# " PREMATURE " DEATH PHASE OF

# LACTOBACILLUS ACIDOPHILUS

# NCFM GROWING AT PH 6.0

By

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# CHAPTER I

## INTRODUCTION

There is an increasing interest in the bioprocessing of foods, mainly those of dairy origin. Lactobacillus acidophilus is one of the microorganisms involved in the bioprocessing of dairy foods. L. acidophilus NCFM of human intestinal origin has been commercially used as a dietary adjunct in the production of a nonfermented dairy product. Production of such products depends on the use of concentrated cultures. It is important to select strains of L. acidophilus which have desirable characteristics such as bile resistance and ability to displace undesirable intestinal organisms (Gilliland and Speck, 1977). It is also desirable to produce a high bacterial population prior to harvesting the cells for preparing frozen concentrated cultures.

A major factor limiting the attainable population of many lactic acid bacteria during growth of cells for concentrated cultures is the accumulation of acid. By maintaining the pH of the growth medium at a favorable level, the maximum population can be increased (Bergere, 1968b). Some starter cultures can grow better when the broth is maintained at pH 6.0 (Pont and Holloway, 1968). However for L. acidophilus NCFM this does not seem to hold true. In some media, L. acidophilus NCFM when growing at pH 6.0 appears to enter a "premature" death phase (Mitchell and Gilliland, 1968). This apparently did not involve limitation of nutrients. Such a phenomenon easily

could limit the maximum attainable population. In experiments in which pepsinized whey base media were evaluated, the "premature" death phase was encountered when the medium contained 7.5% whey solids but not when the medium contained 2.5% or 5.0% whey solids (Mitchell and Gilliland, 1983). Results from other experiments (Pont and Holloway, 1968) suggested that the concentration of yeast extract in media can influence the occurrence of the phenomenon. In a set of experiments involving growth of Streptococcus lactis at pH 6.3 in cheese whey fortified with yeast extract and tryptone, maximum growth ($5.7 \times 10^{10}$ $ml^{-1}$) was attained at 12 hours followed by "premature" death phase (Pont and Holloway, 1968). The reason for the cessation of growth was not clear, however, sodium lactate appeared to be one of the inhibitory factors.

Because agitation is involved in the process of maintaining the pH at desirable levels, hydrogen peroxide and/or other oxygen derived toxic substances have also been implicated in inhibiting the growth of starter cultures (Keen, 1972b). A better understanding of the cause of this "premature" death might permit more efficient use of media in producing cells of L. acidophilus NCFM for concentrated culture.

The objective of this study was to determine which factor(s) was responsible for causing L. acidophilus NCFM to enter a "premature" death phase when being grown at pH 6.0.

# CHAPTER II

# LITERATURE REVIEW

## Importance of Lactobacillus acidophilus as a Starter Culture or Dietary Adjunct

Since 1908 when Metchnikoff postulated that the composition of the enteric microflora might exert important influences in human health, efforts have been made to transform this intestinal flora to have lactobacilli as the predominant species (Kopeloff, 1926; Frost et al., 1931; Rettger et al., 1935; Speck et al., 1976). L. acidophilus has been used singly or in combination with other species of lactic acid bacteria in the production of a variety of fermented and unfermented foods. The value of fermented dairy products for humans is based not only on the nutritive aspect but also on their beneficial effect to the human health (Lang and Lang, 1978; Rasik and Kurman, 1978; Gilliland, 1985a).

The growth of some lactobacilli in milk improves the nutritional value of its protein by making it more digestible (Davis, 1952a; Davis, 1958; Hargrove et al., 1978; Speck and Katz, 1980). Enzymes of lactic acid cultures can partially degrade proteins, lipids and lactose in milk, (Shahani and Chandan, 1979). In general, cultured products contain higher folic acid, niacin, biotin, pantothenic acid, B6 and B12 vitamins than milk (Shahani et al., 1962; Reddy et al., 1976; Ferreira and Bradley, 1982). Another nutritional improvement can result

from the fermentation of lactose into lactic acid which tends to stabilize the more labile vitamins (Davis, 1956).

It is well known that lactose can cause nutritionally oriented problems in some individuals. Milk products containing L. acidophilus or other lactobacilli, because of the ability to hydrolyze lactose have been shown to be beneficial to those people unable to digest this sugar (Gallagher et al., 1974; Speck and Geoffrion, 1980; Kim and Gilliland, 1983; Gilliland and Kim, 1984; Kolars et al., 1984). Cultured products also may provide supplementary quantities of lactase elaborated by the cultures during fermentation (Goodenough and Kleyn, 1976; Shahani and Chandan, 1979). Cultures of L. acidophilus growing in milk normally do not hydrolyze more lactose than they need for growth. However when bile salts were added to milk inoculated with the lactobacilli, more lactose was hydrolized than needed for growth (Kim and Gilliland, 1983). It has been suggested by Gilliland (1985c) that because of the presence of bile salts in the intestinal tract these organisms are able to hydrolize lactose more rapidly than is needed for their growth.

L. acidophilus has been considered responsible for exerting action in the colon to lower the level of bacterial enzymes responsible for the production of carcinogenic amines (Bakke, 1969; Bryan, 1971; Speck et al., 1976; Goldin and Gorbach, 1984). Experiments with mice done by the Texin Enterprise Economique Detat (Anon, 1967) has indicated that various lactobacilli, including a strain of L. acidophilus synthesized components which possessed antitumor activity. Results from other studies have been published indicating inhibition of tumor growth in rats by extracts of L. acidophilus and other lactobacilli (Reddy et al., 1972; Farmer et al., 1975; Bailey and Shahani, 1976).

Consumption of lactobacilli in certain conditions reduces serum cholesterol in humans (Mann and Spoerry, 1974; Mann, 1977; Hepner et al., 1979). A decrease in serum cholesterol has been observed in different experimental animals after being fed fermented milks containing, among others, strains of L. acidophilus (Mott et al., 1973; Tortuero et al., 1975; Grunewald, 1982; Gilliland et al., 1985). Certain strains of L. acidophilus have been shown to have the ability to deconjugate bile salts (Gilliland and Speck, 1977). Recently, Gilliland et al. (1985) reported the ability of specific strains of L. acidophilus to assimilate cholesterol. Their studies revealed that pigs on a high cholesterol diet supplemented with a selected strain of L. acidophilus had lower serum cholesterol levels than did pigs on the same diet without the selected strain of L. acidophilus. Although the mechanism by which uptake of cholesterol occurs in these microorganisms has not been defined, the authors indicated the need to determine whether or not ingestion of cells of selected strains of L. acidophilus could decrease serum cholesterol in humans with primary hypercholesterolemia.

The use of L. acidophilus in correcting some types of intestinal disorders has been highlighted by serveral authors (Gordon et al., 1957; Sandine et al., 1972; Christensen, 1976; Keogh, 1978; Shahani and Chandan, 1979). In general, lactobacilli have the ability to produce a large number of enzymes which split the various substrates they utilize for energy production (Premi et al., 1972; Keogh, 1978; Rasik and Kurman, 1978; Sandine, 1979). This characteristic added to the ability to produce microbial inhibitors (Sabine, 1963; Tramer, 1966; Hamdan and Mikolajcik, 1973; Babel, 1977) which are able to retard the growth of undesirable microorganisms is thought to be important in the

maintenance of a balanced intestinal flora favorable to the health of the host (Davis, 1952b; Speck, 1965; Sandine et al., 1972; Shahani and Chandan, 1979; Hull and Roberts, 1984). Myers, in 1931, stated that to some who would like to take advantage of acidophilus milk therapy, acidophilus milk was distasteful. To overcome this problem he claimed that a product which had the taste of ordinary sweet milk and yet was a means for carrying large number of L. acidophilus into the intestinal tract would meet a real need. His theory was based on the fact that cells of L. acidophilus suspended in milk would be capable of being implanted in the human intestinal tract. The renewal of this theory coupled with the development of procedures to produce stable frozen concentrated cultures of L. acidophilus resulted in the recent commercial development of nonfermented acidophilus milk (Speck, 1975; Manus, 1979). Strains of L. acidophilus have been used as dietary adjunct in a variety of other food products, namely acidophilus milk (Kopeloff, 1926; Kosikowsky, 1977), acidophilus yogurt (Davis, 1970; Jespersen, 1977; Rasik and Kurman, 1978; Hull et al., 1984), acidophilus yeast milk (Keogh, 1978; Subramanian and Shankar, 1985), Bioghurt (Rasik and Kurman, 1970; Hull et al., 1984) and buttermilk (Manus, 1979).

## Production of Cells of Lactobacillus acidophilus for Concentrated Cultures

Since the properties of the starter affect the characteristics of the ultimate cultured product, a good starter has to be maintained in good condition (Klaenhammer, 1982). The traditional method of handling starter cultures in the dairy processing plant in the past involved the holding of the selected stock cultures in freeze dried state. Several

subcultures from these were required for the culture to achieve its best biological activity and to produce enough quantity of the starter to provide the bulk starter for final inoculation of the product vat (Lamprech and Foster, 1963; Lloyd, 1971; Law, 1982; Thunnel et al., 1984). This procedure resulted in problems such as reduction of ability to produce acid through improper handling, contamination with foreign bacteria, attack by bacteriophage and excessive time involved in the building up process (Bergere and Hermier, 1968; Christensen, 1969). Liquid and frozen cultures are other alternatives which do not overcome the building up step to the desired volume of culture for the bulk starter or final inoculation (Lamprech and Foster, 1963).

The ideal method to preserve a culture would permit maintenance of maximum numbers and biological activity for a long period of time. This ideal method for preservation does not exist. Concentrated cultures frozen in liquid nitrogen offer the best method available presently (Efstathiou et al., 1975; Speck and Gilliland, 1975). This method stabilizes the culture's enzyme system in the same state as during active growth keeping loss of viability and activity to a minimum (Cowman and Speck, 1963; Gibson et al., 1974; Reddy et al., 1974).

As defined by Gilliland (1985b), concentrated cultures are those grown under closely controlled conditions, concentrated into very small volumes by centrifugation and frozen or dried for subsequent storage and transportation. This type of culture has been used successfully either to inoculate the bulk starter or the product vat directly for the manufacture of the different bioprocessed foods (Speck and Gilliland, 1975; Klaenhammer, 1982; Gilliland, 1985b). The production of concentrated suspensions of starter culture cells can be achieved either by

batch or continuous systems. Several studies have been done in the area of continuous fermentation (Pont and Holloway, 1968; Linklater and Griffin, 1971a; 1971b; Keen, 1972a; 1972c; Lloyd and Pont, 1973a; 1973b; Rhee and Pack, 1980). However, the production of concentrated starters by this method has not been very successful either because of economic problems or due to problems such as accumulation of variant strains with lower acid-producing ability (Lloyd, 1971; McKay et al., 1972) and "degeneration" of the culture (Lloyd and Pont, 1973b). It is now generally accepted that concentrated cultures are best prepared from batch cultures (Lawrence et al., 1976). The main parameters to be observed in a batch culture which will influence the achievement of the highest activity and biomass are the composition of medium and growth conditions.

## Media Ingredients

Media used for propagation of the lactobacilli must supply a variety of complex nutrients due to the fastidious character of the genus (de Man et al., 1960; Rogosa, 1974; Terzaghi and Sandine, 1975; Klaenhammer, 1982) and should provide the necessary ingredients to ensure maximum growth and to ensure that the resulting culture has the necessary complement of enzymes to permit optimum performance in the manufacture of cultured foods (Klaenhammer, 1982; Gilliland, 1985b). The medium also must facilitate the harvesting of the cells (Stanley, 1977; Gilliland, 1985b). Although milk and milk based media have been traditionally used as growth media for lactic acid bacteria (Speck, 1962; Olson and Qutub, 1970) it makes harvesting of bacterial difficult (Law, 1982). In the production of cells of L. acidophilus milk is not a good

choice because this microorganism does not grow well in milk (Jespersen, 1977; Klaenhammer, 1982) and cells can not be harvest easily from milk and milk-base media without prior casein solubilization (Stadhouders et al., 1969) or hydrolysis (Stanley, 1977).

