale dairy industries.

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8. APPENDICES

APPENDIX I

TRIANGLE TEST

NAME : _____

DATE : _____

PRODUCT : _____

Instructions: Here are three samples for evaluation. Two of these samples are duplicate. Separate the odd sample for difference only.

(1) Sample	(2) Check odd sample
_____	_____
_____	_____
_____	_____

(3) Indicate the degree of difference between the duplicate samples and the odd sample.

Slight : _____ Much : _____

Moderate: _____ Extreme: _____

(4) Acceptability:

Odd sample more acceptable : _____

Duplicate samples more acceptable : _____

(5) Comments: _____

_____

APPENDIX II

RANKING PREFERENCE TEST

NAME : _____

DATE : _____

PRODUCT : _____

You are presented with the following coded samples. Please grant them according to your preference.

Code

First : _____

Second : _____

Third : _____

Fourth : _____

Fifth : _____

Comments: _____

_____

APPENDIX III
STATISTICAL CHART 1

Number of tasters	Two-example test Number of concurring choices necessary to establish significance			Triangle test difference analysis number of correct answers necessary to establish significance		
	*	**	***	*	**	***
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	3	-	-
4	-	-	-	4	-	-
5	-	-	-	5	5	-
6	6	-	-	5	6	-
7	7	-	-	5	6	-
8	8	8	-	6	7	8
9	8	9	-	6	7	8
10	9	10	-	7	8	9
11	10	11	11	7	8	10
12	10	11	12	8	9	10
13	11	12	13	8	9	11
14	12	13	14	9	10	11
15	12	13	11	9	10	12
16	13	14	15	9	11	12
17	13	15	16	10	11	13
18	14	15	17	10	12	14
19	15	16	17	11	11	11
20	15	17	18	11	11	11

* 5% level of significance. ** 1% level. *** 0.1% level.

Extracted from Heatherbell, 1970

APPENDIX IV

STATISTICAL CHART 5

Scores for Ranked Data

The mean deviations of the 1st, 2nd, 3rd...largest members of samples of different sizes: zero and negative values omitted.

Ordinal Number	Size of Sample 2	3	4	5	6	7	8	9	10
1	.56	.85	1.03	1.16	1.27	1.35	1.42	1.49	1.54
2			.30	.50	.64	.76	.85	.93	1.00
3					.20	.35	.47	.57	.66
4							.15	.27	.38
5									.12
	11	12	13	14	15	16	17	18	19	20
1	1.59	1.63	1.67	1.70	1.74	1.76	1.79	1.82	1.84	1.87
2	1.06	1.12	1.16	1.21	1.25	1.28	1.32	1.35	1.38	1.41
3	.73	.79	.85	.90	.95	.99	1.03	1.07	1.10	1.13
4	.46	.54	.60	.66	.71	.76	.81	.85	.89	.92
5	.22	.31	.39	.46	.52	.57	.62	.67	.71	.75
6		.10	.19	.27	.34	.39	.45	.50	.55	.59
7				.09	.17	.23	.30	.35	.40	.45
8						.08	.15	.21	.26	.31
9								.07	.13	.19
10										.06

Extracted from Heatherbell, 1970