As stated by Law (1982) media formulation for production of concentrated starter cultures are often commercial secrets. These media are generally based either on digests of whey supplemented with peptone and/or yeast extract or digests of skim milk. The advantage of digesting the proteins in milk and/or whey by means of a suitable proteolytic enzyme (trypsin, pepsin, papain) are twofold. Peptides are readily available sources of nitrogen to the starter hence promoting growth. Secondly the harvesting of bacteria by centrifugation is much easier. The addition of citrate to a starter culture grown in milk medium to "solubilize" the casein is beneficial in the latter respect (Stanley, 1977). Cowman and Speck (1963), grew Streptococcus lactis in a medium composed of casein, yeast extract and lactose. Pont and Holloway (1968) suggested the use of cheese whey fortified with yeast extract as growth medium for cheese starter culture concentrates. Bergere, (1968a) devised a medium for production of concentrated cells of lactic streptococci. In his study he utilized S. lactis C10 growing in a medium which consisted of tryptone, yeast extract, lactose, and phosphate buffer, pH 7.0. Although with this medium it was possible to obtain high yields of biomass, the cost of components made it uneconomical for large scale preparation of starter culture. In another publication (Bergere, 1968b) the author suggested the use of a medium composed of spray-dried whey, casein and corn-steep liquor which would be cheaper and still support high cell populations. Stadhouders et al. (1969)

grew a mixed starter culture in steamed skim milk which was then neutralized with 33% sodium hydroxide. Sodium citrate at the concentration of 1% was added to facilitate harvesting. Peebles et al. (1969) grew cremoris in a tryptone, yeast extract, lactose, and glucose medium. Keogh (1970) used a medium composed of trypsin-digested whey and yeast extract for growing cells of S. lactis C2 and the derived concentrates were used for direct vat inoculation to produce good quality cheddar cheese. Gilliland et al. (1970) grew strains of Leuconostoc citrovorum in a medium containing tryptone, yeast extract, glucose and sodium citrate at a concentration of 0.5% added to enhance flavor production by the starter during its use in manufacturing cultured products. A whey based medium was used by Duggan (1959) to produce concentrated cultures of L. acidophilus. Although this medium supported high biomass of this microorganism, better growth would have been possible if the whey protein had been hydrolized. Based in this principle, Mitchell and Gilliland (1983) utilized a pepsinized sweet whey medium for growing L. acidophilus for concentrated cultures. Commercial complex formulations such as the lactobacilli de Man, Rogosa, Sharpe (MRS) medium and All Purpose Tween (APT) broth are available for growth of lactic acid bacteria.

From this review it is clear that a variety of different media can be used for the production of concentrated suspensions of cells of lactic acid bacteria. Whey-based and peptone-based media have the advantage of facilitating the harvesting of the cells. However, in many cases skim milk has been the choice medium for producing concentrated cells of lactic streptococci mainly because it represents the same composition of the medium that will be used in the manufacture of the

ultimate cultured product (Stadhouders et al., 1969; Gilliland, 1985b). It has been advocated that for dairy starter cultures at least some milk solids should be included in the growth medium. This practice will help to ensure that the concentrated culture will have the necessary enzymes to act upon the milk into which it will be inoculated. It also is essential to maintain the balance among strains in multiple strain starter cultures of lactic streptococci (Stadhouders et al., 1969; Gilliland, 1971). Thus the factors that control the choice of the growth medium are the ability to produce high numbers of bacterial cells containing suitable activity, the cost, and the ease with which the cells can be harvested (Gilliland, 1985b).

Growth Conditions

When considering the growth conditions to be used in producing cell crops, the optimum growth temperature of the organism and the temperature at which the concentrated culture will be used must be taken into account (Gilliland, 1985b). When considering mixed cultures it is important to ensure that conditions which give maximum yields of mixed cultures do not upset the balance of individual species (Gilliland, 1971; Gilliland and Speck, 1974; Efstathiou et al., 1975).

As far as pH is concerned, the superiority of automatically maintaining the pH at a desired level for growth over the production of cultures without pH control is well documented in the literature (Bergere and Hermier, 1968; Pont and Holloway, 1968; Lloyd, 1971; Gilliland, 1971; Irie et al., 1974; Jonas, 1977). Control of pH is generally accomplished with alkaline neutralizers, typically sodium hydroxide, ammonium hydroxide and/or calcium hydroxide (Lamprech and

Foster, 1963; Bergere and Hermier, 1968; Gilliland and Speck, 1974; Kosikowski, 1977; Gilliland, 1985b). Populations of lactic streptococci growing at pH 6.0 with ammonium hydroxide as a neutralizer have been indicated to be twice those obtained using sodium hydroxide (Peebles et al., 1969; Lloyd and Pont, 1973b; Gilliland and Speck, 1974; Efstathiou et al., 1975). However the cells grown with the ammonium hydroxide neutralizer seem to be less active (Peebles et al., 1969; Lloyd and Pont, 1973b) but more resistant to freezing (Efstathiou et al., 1975) than those grown with sodium hydroxide. In the preparation of cell crops of L. acidophilus a combination of 20% $Na_2CO_3$ in 20% NaOH was successfully used to maintain pH 6.0 (Mitchell and Gilliland, 1983). As indicated by Gilliland (1985b) the slow release of carbon dioxide by $Na_2CO_3$ at 6.0 appears to benefit the growth of L. acidophilus. When studying factors involved in the production of maximum cell growth, the factors that limit the achievement of maximum cell numbers must be considered as well.

## Factors That Limit Maximum Cell Population in Cultures of Lactobacilli

Efficiency in the production of cell crops for concentrated starter cultures can be achieved through the monitoring of the factors involved in the maximum cell production per unit of volume of growth medium. The basic factor is to avoid nutrient limitation. However other factors that limit growth of bacteria have to be considered as well (Gilliland, 1985b).

Although milk is traditionally used for growing lactic acid

bacteria it must be supplemented sometimes to ensure production of maximum cell numbers. This is not only because it varies in composition but also because not all lactobacilli grew well in it (Speck, 1962; Mocquot and Hurel, 1970; Olson and Qutub, 1970). The benefits of high heat treatments to milk prior to inoculation are well recognized in the literature. Among these benefits is the partial hydrolysis of casein which provides readily available nitrogen to the medium (Foster, 1952) and sulphydryl groups (Mocquot and Hurel, 1970). Supplementation of milk with different extracts of plants, animal tissues and hydrolysates of proteins is a practice advocated by many (Anderson and Elliker, 1953; Kennedy, 1955; Garvie and Mabbit, 1956; Speck et al., 1958). Mineral solutions may also play an important role in improving starter growth. Olson and Qutub (1970) indicated that the addition of iron, magnesium and selenium improved growth of lactic streptococci in milk. Addition of vegetable extracts to a growth-sustaining medium other than milk for subsequent enhancement of growth of many lactic acid bacteria has been employed in laboratories for many years. These extracts appear to serve as donors of inorganic ions (Stamer et al., 1964). Additions of crystalline vitamin B12 and liver extract stimulated growth of L. lactis (Shorb, 1947 and 1948). Stamer et al (1964) suggested that a stimulatory factor in tomato juice was manganese. Many studies have shown the importance of manganese in metabolism and growth of lactic acid bacteria (Woolley, 1941; MacLeod and Snell, 1947; Stamer et al., 1946; Wright and Klaenhammer, 1983; Raccah, 1985).

Excess and/or imbalance of nutrients has also been found to preclude the culture from achieving maximum growth. The high concentration of lactose in milk contributes to making it an undesirable

culture medium. It is known that when the cells of some species reach the maximum stationary phase as a result of exhausting one or more nutrients in milk, the resting cells continue to produce acid from lactose to their own detriment (Sandine, 1976). Mitchell and Gilliland (1983), growing L. acidophilus NCFM in pepsinized sweet whey medium fortified with 2.5%, 5% and 7.5% yeast extract, with pH controlled at 6.0, observed that maximum growth was inversely proportional to the concentration of yeast extract used. Thus, high levels of an ingredient such as yeast extract which is a very rich and complex nutrient source might result in "imbalance" of nutrients, precluding the achievement of maximum growth of starter culture.

Gilliland and Speck (1967) indicated that when a mixed strain starter culture of lactic streptococci was grown in a broth medium containing tryptone, yeast extract, lactose and glucose at pH 6.0 (automatically controlled) the maximum population was obtained after 11-12 h. The spent broth from such cultures was markedly inhibitory toward the starter culture. Although the inhibitory action was greater at pH 6.0 or below, inhibition also occurred at higher pH levels. They indicated that the inhibitor was apparently formed during the growth of the culture. Although at that time the identity of the inhibitor was not defined, in a later publication (Gilliland and Speck, 1968) one inhibitor was characterized as D-leucine. The mechanism whereby D-aminoacids inhibit bacteria has not been elucidated. It has been suggested that the inhibition of lactobacilli by D-forms of leucine, valine, methionine and serine was not due to true competitive inhibition for the L-forms (Teeri, 1954). Grula (1960) indicated that cell division in species of Erwinia was inhibited by D-forms of serine, methionine, phenylalanine,

threonine, tryptophan and histidine. Gilliland and Speck (1968) concluded that although there was a possibility for D-leucine to be competing with the L-form which is required for the streptococci (Anderson and Elliker, 1953) a combination of factors rather than the D-leucine alone, was responsible for the observed cessation of growth of the lactic streptococci at pH 6.0.

One of the major factors that limit maximum growth of lactic acid bacteria is the lower pH resulting from the fermentation by these bacteria during growth (Harvey, 1965). Thus, control of the pH of growth medium at a favorable level should increase the yield of cells from a given volume of growth medium.

Unbuffered media for growing starters, including milk, are responsible for damage suffered by organisms exposed to high hydrogen ion concentration (Lawrence et al., 1976). Harvey (1965) found that incubation of S. lactis in a broth medium below pH 5.0 resulted in a marked reduction in the specific activity of cytoplasmic enzymes. Marquis et al. (1973) indicated that derangement of membrane structures and solute leakage occur when pH drop below 5.0 in cultures of S. faecalis. During glycolysis, streptococci maintain an internal pH more alkaline than that in the growth medium. This pH gradient is not maintained by non-glycolysing cells (Harold et al., 1970). When growing in unbuffered medium, lactic streptococci cease fermentation at pH 4.2 - 4.4 and presumably they are not able to maintain an energy-dependent pH gradient. Eventually the exposure of the cytoplasmic components to low pH causes death of the cell (Tanford, 1968). Thus, unless the medium is adequately buffered, hydrogen ion concentration becomes growth-limiting near pH 5.0. This inhibition can be prevented

either by providing a growth medium with an effective buffering system or by controlling pH automatically during growth. An internal pH-control medium has been described (Thunnel et al., 1984) which has the unique property of continually neutralizing acid produced by actively metabolizing bacteria, thus maintaining the pH of the growing culture sufficiently high to prevent acid damage to the bacteria. Pont and Holloway (1968) indicated that lactic streptococci growing without pH control reached maximum cell counts of 1 x $10^{10}$ $ml^{-1}$ at about 12 h. and counts declined rapidly from this level. Under controlled pH, this maximum count remained constant until the lactose was exhausted, approximately at 36 to 46 hours.

Gilliland et al. (1970) grew cells of L. citrovorum with pH controlled at 6.0, 6.5, and 7.0 using $NH_4OH$ or NaOH as neutralizer. They observed that although the neutralizer had no effect on maximum cell numbers the level that permitted the cell crops to produce highest concentrations of diacetyl was pH 6.0. The cell numbers at this pH were approximately twice those obtained without pH control. The control of the pH at desirable levels is now a common practice in the production of cells for concentrated cultures. This practice should increase the yield of cells from a given volume of growth medium.

Lactic acid bacteria produce predominantly lactic acid during growth on either lactose or glucose. The rate of lactic acid formation may be as much as 1.2 moles/mg dry wt. bacteria/min., or more than 10% of their weight per minute (Thomas and Batt, 1969). Despite this massive generation of acid, the cytoplasmatic pH may be more alkaline than that of the growth medium (Harold et al., 1970) and relatively little lactate accumulates in the cytoplasm (Zarlengo and Schultz, 1966).

In 1963, Lamprech and Foster were unable to obtain a yield above $1 \times 10^{10}$ cells/ml of S. lactis C2 even when pH was kept above 5.5. They suggested that the concentration of lactate or other growth products might have been the limiting factors. Bergere and Hermier (1968) demonstrated by using dialysis techniques that lactic acid accumulating in the medium was a limiting factor in achieving high cell yields of lactic streptococci growing under pH control. Bergere (1968a,b) suggested that the factor limiting maximum cell populations in cultures of S. lactis C1 and C3 was their greater sensitivity to lactate when compared to S. lactis C2. Pont and Holloway (1968) growing cells of S. lactis in media containing whey fortified with tryptone and yeast extract obtained yields of $5 \times 10^{10}$ cells/ml which could not be increased further. They observed that a concentration of 2% of sodium lactate present at the end of the exponential phase and of 3.5% at the level of maximum growth were inhibitory to the growth of this organism. Stanley (1977) grew lactic streptococci in a protease treated skim milk medium to a maximum total cells/ml of $1 \times 10^{10}$. The further addition of nutrients did not increase the cell yield and it was assumed that lactate ions were responsible for the inhibition. Producing frozen concentrated cheese starter by diffusion culture, Osborne (1977) indicated that the most important factor limiting growth of lactic streptococci was lactic acid or lactate salts. In his experiments S. cremoris strains having total cell counts of between 3 and $5 \times 10^{10}$ $ml^{-1}$ were obtained. Analysis of the spent broth from such fermentor cultures for L-lactate indicated levels inhibitory to the growth of the culture.

To maintain the pH at desired levels a suitable neutralizer is used. The earliest work using neutralization of the growth medium to

increase biomass appears to be that of Kosikowski (1966) who described experiments carried out in 1961 in which a starter culture was grown in skim milk. During the fermentation period neutralization of NaOH occurred three times. Lamprech and Foster (1963) were the first workers to combine the principles of neutralization and centrifugal separation to produce concentrated cultures. Bergere and Hermier (1968) confirmed the increase in yield of biomass when neutralization was used. In their experiment S. lactis C10 growing in a tryptone, yeast extract, and lactose medium exhibited increased cell yield from $1 \times 10^9$ $ml^{-1}$ to $3.6 \times 10^9$ $ml^{-1}$ when NaOH was used to maintain pH at 6.5. Stadhouders et al. (1969) grew lactic streptococci in steamed skim milk and the acid produced was neutralized to pH 6.6 by adding $Ca(OH)_2$ slurry to the medium. They indicated that the use of calcium hydroxide as a neutralizing agent is advantageous since the inhibitory effect of calcium lactate is less when compared to ammonium and sodium lactate. Peebles et al. (1969) growing cells of S. cremoris under pH controlled at 5.5, 6.0 and 7.0 indicated that although no difference existed in the maximum cell populations attained at these different pHs, the maximum populations were higher when $NH_4OH$ was used for neutralizing than when NaOH was used. However, for one strain (S. cremoris MC1) there was some reduction in the acid-producing ability with $NH_4OH$. The same methods were used to investigate the production of concentrated cultures of L. citrovorum (Gilliland et al., 1970) and little difference in the maximum population was observed when cultures were grown at pH 6.0, 6.5, or 7.0 either with NaOH or $NH_4OH$ as neutralizers. Blaine et al. (1970) produced cells of lactic streptococci in 11% skim milk with the pH maintained at 6.3 with various neutralizers. They found that both

$NH_4OH$ and $Na_2CO_3$ gave higher yields than those obtained with NaOH, KOH, or $Ca(OH)_2$. However the maximum growth rate of cultures grown with $Na_2CO_3$ was only 0.74 compared to 0.95 when $NH_4OH$ was used. Thus the type of neutralizer is a factor which can influence the maximum cell growth of starter culture bacteria.

To maintain the pH at desirable levels the addition of neutralizer requires agitation. Unless the medium is sparged with gas such as carbon dioxide or nitrogen, oxygen can be incorporated (Gilliland, 1985b). When this occurs, another source of inhibition becomes available, namely hydrogen peroxide or the oxygen derived toxic substances to which the sensitivity of lactic acid bacteria varies.

Oxygen is a potent regulator of microbial metabolism (Wimpenny, 1969). A classical example is the well known Pasteur effect in which facultative anaerobes transferred from anaerobic to aerobic environments demonstrate a sparing effect, increasing the yield of cells with a simultaneous decrease in the rate of $CO_2$ produced and glucose consumed. As indicated by Gotlieb (1981), the main factors that affect the growth of an organism in the presence of oxygen are the hydrogen ion concentration, $CO_2$, partial pressure of $O_2$, nutritional status of the medium, and temperature. Also important are the physiological and biochemical attributes, the genetic make up, and the nutritional status of the organism. During growth, bacteria develop reduced environments generating the lowest oxidation-reduction (redox) potentials during the logarithmic phase of growth (Gotlieb, 1981). This alteration of redox potential is the result of many complex interactions. As oxygen tensions decrease, the redox potential changes also, which can result in different metabolic products. The accumulation of these metabolic

products can exert different effects on the growing population. This constant change in redox is one of the main events which affect the behavior of batch culture growing in the presence of oxygen. Thus, the medium composition is constantly being altered not only by metabolic products but by cellular constituents as well. The size of the inoculum, the age and immediate previous growth history of the organism will affect its response to this constant change in environment (Tempest, 1969; Brown and Johnson, 1971; Gotlieb, 1981).

While molecular oxygen is not exceedingly reactive in biological systems, its reduced form yields toxic intermediates (Gerschman, 1981; Gotlieb, 1981). It is these intermediates that are involved in phenomena such as enzyme inactivation, denaturation of proteins, lipid peroxidation and structural changes in nucleic acids (Fridovich, 1977; Gotlieb, 1981). The first report in the literature relating the effect of oxygen poisoning to generation of free radicals was published by Gerschman et al. (1954). After this work, much has been published about free radicals including superoxide, hydroxyl radicals and derivatives of these radicals such as hydrogen peroxide ($H_2O_2$) and singlet oxygen (Forman and Fisher, 1981). It is now known that these partially reduced species of oxygen are generated normally during cellular metabolism (Collins and Aramaki, 1980; Forman and Fisher, 1981; Grufferty and Condon, 1983).

Microorganisms have evolved different mechanisms to resist the destructive capability of the oxygen in the air (Gerschman, 1981; Mattilla, 1985). Some simply avoid contact with molecular oxygen by living in anaerobic environments. These obligate anaerobes die when exposed to air. Most aerobic organisms, because of their metabolic

requirement of oxygen as the final electron acceptor for substrate oxidation and energy extraction, resort to other means of defense against oxygen toxicity (Gottschalk, 1979). Some are aerotolerant and do not use molecular oxygen as an oxidant. It is in this category that the lactic acid bacteria are placed. Sequential one electron reduction of oxygen produces superoxide anions, hydrogen peroxide, hydroxyl radical and water. A general mechanism of protection of biological systems against the potential hazards of superoxide, hydrogen peroxide and hydroxyl radical is to convert them either to oxygen by oxidation or to water by reduction (Forman and Fisher (1981) and Figure 1).

For some time the deleterious effect of oxygen was related exclusively to the accumulation of hydrogen peroxide which is formed when activated hydrogen molecules on reduced flavoproteins or reduced non-sulphur proteins come together with oxygen. The reaction is catalyzed by oxidases that are present in all organisms (Gottschalk, 1979). A general reaction is as follows:

$$FADH_2 + O_2 \xrightarrow{\text{oxidase}} FAD + H_2O_2$$

As a mechanism to destroy the toxic $H_2O_2$, aerobes contain catalase (an heme protein) which converts $H_2O_2$ to oxygen and water in a reaction such as:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Superoxide radical ($O_2$) is formed by univalent reduction of oxygen with reduced flavins, quinones or other electron carriers (Gottschalk, 1979; Forman and Fisher, 1981). Aerotolerant microorganisms contain the enzyme superoxide dismutase (SD) which converts the radical to hydrogen

peroxide and oxygen, by the following type of reaction:

$$O_2^- + O_2^- + 2H \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

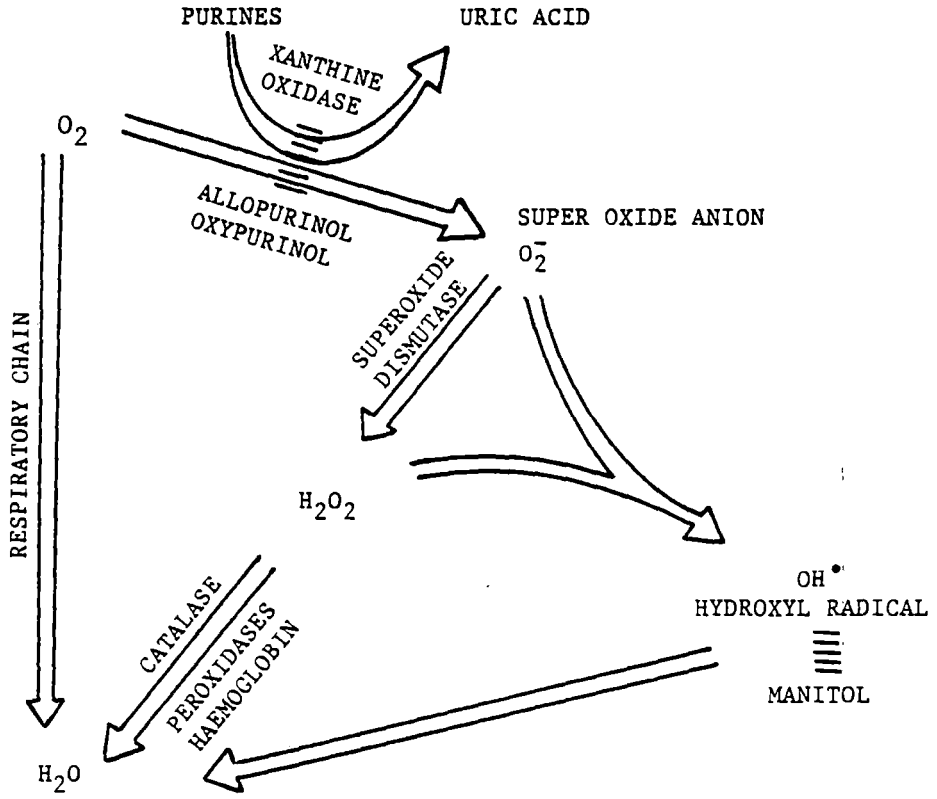


Figure 1. Pathways of one-electron reduction of oxygen into water and the detoxification of the oxygen intermediated by the cooperative action of superoxide dismutase, catalase and peroxidase (Mattilla, 1985).

This reaction catalyzed by superoxide dismutase removes one potentially dangerous species ($O_2^-$) while producing another ($H_2O_2$). Other enzymes must then remove hydrogen peroxide from the cell. Lactic

acid bacteria contain superoxide dismutase but lack true catalase. (Strittmatter, 1959, Rogosa, 1974; Gottschalk, 1979). Hence these bacteria can produce $H_2O_2$ by this mechanism in addition to the flavoprotein mediated route. Peroxide produced by either process can limit the growth of lactic acid bacteria.

The peroxidases, enzymes which remove hydrogen peroxide through peroxidative mechanisms catalyze reactions of the type:

$$H_2O_2 + H_2A \xrightarrow{\text{peroxidase}} 2H_2O + A$$

Where $H_2A$ represents the hydrogen donor (Forman and Fisher, 1981). Although both catalase and peroxidase catalyze reactions of removal of hydrogen peroxide, the peroxidases are different from catalase in their necessity for specific hydrogen donors (Brock, 1979; Forman and Fisher, 1981).

Many lactic acid bacteria, including L. acidophilus have been shown to produce hydrogen peroxide under aerobic conditions (Dahiya and Speck, 1968; Gilliland and Speck, 1969; Price and Lee, 1970; Collins and Aramaki, 1980). Lactic streptococci growing in liquid medium with pH automatically controlled at pH 6.0 showed some reduction in growth due to accumulation of hydrogen peroxide (Keen, 1972c). Gilliland and Speck (1969) indicated that addition of filter sterilized catalase or other compounds (such as reducing agents, pyruvate, ferrous sulfate) which have shown to prevent $H_2O_2$ accumulation might overcome the problem. The accumulation of $H_2O_2$ and/or other toxic radicals in the culture of lactic acid bacteria also can be affected by composition of media in which the organism is growing (Farrel, 1935; Anders et al., 1970).

Gilliland (1985b) indicated that sparging the medium with $CO_2$ may

have a twofold benefit. One of overcoming the effect of excess aeration and another of supplying $CO_2$ that has been shown by some authors (Pont and Holloway, 1968; Linklater and Griffin, 1971a), to be necessary for optimum growth of lactic streptococci.

Another point of importance in achieving the maximum cell growth for production of concentrated cultures is the genetic make up of the culture involved. In this respect it is of importance that the relative amounts of NADH oxidase which is responsible for $H_2O_2$ production and NaDH peroxidase which splits the peroxide (Strittmatter, 1959; Collins and Aramaki, 1980; Grufferti and Condon, 1983) be carried out by the species involved.

It is well known that cow's milk inhibits a number of bacterial species (Wright and Tramer, 1958). Milk contains a peroxidase-thiocyanate-hydrogen peroxide (LP-SCN-$H_2O_2$) system which when activated abruptly halts lactic acid production by sensitive strains of lactic streptococci (McKay, 1985). The understanding of this system began when in 1963, Reiter et al. confirmed that lactoperoxidase inhibited some strains of S. cremoris in milk but not in dialyzed milk or synthetic media. The dialyzed factor was identified as thiocyanate anion (SCN). The inhibition required peroxidase, SCN and $H_2O_2$ which was produced under aerobic conditions by the lactic streptococci. Cell multiplication, lactic acid production and $O_2$ uptake by resting cells were all found to be inhibited. Auclair and Vassal (1963), indicated that most starter cultures were resistant to that peroxidase system, although they could give rise to sensitive mutants when subcultured in autoclaved milk. Further publications (Oram and Reiter 1966a and 1966b; Reiter and Oram, 1967) indicated that lactoperoxidase in the presence of SCN

and $H_2O_2$ completely inhibited hexokinase and partially inhibited other glycolytic enzymes of resting streptococci. Moreover, strains of streptococci which resisted that inhibition possessed an enzyme which catalyzed the reduction of the intermediate oxidation products of SCN. They indicated that the intermediate inhibitor was very unstable in milk. It was also established that the sensitive strains could have lost the enzyme that reduces the inhibitory intermediate.

Recently, McKay (1985) speculated that because resistant cultures are unstable with respect to the lactoperoxidase system it might be possible that plasmid DNA be responsible for producing the enzyme that allows the resistant strains to overcome the LP system. Thus, the growth of lactic acid bacteria in milk could be inhibited when this system is activated and the growing culture has lost the plasmid and thus does not have the respective complement enzymes to overcome it.

Antibiosis is well established property shared among starter cultures. As stated by Babel (1977), antibiosis is an antagonistic association between microorganisms to the detriment of one of them. When inhibiting undesirable organisms, the association is beneficial. However when producing mixed starter cultures this detrimental effect among the organisms involved must be avoided. Some of the substances exhibiting this inhibitory effect have been identified as organic acids or hydrogen peroxide. Fermentation products of lactic acid bacteria have been shown to exhibit inhibition not only against the undesirable organisms but against the producer strains as well. Pinheiro et al. (1968) indicated that the inhibitory effect of metabolically produced acetic acid, glyoxylic acid, melonic acid and alpha-ketoglutaric acid may have additive effect. Thus, low concentration of several

compounds may together result in creating an inhibitory environment. The first observation of antibiosis among lactic acid bacteria was made by Rogers (1928). He indicated that some strains of S. lactis were more inhibitory to L. bulgaricus than others. The observation was restricted to the live cells. He partially characterized the principle responsible for the antibiosis as being heat stable and dialyzable.

In 1944, Oxford extracted an inhibitory agent from cultures of streptococci which resembled S. cremoris. He called the substance diplococcin which was a water soluble protein, insoluble in ethanol and heat stable in acid media. This substance was more active against S. cremoris than to S. lactis. It had some effect against Staphylococcus aureus but none against E. coli. Mattick and Hirsch (1944) concentrated an inhibitory substance produced by S. lactis which even in considerably dilute solutions completely inhibited several organisms. In a later publication (Mattick and Hirsch, 1947) the inhibitory substance was named "nisin", which was further characterized by Berridge (1947; 1949). It is heat stable at acid pH, protein in nature and primarily active against Gram positive organisms. These two antibiotic substances, nisin and diplococcin, have received the most attention among the inhibitory substances produced by starter cultures. Other inhibitory principles have been found among starter cultures, however they have not been well characterized.

Degrees of antibiosis have also been observed among different combinations of starter cultures. Hoyle and Nichols (1948), studying various strains isolated from starter cultures, observed that in mixed strains cultures, the inhibitory strains always dominated. Czulak and Hammond (1954) blended pure cultures of S. cremoris, S. lactis and

S. lactis subsp. diacetylactis and studied activity of different combinations of these strains. They found that sometimes the activity of a mixture was greater than that of the component strains and the mixture of the three types were most stable. Collins (1961) investigating domination among strains of lactic streptococci using several strains of S. cremoris, S. lactis, and S. lactis var. diacetylactis observed that three strains of the S. lactis var. diacetylactis and five of the S. cremoris produced one type of antibiotic and two strains of the S. lactis produced a different type of antibiotic. He also noted that the antibiotic resistance of cultures was rare with exception that each antibiotic producing culture was resistant to the antibiotic it produced. Several investigators have reported the production of antibiotic substances by lactobacilli or the presence of antibiotic principle in their culture filtrates (DeKlerk et al., 1961; Sabine, 1963; Tramer, 1966; Shahani et al., 1976 and 1977). Kodama (1952) reported the presence of "lactolin" in lactic acid bacteria. "Lactobrevin" was the name given to the antibiotic like substance produced by L. brevis (Kavanikov and Sudenko, 1967). Mikolajcik and Hamdan (1975a and 1975b) described "acidolin", an antimicrobial substance produced by strains of L. acidophilus. In a series of experiments, Shahani and co-workers reported the cultural conditions and isolation of an antibiotic principle from L. bulgaricus, "bulgarican", and "acidophilin" from L. acidophilus (Shahani et al., 1976; 1977; Reddy et al., 1984). Acidolin and acidophilin share in common the ability to be produced when L. acidophilus is growing in milk and the ability to be active against Gram negative organisms. Babel (1976) emphasized that because inhibitory substances are produced by various strains of S. cremoris, S. lactis and

leuconostocs, care must be taken when selecting strains for lactic cultures, particularly in composing multiple-strains cultures. This suggestion could be extrapolated further to include the lactobacilli once antagonism among these has also been observed and might be a factor that must be taken into account when selecting cultures for production of cells for concentrated cultures.

Bacterial antagonism within the group D streptococci has long been recognized as a widespread phenomenon and many of the agents responsible have been classified as bacteriocins, bacteriocin-like agents, haemolysins or defective phages (Tagg et al., 1976; Tagg and Russel, 1981). These bacteriocins are antibiotic-like substances of protein nature, which differ from the classical antibiotics in that their action is limited within members of the same or closely related families (DeKlerk, 1967; Tagg et al., 1976). Bacteriocin-like substances also have been found among the lactobacilli. Vincent et al. (1959) purified a bacteriocin-like substance produced by strains of L. acidophilus which originated from human intestine and from several common laboratory animals. They called this substance lactocidin. The spectrum of 12 spent media were tested on other members of the Lactobacillaceae, various enterobacteria and staphylococci. The action of the antibiotic-like material was restricted to the Lactobacillaceae, in much the same way as the action of colicins (produced by E. coli) is confined to the Enterobacteriaceae. Experiments done by DeKlerk (1967) and DeKlerk and Smith (1967) indicated the presence of bacteriocigenicity among strains of L. fermenti. One hundred and twenty-one strains of L. fermenti isolated from many different sources of human saliva were tested. The media used were MRS broth and agar (DeMan et al., 1960). Bacteriocin

production was investigated by stabbing single colonies of the 121 strains into fresh plates. After overnight incubation, the plates were sterilized with chloroform vapor and layered with soft agar seeded with an indicator strain and reincubated overnight. Twenty-five of the strains showed clear rings of inhibition of indicator organisms. The inhibitory activity could be concentrated by precipitation with saturated ammonium sulphate. The inhibitory agents diffused more than 1 cm from a central hole in agar which had been filled with a concentrated solution of it and covered with soft agar containing the indicator. At this time the investigator indicated that these properties labeled the inhibitory agents as bacteriocins. The classification of these bacteriocins could not be done because it was not possible to obtain resistant mutants of the indicator strains precluding the use of cross-resistance tests. Isolation and characterization of a bacteriocin from a homofermentative species of Lactobacillus was published by Upreti and Hinsdill (1973). The same authors in 1975 reported the production and mode of action of lactacin 27, a bacteriocin from L. helveticus strain LS18. More recently Barefoot and Klaenhammer (1983) detected lactacin B, a bacteriocin produced by L. acidophilus. Inhibitory activity of L. acidophilus has been identified towards other members of Lactobacillaceae and group D streptococci. The inhibitory interactions included among others that of L. acidophilus N2 toward L. leichmannii, L. bulgaricus, L. lactis and L. helveticus. Thus, there is a potential for bacteriocin production among the lactobacilli. When producing mixed cultures of these organisms, or cultures with mixed strains of the same organism, bacteriocin production could be a factor causing one strain to dominate.

# CHAPTER III

## METHODS AND PROCEDURES

### Source and Maintenance of Cultures

L. acidophilus NCFM (of human origin) was obtained from the stock collection from the Dairy Food Microbiology Lab, Department of Animal Science at the Oklahoma State University, Stillwater. The strain was subcultured weekly in sterile 10% non-fat milk solids (NFMS) using a 1% inoculum and 18 h. incubation at 37°C. The culture was stored at 0-5°C between transfers. Before use it was propagated in lactobacilli MRS (de Man, Rogosa, Sharpe) broth (DIFCO Laboratories, Detroit, Michigan) using a 1% level of inoculum and incubation at 37°C for 18 hours. Three subcultures were done to ensure that the culture was fully activated.

The other microorganisms used within this work were all obtained from the stock culture collection from the Dairy Food Microbiology Laboratory, Oklahoma State University. The procedure for propagating these cultures were the same as stated initially with the following modifications. i) S. lactis strains were incubated at 30°C. ii) E. coli B44, E. coli 026:k60, Enterobacter aerogenes, Salmonella typhimurium, Staphylococcus aureus B 925 and Salmonella pullorum, were subcultured weekly in Trypticase Soy Agar (TSA) from BBL (Baltimore Biological Laboratories, Cockeysville, Maryland) and before use they were subcultured in Trypticase Soy Broth (TSB, BBL) using a 1% inoculum and incubation at 37°C. iii) The Psychrotroph HSK was propagated weekly in

TSA and subcultured in TSB using a 1% inoculum and incubation at 30°C. iv) Streptococcus lactis AC2 was propagated in MRS Agar (DIFCO), subcultured in MRS broth (DIFCO) and incubated 18 h. at 30°C.

A miniturized system for identifying bacteria, the Minitek System (BBL) was used as described by Gilliland and Speck (1977b) to confirm the identity of the lactobacillus cultures. Gram stain, catalase test and growth at 15°C and at 45°C were also done to complete the identity of the cultures.

## Growth of Cultures of Lactobacilli

Portions (1000 ml) of MRS broth were sterilized in Erlemeyer flasks by heating 15 minutes at 121°C just prior to use. The sterile broth was tempered to 37°C and aseptically transferred to previously sterilized 1000 ml fermentors. Each fermentor was equiped with an Ingold combination pH electrode, magnetic stirrer bar and ports for neutralizer addition and sampling. The broth was adjusted to pH 6.0 $\pm$ 0.1 with sterile 20% lactic acid prior to inoculation. The fermentors were placed into a water bath where the temperature (37°C) was maintained by a thermistor. Each fermentor was attached to a pH controller Model 5997 (Horizon Ecology Company, Chicago, Illinois) connected to a Masterflex peristaltic pump (Cole-Parmer Company, Chicago, Illinois) which automatically discharged the neutralizer into the fermentor as needed to maintain the medium at pH 6.0 $\pm$ 0.2.

The neutralizer was prepared by dissolving 58.5g of $Na_2CO_3 \cdot H_2O$ (Baker Analyzed, J. T. Baker Chemical Company, Phillipsburg, New Jersey) in 417.5 ml distilled $H_2O$. The solution was autoclaved at 121°C for 15 minutes and cooled at room temperature. Just prior to use 82.5 ml

of concentrated $NH_4OH$ (Baker Analyzed) was added. This yielded a 10% $Na_2CO_3$ in 10% $NH_4OH$ neutralizer.

Each fermentor was inoculated (1%) with a freshly prepared MRS broth culture for the lactobacilli. The fermentation was allowed to proceed for 24 hours. During incubation the culture was agitated moderately with a magnetic stirrer. To evaluate the lack of pH control, the culture also was grown in fermentors with agitation but without pH control and statically.

Samples were taken aseptically at time zero, and every 3 hours thereafter (or as otherwise indicated) for 24 hours. The samples were placed into sterile screw cap tubes contained in an ice-water bath. They were analyzed for viable populations by plate count immediately after sampling or within 8 hours.

To prepare cell free spent broth (Filtrate) approximately 20 ml portions of each sample from the fermentor culture were centrifuged at 4,800 - 5,000 x g at 0 - 1°C for 10 minutes. The supernatant fluid was collected and sterilized by passing through a sterile 0.45$\mu$ acrodisc filter (Gelman, Ann Arbor, Michigan) into a sterile screw cap test tube. The cell free filtrates were maintained under refrigeration (0 - 5°C).

## Enumeration of Lactobacilli

Serial dilutions were prepared using 99 ml dilution blanks with a composition of 0.1% peptone (DIFCO Laboratories, Detroit, Michigan) and 0.001% Antifoam A Emulsion (Sigma Chemical Company, St. Louis, Missouri) in distilled water. The dilution blanks were sterilized by heating 15 minutes at 121°C. Whenever populations of the Lactobacilli were assessed we followed the procedures described in the Standard Methods

for the Examination of Dairy Products (Marth, 1978) for preparing required dilutions. Lactobacilli MRS agar was used to determine total populations. This medium was prepared by dissolving 1.5% agar in lactobacilli MRS broth (DIFCO) prior to sterilizing. The pour plate method was used and each dilution was made in duplicate. After solidification, the plates were inverted and incubated at 37°C for 48 hours. All colonies visible with the aid of a Quebec Colony Counter were counted.

## Assay for Inhibitory Action

### Tube Assay

Fifty ml portions of lactobacilli MRS broth adjusted to pH 6.0 with sterilized lactic acid solution (20%) were inoculated with 0.5 ml of freshly prepared MRS broth culture (18 h.) of L. acidophilus NCFM or other selected lactobacilli. After mixing, 5 ml portions were aseptically dispensed into small sterile screw cap tubes (13 x 100 mm) containing 0.1 ml sterile distilled water (control) or 0.1 ml of spent broth (or as directed). Incubation followed at 37°C in a water bath and growth was monitored turbidimetrically ($A_{620}$ nm) hourly, for 6 - 8 hours (or as directed) in a Spectronic 21 colorimeter (Bausch and Lomb, Rochester, New York).

### Disc Assay

Filter paper discs (Schleicher and Schvell, Incorporated, Keene, New Hampshire) 12.7 mm diameter were sterilized at 121°C for 30 minutes and used throughout this work. Whenever the spent broth was tested through this method it was always adjusted to pH 6.0. This precaution was undertaken to avoid pH effect on the inhibitory response of the

tested organism. The discs were saturated with the appropriate sample by aseptically dipping them with sterile forceps into the sample. The discs were placed upon freshly seeded and solidified agar and incubation followed with the plate upright at 37°C (or as indicated) for 24 hours. At the end of the incubation period the plates were observed for zones of inhibition (clear halo around the disc). The presence of this halo was marked as a positive result, likewise its absence was an indication of a negative result.

## Influence of Catalase on Growth in Fermentor Cultures

A concentrated filter sterilized solution of catalase (E. C. I. II. I. 6) from bovine liver (Sigma Chemical Company, St. Louis, Missouri) was used to yield a concentration of 0.001% in 1000 ml of MRS broth in the fermentor, as indicated by Wheater et al. (1952). Heat inactivated catalase (heated at 121°C for 15 minutes) was used at the same concentration as the active solution, for a control in a second fermentor. The fermentors containing MRS broth with active and inactive catalase were used in the same manner as described in the section "Growth of Lactobacilli in the Fermentor". Samples were taken and viable counts were determined in the usual manner.

## Assay of Spent Broth for Lactate

### Total Lactate Determination

The colorimetric method for the determination of total lactate described by Barker and Summerson (1941) was used with a slight modification to fit the conditions dictated by the test material. The step

involving preliminary protein precipitation was eliminated due to the nature of the samples. For preparing the stock lactic acid solution five samples of concentrated lactic acid solution (Baker Analyzed Reagent) were weighed and titrated with 0.1 N NaOH (Baker Analyzed Reagent) to determine the percentage of lactic acid. Based on the titration values, a stock solution was prepared containing 20,000 μg lactic acid/ml. Standards for the assay were prepared from this stock solution by making the appropriate dilutions. Total lactate in samples were calculated by the following formula:

(μg total lactate from standard curve)(dilution factor)

= μg total lactate/ml sample

L(+) Lactate Determination

L(+) lactate was determined using an enzymatic kit obtained from Sigma Chemical Company (St. Louis, Missouri). The assay was conducted according to the manufacturer's directions, with slight modification to fit the nature of the samples. The step involving protein precipitation was eliminated. L(+) lactate in the samples was calculated following the formula:

(μg of L(+) lactate from standard curve)(5*)(Dilution factor)

= μg L(+) lactate/ml sample

(*) Factor used to convert the 0.2 ml sample used to one milliliter.

Influence of pH on Inhibitory Activity

of the Cell Free Spent Broth

Cell free spent broth from samples taken from the fermentor

maintained at pH 6.0 after 12 hours of incubation (AF12) were used in a 1:2 dilution. The pH of 20 ml portions of MRS broth previously autoclaved was adjusted to the following levels: P-(5.0); Q-(5.5); R-(6.0); S-(6.5); T-(7.0). Each portion was filter sterilized by passage through a sterile 0.45$\mu$ acrodisc filter (Gelman).

The assay for detecting the effect of pH on activity of the inhibitor was as follows: i) Into appropriately labeled sterilized tubes, 5 ml MRS broth at each pH level, were added, followed by additions of the indicated amounts of the diluted spent broth or sterile distilled water as indicated in Table I. The pH of the spent broth was not adjusted to each level because 0.2 ml of a 1:2 dilution of it did not alter the pH of the 5 ml portions of MRS broth.

Each tube was inoculated with 0.1 ml of a 1:2 dilution of a freshly prepared MRS broth culture of L. acidophilus NCFM and incubated in 37$^{o}$C water bath. Growth was monitored by measuring the $A_{620}$ nm during the 10 hours of incubation.

## Effect of Heat on Inhibitory Action of the Spent Broth

Fifteen milliliter portions of the inhibitory spent broth were heated at 121$^{o}$C for 15 minutes, cooled at room temperature and assayed for inhibitory action by the tube assay. A 0.1 ml aliquot of the heated and unheated spent broth (positive control) in 5 ml MRS broth inoculated with L. acidophilus NCFM (1%) was used. The procedure for monitoring growth was followed as described previously.

## Ion Exchange Fractionation of Spent Broth

Twenty-five milliliter aliquots of the inhibitory spent broth were passed over a Dowex 50 W (Bio-RAD Laboratories, Richmond, California) cation ($H^+$ form) exchange column (Bed volume, 50 ml). The samples were washed through the column with distilled $H_2O$ until the effluents were negative to the Molish test (Rendina, 1971).

TABLE I

PREPARATION OF TUBES FOR DETECTING EFFECT OF PH ON INHIBITORY ACTION OF THE CELL FREE SPENT BROTH

| | | Additions | |
|---|---|---|---|
| Sample | pH | 0.2 ml Sterilized Distilled Water | 0.2 ml Cell Free Spent Broth (1:2 diluted) |
| P-1 | 5.0 | v* | - |
| P-2 | | - | v |
| Q-1 | 5.5 | v | - |
| Q-2 | | - | v |
| R-1 | 6.0 | v | - |
| R-2 | | - | v |
| S-1 | 6.5 | v | - |
| S-2 | | - | v |
| T-1 | 7.0 | v | - |
| T-2 | | - | v |

* = indicated additions into assay tubes

The column was then eluted with 6.5 bed volumes of 2N $NH_4OH$. The $NH_4OH$ was removed from the eluate by repeated evaporation (at 45°C under vacuum). Both effluent and eluate were concentrated by evaporation under reduced pressure to a volume approximately of 10 ml. The pH was adjusted to 6.0 and the final volume adjusted to 25 ml with distilled water. Both samples were passed through a sterile membrane filter (0.45 $\mu$ pore size, Gelman) and stored at 5°C in a sterile container.

Fifteen milliliters of the effluent fraction from the cation exchange column was applied to a Dowex-1 anion ($Cl^-$ form, Bio-Rad, Laboratories) exchange column having a 50 ml bed volume. The sample was washed through the resin with distilled water until the effluent was negative to the Molish test (Rendina, 1971). The effluent fraction was concentrated to 10 ml and adjusted to pH 6.0. The column was eluted with 10 bed volumes of 2N acetic acid. The acetic acid was removed by repeated evaporation at 45°C under vacuum. It was finally concentrated to 10 ml and the pH adjusted to 6.0. Both fractions (anion effluent and eluate) were adjusted to a final volume of 15 ml with distilled water and filter sterilized by passing through sterile membrane filters (0.45$\mu$ pore size, Gelman) into sterile containers and stored at 5°C.

To verify which of the different fractions or combinations of them retained inhibitory activity, a tube assay was employed. The overall procedure was the same as indicated before. To 5 ml of MRS inoculated with L. acidophilus NCFM was added sterilized distilled water and/or fractions or combination of fractions as indicated in Table II.

The turbidity of the tubes prepared as indicated in Table II was followed at $A_{620}$ nm hourly for 6 to 8 hours at 37°C and at 24 hours.

TABLE II

Preparation of the Tubes for Assay of Ion Exchanged Fractions of Cell-Free Spent Broth

| Tube # | $H_2O$ | Fractions | | | | |
|---|---|---|---|---|---|---|
| | | Cation Effluent | Cation Eluate | Anion Effluent | Anion Eluate | Spent Broth |
| 1 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 |
| 3 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |
| 4 | 0.0 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 |
| 5 | 0.1 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 |
| 6 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| 7 | 0.2 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| 8 | 0.2 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 |
| 9 | 0.2 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |

## Isolation of Strains From L. acidophilus NCFM Resistant to the Inhibitory Principle

In the tube assay for inhibitory action the tube which contains the 12 h. inhibitory spent broth usually shows a long lag phase at the first part of the incubation period of the assay. Toward the end of the assay period turbidity is detected and at 24 h. of incubation this turbidity is such that it is indistinguishable from that of control tubes. This indicates that the microorganisms appear to have overcome the inhibitory

material or a new strain has increased in numbers to overshadow the inhibition observed in the earlier part of the assay. Thus theoretically the growth observed at the end of the 24 h. incubation period may have been accomplished by a low level population of strains present in L. acidophilus NCFM that are resistant to the inhibitory substance in the spent broth. To isolate such cultures, two approaches were taken. i) Tube Assay Method. ii) Direct Plate Method.

In the Tube Assay Method the 24 h. assay tube containing the 12 h. spent broth was used to inoculate (1%) MRS broth containing 2% of the inhibitory spent broth. After 24 h. incubation at 37°C another tube of MRS broth containing 2% spent broth was inoculated with the material from a previous culture, incubation followed for 24 h. at 37°C. This procedure is repeated through four subcultures (Treatment A). The same procedure (1% of 24 h. assay tube containing the 12 h. spent broth) also was done using MRS broth only for the four subcultures (Treatment B). Additionally the culture was subcultured in MRS containing no inhibitor and in MRS containing the inhibitor, intermittently, for four subcultures (Treatment C). After the 24 h. incubation of the fourth subculture in each case, the resulting cultures were used to inoculate MRS broth with and without the spent broth to test for their susceptibility to the inhibitory material. As control, a fresh culture (18 h.) of the original L. acidophilus NCFM was also used to inoculate tubes containing MRS broth with and without the spent broth. After 6 h. of incubation the $A_{620}$ nm of tubes from the three treatments (A,B,C) and control were plotted (Increase $A_{620}$ nm against time). The control usually exhibited little or no growth in the presence of the spent broth during the 6 h. incubation indicating sensitivity of the culture to the inhibitory

principle in the spent broth. The tubes containing inoculum from treatments A, B, and C on the other hand exhibited the same $A_{620}$ nm in MRS broth with or without the spent broth indicating that the treatments resulted in selection of a resistant strain(s) or development of resistance to the inhibitor. Cultures from treatments A, B, and C were streaked onto MRS agar for isolated colonies. Isolated colonies were re-streaked in a second set of MRS agar plates to ensure pure cultures. The isolated cultures were propagated and maintained on MRS agar slants and in 10% NFMS using incubation at 37°C.

In the Direct Plate Method the stock culture of L. acidophilus NCFM was plated on MRS agar without and with several concentrations of inhibitory spent broth using the pour plate method with overlay. Set A was plated in MRS agar only (dilutions $10^{-5}$ to $10^{-7}$). Sets B, C, and D contained MRS broth with 2%, 5% and 10% spent broth, respectively and dilutions were from 0 to $10^{-7}$. From set A, isolated colonies from plates with highest dilutions were selected and subsequently streaked in MRS agar. This procedure was repeated twice and isolated cultures were propagated and maintained on MRS agar slants and in 10% sterilized NFMS. From sets B, C, and D colonies were isolated from plates of the highest dilutions exhibiting well isolated colonies. The isolated cultures were propagated and maintained as above.

A disc assay was used to test resistance or sensitivity of the isolated cultures to the inhibitory spent broth from fermentor maintained at pH 6.0.

## Effect of Catalase, Trypsin and Pepsin on Inhibitory Activity of the Spent Broth Treated with Enzymes

Thirty milliliter portions of spent broth collected after 12 h. of growth of L. acidophilus NCFM in MRS broth at pH 6.0 were adjusted to the following pH values: A) pH 6.0; B) pH 8.0; C) pH 3.0.

To adjust the pH, HCl and NaOH of sufficient concentration (8%) were used so as not to cause much dilution of the samples. The amounts used were measured and compensated with distilled water in the other two tubes so the final volumes of all three flasks were equal. Each of these samples were then "filter sterilized".

The enzymes used in the preparation of the solutions were: (A) catalase (Hydrogen-peroxide; Hydrogenperoxide oxidoreductase; E.C. NO. I. II. I. 6) from bovine liver, (B) Trypsin (type II, crude), and (C) Pepsin (E.C. NO. 3.4.23.I), 1:10,000. All enzymes were obtained from Sigma Chemical Company and the solutions of each were prepared just prior to use. Twenty-five milligrams of each enzyme was added to a test tube containing 5 ml of cold distilled water, mixed well on a vortex tube mixer, "filter sterilized" and kept in ice water for immediate use. A 10 ml aliquot of spent broth at each pH level, (A) pH 6.0; (B) pH 8.0; (C) pH 3.0, was placed into each of two sterile tubes for each sample. Sterile distilled water (0.5 ml) was added to the appropriately labeled tubes such that a water control was prepared for each pH level (A,B,C). Catalase (0.5 ml) was added to one tube containing spent broth at pH 6.0 (tube A). Trypsin (0.5 ml) and Pepsin (0.5 ml) were added to tubes B (pH 8.0) and tube C (pH 3.0), respectively. All tubes were incubated in a water bath at 37$^{o}$C for 30 minutes. The contents of each tube was

adjusted to pH 6.0 using the appropriated acid (HCl) or alkali (NaOH). The volume of alkali and/or acid added were measured and compensated with distilled water in the appropriated control tubes such that the control and enzyme tubes for each of the three treatments would have equal volumes. Each sample was filter sterilized again (Gelman acro-disc, 0.45$\mu$ membrane pore) added into sterile tubes and used for disc assay as indicated previously. A tube assay for inhibitory action of the enzyme treated spent broths also was run along with the disc assay. Samples for the tube assay were taken immediately after the enzyme treatment, prior to the readjustment of pH 6.0. A 0.1 ml aliquot of each enzyme treated and untreated spent broth (A, B, and C) were assayed following the same procedure described under the section "Assay for Inhibitory Action", tube assay method.

## Statistical Analyses

Analysis of Variances and the Least Significant Difference (LSD) methods utilized to evaluate the data are outlined in Principle and Procedures of Statistics (Steel and Torrie, 1980).

# CHAPTER IV

# RESULTS

## Confirmation of Identity of Culture

The results of tests to confirm the identity of Lactobacillus acidophilus NCFM and the strains derived from it, used throughout this work are presented in TABLE III. The characteristics of the cultures matched those of L. acidophilus as described in Bergey's Manual of Determinative Bacteriology, 8th Edition (Rogosa, 1974).

## Growth of Lactobacillus acidophilus NCFM in MRS Broth With and Without Control at pH 6.0

L. acidophilus NCFM was grown in 1000 ml volumes of MRS broth in fermentors maintained at pH 6.0 (A), without pH control but with agitation (B) and statically (C). Figure 2 compares the plate counts (averages from 5 trials) obtained from samples taken at time zero and thereafter at every 3 hours for a period of 24 h. for the culture growing under the three fermentation conditions described. (The data from all trials are in Table XII, in Appendix A).

The "premature" death phase occurred immediately after 12 h. in the fermentor being maintained at pH 6.0. Analysis of the data from 12 and 24 h. of incubation (TABLES XIII and XIV in Appendix A) indicated that at 12 h. there were no significant differences ($p > 0.05$) among populations in the fermentors at pH 6.0 (A), with agitation only (B) or under

TABLE III

BIOLOGICAL CHARACTERISTICS OF STRAINS OF L. ACIDOPHILUS

| Test | Lactobacillus acidophilus Strain | | | | |
|---|---|---|---|---|---|
| | NCFM | NCFM-F | NCFM-L | NCFM-S | BERGEY'S[A] |
| Gram Stain | + | + | + | + | + |
| Cellular morphology | rods | rods | rods | rods | rods |
| Catalase | - | - | - | - | - |
| Growth at 15°C | - | - | - | - | - |
| Growth at 45°C | + | + | + | + | + |
| $NO_3$ from Arginine | - | - | - | - | - |
| Acid from: | | | | | |
| Amygdalin | + | + | + | + | + |
| Arabinose | - | - | - | - | - |
| Cellobiose | + | + | + | + | + |
| Esculin | + | + | + | + | + |
| Galactose | + | + | + | + | + |
| Glucose | + | + | + | + | + |
| Lactose | + | + | + | + | + |
| Maltose | + | + | + | + | + |
| Mannitol | - | - | - | - | - |
| Mannose | + | + | + | + | + |
| Melezitose | - | - | - | - | - |
| Melibiose | + | + | + | + | ± |
| Raffinose | + | + | + | + | ± |
| Rhamnose | - | - | - | - | - |
| Salicin | + | + | + | + | + |
| Sorbitol | - | - | - | - | - |
| Sucrose | + | + | + | + | + |
| Trehalose | + | + | + | + | + |
| Xylose | - | - | - | - | - |

[A] Characteristics of L. acidophilus as indicated in Bergey's Manual of Determinative Bacteriology, 8th Edition (Rogosa, 1974).

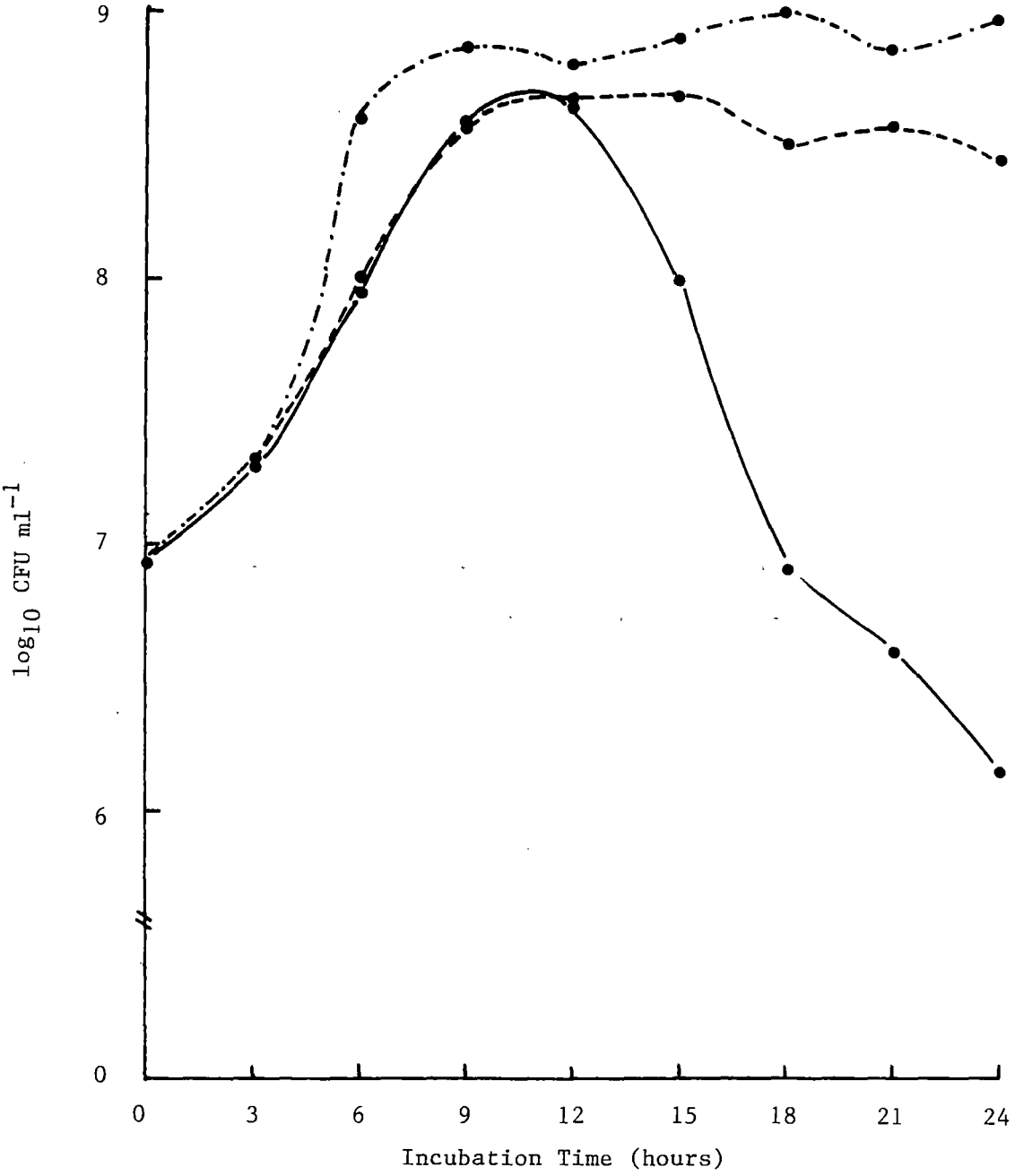


Figure 2. Growth of L. acidophilus NCFM in MRS broth in fermentors •—— with pH 6.0; •--- with agitation only and •-.-. statically.

static conditions (C). However, at the end of the 24 h. fermentation period significant differences ($p < 0.05$) existed among the three treatments. The population in the fermentor maintained at pH 6.0 was significantly lower than those in the other two fermentors ($p < 0.005$). However no difference existed between the population in the fermentor with agitation only and the static one ($p > 0.05$).

## Influence of Catalase on Growth of *L. acidophilus* NCFM in MRS Broth Maintained at pH 6.0

Figure 3 shows the growth of *L. acidophilus* NCFM in fermentors maintained at pH 6.0, in MRS broth with active and inactive catalase. Analysis of the data at 12 and at 24 h. of incubation (TABLES XV, XVI, and XVII in Appendix B) did not indicate significant differences ($p > 0.05$) in growth of *L. acidophilus* NCFM when active and inactive catalase were added to the fermentor at a concentration of 0.001%.

## Assay for Inhibitory Action of Spent Broth Taken During Growth of *L. acidophilus* NCFM in Fermentors Maintained Under Different Conditions

The effect of spent broth taken during growth of *L. acidophilus* NCFM in MRS broth under different conditions on growth of a fresh culture of *L. acidophilus* NCFM was tested using a tube assay. The results are summarized in Figure 4 (data are in TABLE XVIII of Appendix C). Results are presented as the increase in $A_{620}$ nm following 6 h. of incubation in the presence and absence of the spent broth samples.

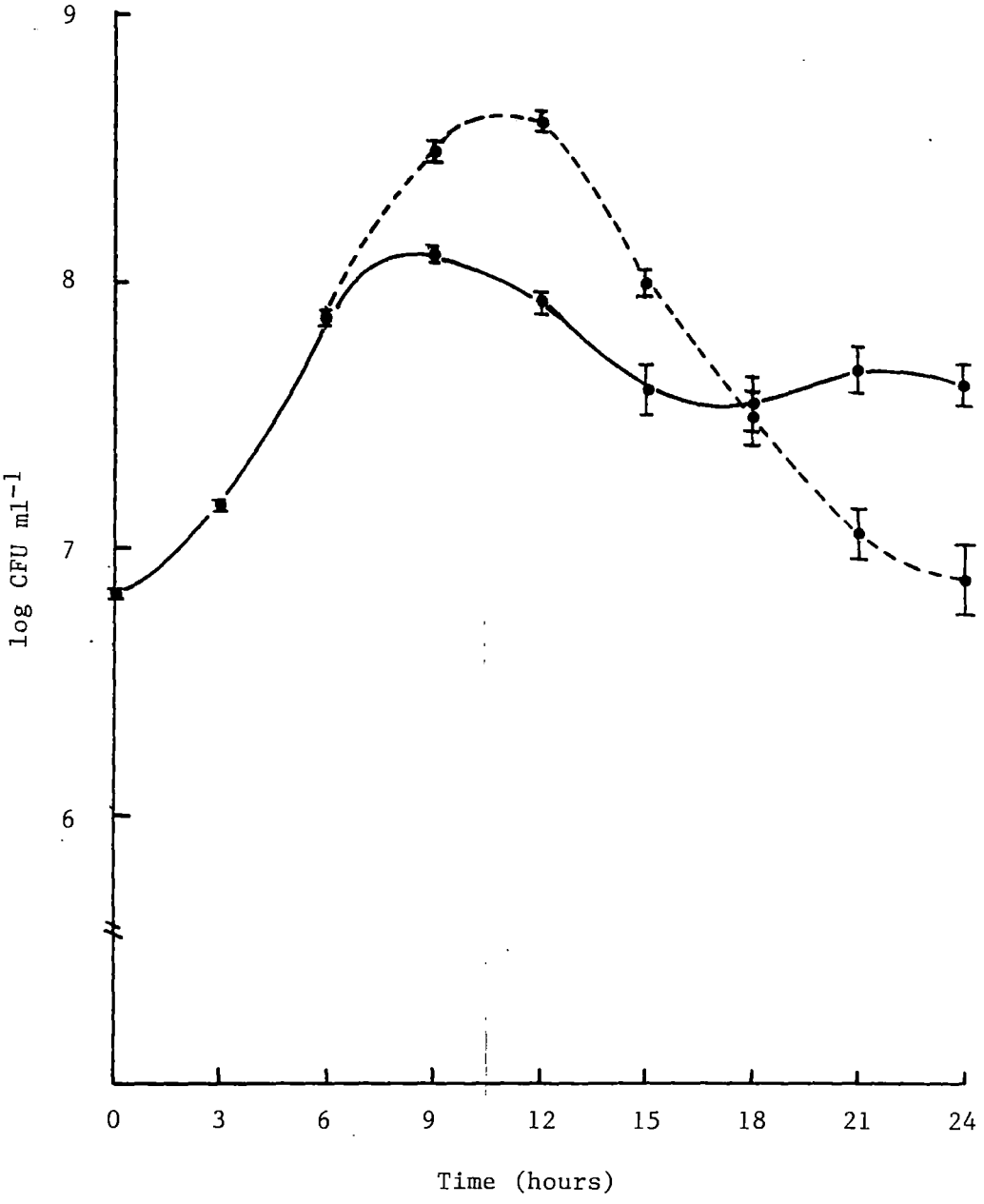


Figure 3. Growth of L. acidophilus NCFM in MRS broth at ●— pH 6.0 with catalase added and ●--- with inactive catalase added. Average of five trials (I represents standard deviations at each level).

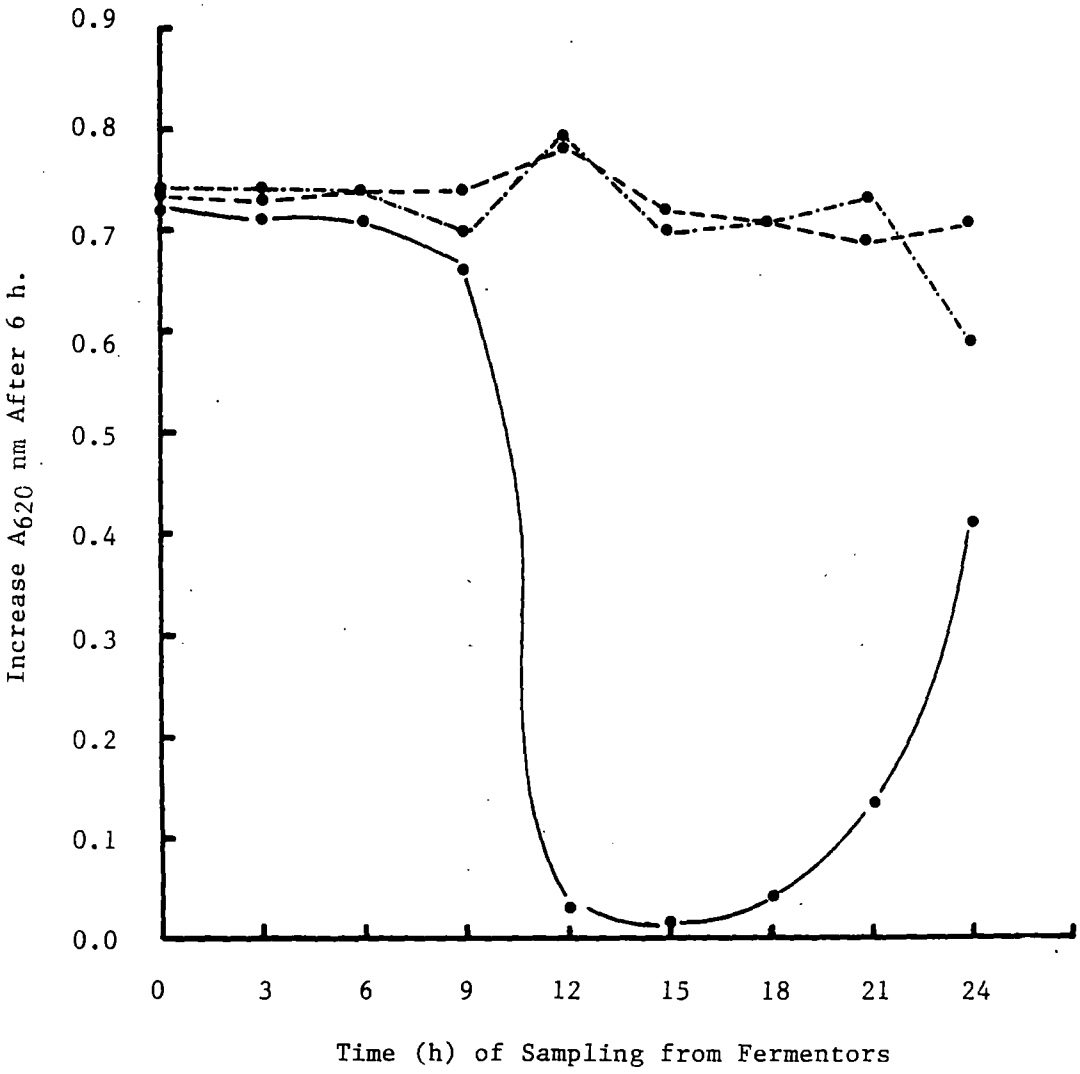


Figure 4. Tube assay for inhibitory action of spent broth from L. acidophilus NCFM growing in fermentors maintained under different conditions •—— pH 6.0; •---- agitated; •-·-· static. For assays, .1 ml of spent broth was added to 5 ml fresh MRS broth. Each value is the average of at least three trials.

These results show clearly the practical nonexistance of inhibitory action of spent broths from fermentors without pH control (agitated only and static). They also indicate inhibition from spent broth collected after 6 hours from fermentor maintained at pH 6.0. Maximum inhibition was caused by the spent broth taken at 15 hours. The inhibitory action declined continuously beyond the 15 hour sampling period.

When the numbers of L. acidophilus NCFM $ml^{-1}$ present in the fermentor at pH 6.0 at each time of sampling were superimposed over the inhibitory action of the spent broth samples it appeared that the maximum inhibition coincided with the maximum population (Figure 5). Although the number of viable cells continued decreasing in the fermentor beyond 12 h., the potency of the inhibitory substance in the spent broth decreased as fermentation proceeded.

The influence of spent broth during extended incubation in the tube from samples taken at 12 h. from fermentors maintained under the different conditions on growth of L. acidophilus NCFM is shown in Figure 6. (Data of each trial are in TABLE XVIII, Appendix C.) Summary of the 6 h. incubation of the 12 h. spent broths in tube assays are in TABLE IV. Analysis of variances and the least significant difference analysis (TABLE XIX, Appendix C) indicated that the 12 h. spent broth from the fermentor maintained at pH 6.0 was highly significantly inhibitory ($p < 0.01$) compared to the control. However no difference was observed in growth in the presence of spent broths from the agitated and static fermentors, and control tubes ($p > 0.05$). No difference in the amounts of growth was evident at the end of the 24 h. incubation in the tube assay. Thus this series of experiments indicated that the 12 h.

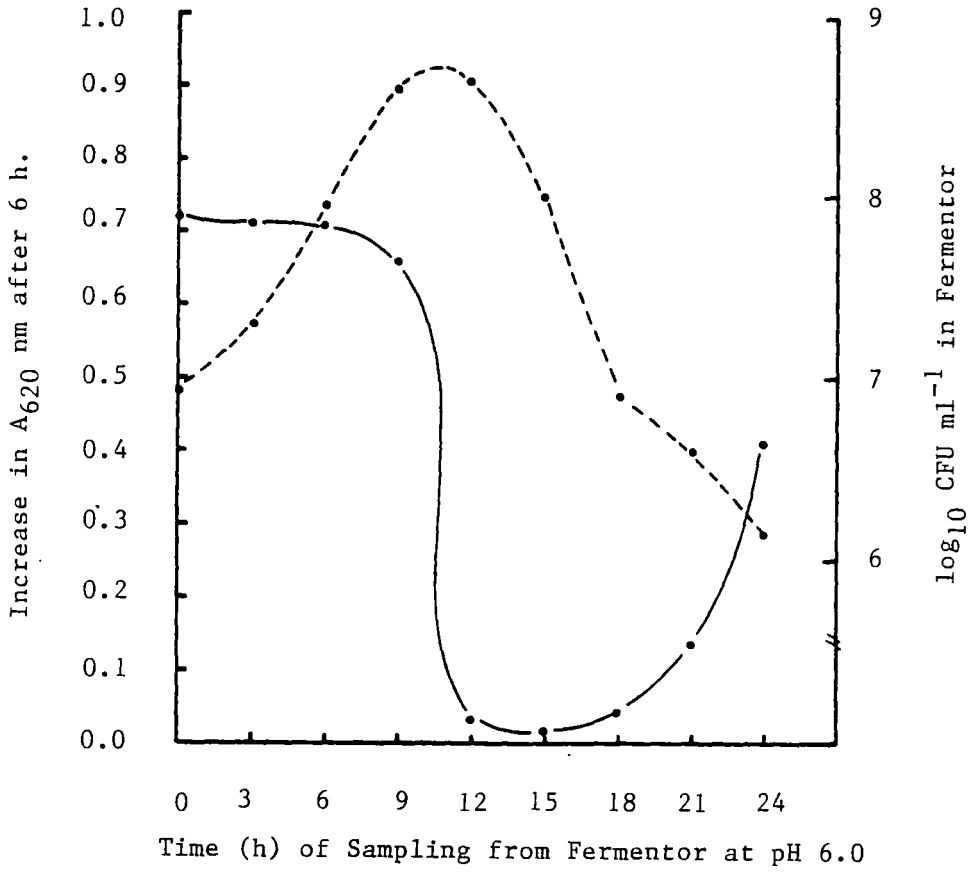


Figure 5. Comparison of growth of L. acidophilus NCFM at pH 6.0, and intensity of inhibitory action of spent broth. (•—— tube assay for inhibition expressed as increase $A_{620}$ nm;•--- growth in fermentor.

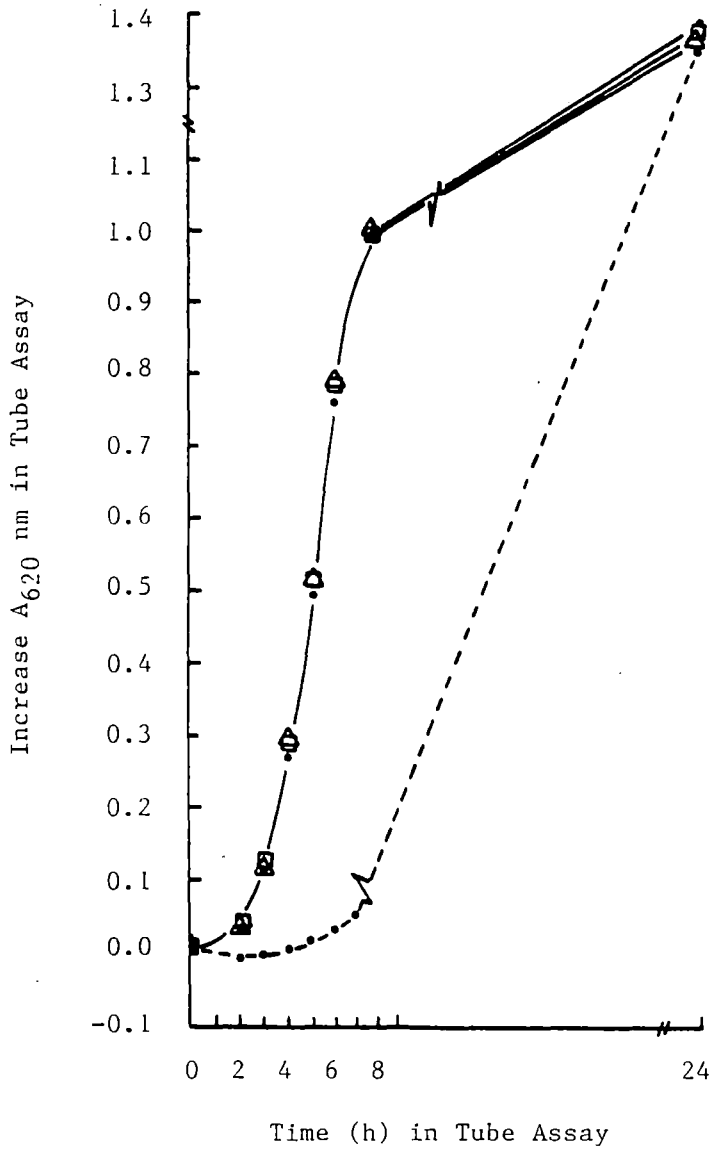


Figure 6. Effect of 12 h. spent broth from L. acidophilus NCFM growing in MRS under different conditions on a fresh culture of L. acidophilus NCFM in tube assay. •—— control; • -- pH 6.0; △— agitated; □—— static. Each value is the average of at least three trials.

spent broth from the fermentor maintained at pH 6.0 was very inhibitory to L. acidophilus NCFM while spent broth from the culture grown without pH control was not. However, as indicated by the $A_{620}$ nm readings following 24 h. incubation in the tube assay, the culture appeared to overcome the inhibition.

TABLE IV

ASSAY FOR INHIBITORY ACTION OF SPENT BROTH FROM 12 H. GROWTH OF L. ACIDOPHILUS NCFM GROWING IN MRS IN FERMENTORS KEPT UNDER DIFFERENT CONDITIONS

| Sample | Increase $A_{620}$ nm[A] |
|---|---|
| Control | 0.76[C] |
| pH 6.0[B] | 0.03[D] |
| Agitation[B] | 0.78[C] |
| Static[B] | 0.79[C] |

[A] Each value is the mean increase in $A_{620}$ nm during 6 h. tube assay from at least three trials.

[B] Spent broth (0.1 ml added to 5 ml MRS in tube assay) from indicated fermentor.

[C,D] The means with different superscript are significantly different ($p < 0.05$).

## Total and L(+) Lactate Produced by L. acidophilus NCFM During Growth in MRS Broth With and Without pH Control

TABLE V summarizes the concentrations of total and L(+) lactate present in fermentor cultures of L. acidophilus NCFM maintained under different conditions at 12 h. (Data of the three trials are in TABLE XX, Appendix D.) Analysis of variances and calculation of the Least Significant Difference (LSD) from the data (TABLES XXI and XXII, from Appendix D) indicated that the concentrations of both the total and L(+) lactates were significantly higher in the fermentor maintained at pH 6.0 than in either the agitated or static fermentors.

TABLE V

TOTAL AND L(+) LACTATE CONCENTRATION[A] AT 12 H. GROWTH OF L. ACIDOPHILUS NCFM IN MRS IN FERMENTORS UNDER DIFFERENT CONDITIONS

| Fermentor Conditions | Total Lactate $\mu g\ ml^{-1}$ | L(+) Lactate $\mu g\ ml^{-1}$ |
|---|---|---|
| pH 6.0 | 17,458[B] | 15,437[B] |
| Agitated | 9,958[C] | 7,104[C] |
| Static | 11,292[C] | 8,792[D] |

[A] Results are average of three trials.

[B,C,D] Means with different superscript are significantly different ($p < 0.05$).

The amounts of total and L(+) lactate in the fermentor maintained at pH 6.0 remained about the same during the remainder of the 24 h. incubation period (TABLE VI). The amounts of total lactate in the culture with agitation only, appeared to remain unchanged during the extended incubation. However the amount of L(+) lactate appeared to increase some during this period. In the static fermentor the levels of total and L(+) Lactates both continued to increase during the remainder of the 24 h. incubation period.

TABLE VI

CONCENTRATION OF TOTAL AND L(+) LACTATE IN SPENT BROTH DURING GROWTH OF L. ACIDOPHILUS NCFM IN MRS IN FERMENTORS WITH DIFFERENT CONDITIONS

| Time of Sampling | Total Lactate[A] $\mu g\ ml^{-1}$ | | | L(+) Lactate[B] $\mu g\ ml^{-1}$ | | |
|---|---|---|---|---|---|---|
| | pH 6.0 | Agitated | Static | pH 6.0 | Agitated | Static |
| 0 | 2,400 | 2,200 | 2,005 | 1,800 | 1,600 | 1,600 |
| 3 | 3,250 | 2,200 | 2,550 | 1,800 | 2,000 | 2,400 |
| 6 | 5,500 | 5,500 | 6,500 | 4,000 | 4,000 | 5,000 |
| 9 | 10,000 | 6,500 | 9,250 | 8,500 | 5,500 | 7,500 |
| 12 | 17,750 | 10,000 | 11,000 | 13,500 | 6,500 | 8,500 |
| 15 | 17,250 | 8,250 | 12,750 | 16,500 | 6,000 | 9,000 |
| 18 | 17,250 | 10,000 | 17,250 | 13,000 | 8,000 | 8,500 |
| 21 | 17,750 | 10,500 | 16,500 | 13,500 | 7,000 | 11,000 |
| 24 | 18,250 | 10,000 | 16,500 | 13,500 | 7,500 | 13,500 |

[A] Results are average of two replicates

[B] One determination only

## Influence of pH on Inhibitory Activity of Cell-Free Spent Broth

The results of increase in $A_{620}$ nm during growth of L. acidophilus NCFM in tube assays in MRS broth adjusted to pH 5.0; 5.5; 6.0; 6.5 and 7.0 are in TABLE XXIII, Appendix E.

Average results of increase in $A_{620}$ nm at 6 hours of incubation is depicted in Figure 7. This figure indicates that in the absence of the spent broth, L. acidophilus NCFM grew better at pH 6.0. However, the MRS broth at pH 6.0 with the spent broth present appeared to be more inhibitory to the growth of this microorganism.

## Effect of Heat Sterilization (121°C for 15 Minutes) on Activity of the Cell-Free Spent Broth

The results of increase in $A_{620}$ nm during growth of L. acidophilus NCFM in assay tubes containing heated and unheated spent broths, and controls are in TABLE XXIV (Appendix F) and the analysis of variances in TABLE XXV (Appendix F).

Figure 8 shows the inhibitory action of the heated and unheated spent broth. The amount of inhibition was almost undistinguished between the two compared to the control. Average of increase $A_{620}$ nm at 6 h. incubation are in TABLE VII. Analysis of variances of data from 6 h. incubation (TABLE XXV, Appendix F) indicated that heat sterilization treatment (121°C for 15 minutes) did not significantly affect the inhibitory activity of the spent broth ($p > 0.05$). The control tubes (with no spent broth) exhibited growth significantly higher ($p < 0.01$) than the tubes containing aliquots of the unheated and heated spent broth.

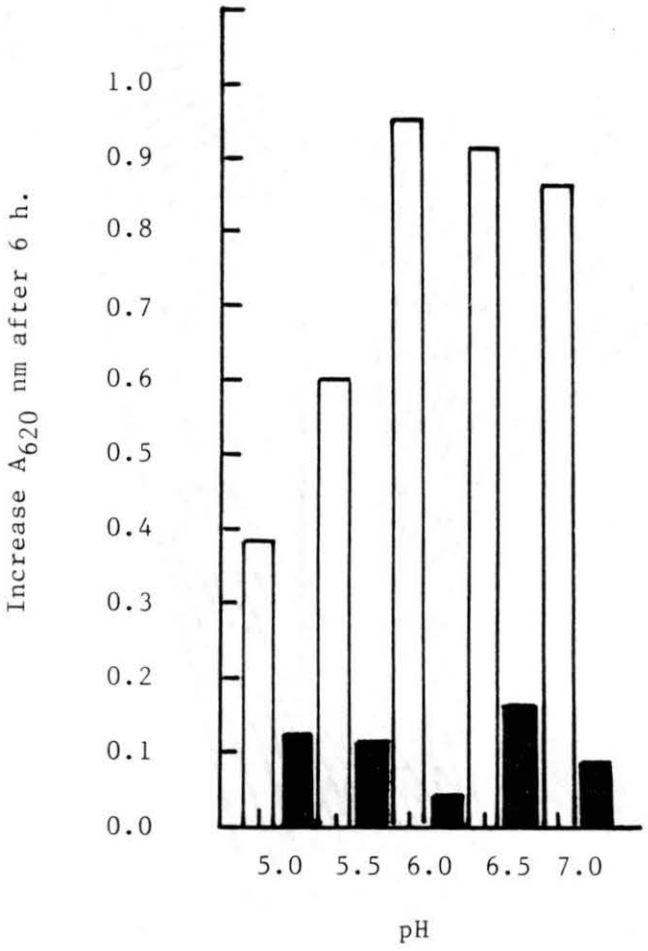


Figure 7. Influence of pH on inhibitory activity of 12 h. spent broth from fermentors maintained at pH 6.0. Open bars = controls (no spent broth); closed bars = spent broth. Each value is an average from two trials.

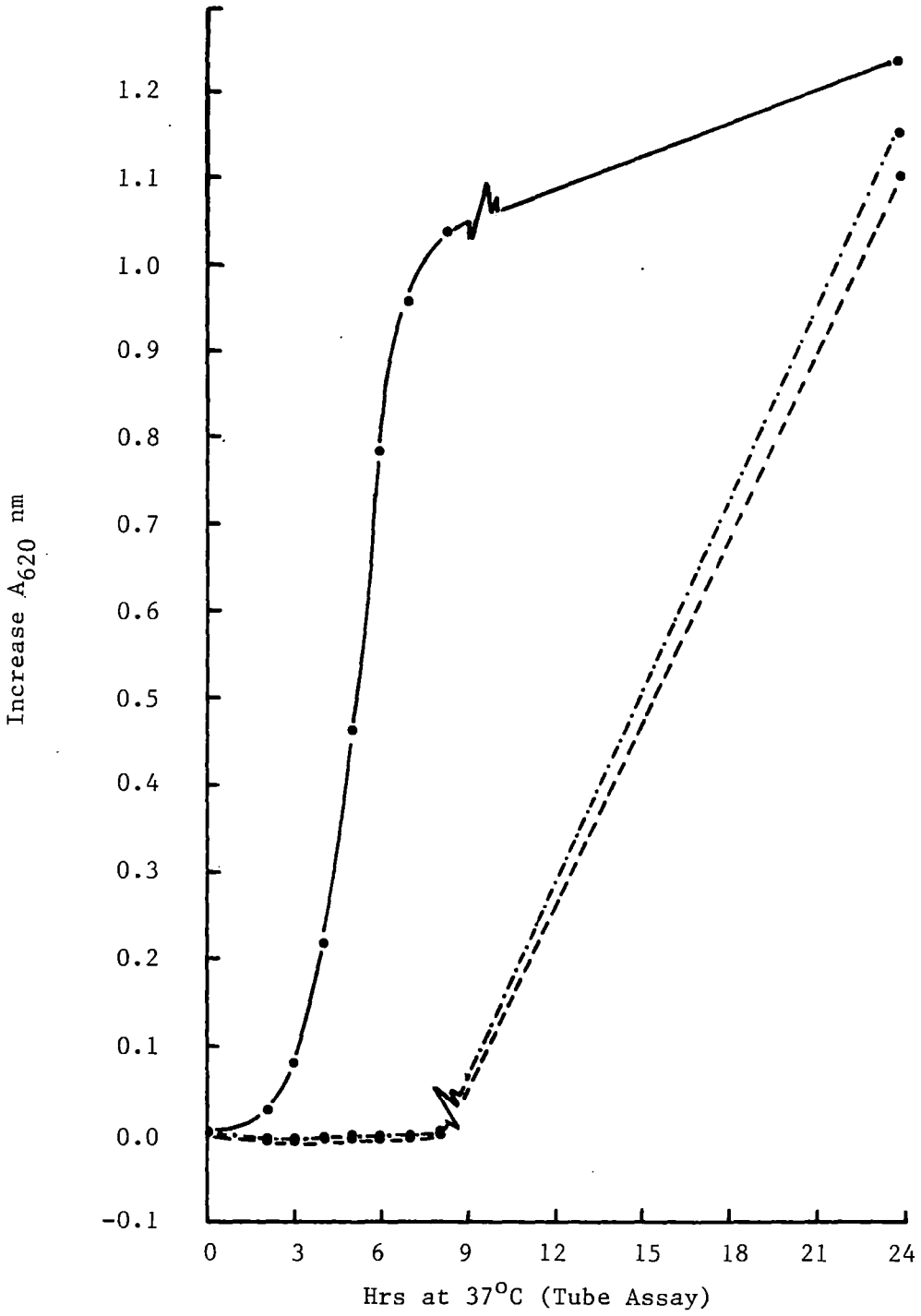


Figure 8. Effect of heat (121°C for 15 minutes) on inhibitory action of spent broth (control •——; unheated spent broth •---; heated spent broth •-•-•. Each value is an average from three trials.

TABLE VII

EFFECT OF HEAT STERILIZATION ON ACTIVITY OF INHIBITORY PRINCIPLE PRESENT IN THE CELL-FREE SPENT BROTH

| Sample | Increase in $A_{620}$ nm at 6 h. Incubation in Tube Assay[A] |
|---|---|
| Control | 0.775[C] |
| Unheated spent broth | -0.008[D] |
| Heated spent broth[B] | -0.007[D] |

[A] Each value is an average of three trials.

[B] Heated 15 minutes at 121°C.

[C,D] Means with different superscript are significantly different ($p < 0.05$).

## Attempt to Purify the Inhibitory Substance Present in the Inhibitory Spent Broth by Ion Exchange Chromatography

The fractions of cell-free spent broth resulting from ion exchange chromatography were analyzed for their inhibitory activity. None of the fractions or their combinations exhibited inhibitory action (TABLE VIII). The only sample exhibiting inhibitory activity was the cell-free spent broth.

TABLE VIII

ASSAY FOR INHIBITORY ACTION OF FRACTIONS OF ION EXCHANGE CHROMATOGRAPHY OF SPENT BROTH

| Sample | Increase in $A_{620}$ nm at 6 h. Incubation in Assay Tubes[A] |
|---|---|
| Control | 1.046[B] |
| Spent Broth | 0.119[C] |
| Cation Effluent + Cation Eluate | 1.043[B] |
| C. Effluent + Anion Effluent + A. Eluate | 1.024[B] |
| A. Effluent + A. Eluate | 1.042[B] |
| Cation Effluent | 1.046[B] |
| Cation Eluate | 1.096[B] |
| Anion Effluent | 1.078[B] |
| Anion Eluate | 1.052[B] |

[A] Results are average of two trials.

[B,C] Means with different superscript are significantly different ($p < 0.05$).

## Isolation of a Strain of L. acidophilus NCFM Resistant to the Inhibitor

In the tube assay, even though the spent broth collected at 12 h. from the fermentor culture maintained at pH 6.0 was very inhibitory, the culture appeared to overcome the inhibition in that growth was evident upon extended incubation (Figure 6). The culture resulting from the tube assay following 24 h. incubation appeared to be resistant to the

inhibitory principle present in the spent broth. Resistance was retained when a) it was subcultured four times in MRS broth containing 2% inhibitory spent broth, b) when it was subcultured in MRS alone, or c) when it was subcultured intermittently in MRS broth with and without spent broth (TABLE IX). Thus, the strain(s) which overcome inhibition (observed in the tube assay for inhibitory action) are resistant to the inhibitory principle present in the spent broth. These strains exhibited the same level of growth whether the inhibitor was present or not. Although these strains do not recognize the inhibitor they exhibit a slower growth pattern when compared to the parent culture of L. acidophilus NCFM.

TABLE IX

ASSAY FOR INHIBITORY ACTION OF SPENT BROTH TREATED TOWARD CULTURES WHICH APPARENTLY DEVELOPED RESISTANCE DURING THE TUBE ASSAY

| Organism | Increase $A_{620}$ nm[A] | |
|---|---|---|
| | Control | 12 h. Spent Broth |
| L. acidophilus NCFM | 0.838 | -0.002 |
| Inoculum from Treatment A[B] | 0.303 | 0.330 |
| Inoculum from Treatment B[B] | 0.300 | 0.301 |
| Inoculum from Treatment C[B] | 0.301 | 0.329 |

[A] Results are average increase $A_{620}$ nm during 6 h. tube assay from two trials.

[B] Treatment A (four subcultures in MRS + 2% inhibitor); Treatment B (four subcultures in MRS only); Treatment C (four subcultures in MRS broth intermittently with and without the inhibitor).

Spent broths prepared from an 18 h. static culture of the resistant strains exhibited action similar to that observed from the spent broth of L. acidophilus NCFM grown at pH 6.0 in the fermentor (data not shown). When the resistant culture was plated on MRS agar, large and small colonies were observed. With this procedure, cultures designated L. acidophilus NCFM-L and L. acidophilus NCFM-S which produce large and small colonies in MRS agar respectively, were isolated for further study. Both strains were checked with respect to their sensitivity to the inhibitory substance in the spent broth by the disc assay method (TABLE X, next section), and found to be resistant.

Another approach to isolating resistant and sensitive strains was to plate the original or parent L. acidophilus NCFM on MRS agar and on MRS agar containing added inhibitory spent broth (2%). From this procedure cultures designated as L. acidophilus NCFM-D; -E; -F; -G isolated from agar plates were shown to be sensitive to the inhibitory spent broth and L. acidophilus NCFM-U isolated from MRS agar plus spent broth was shown to be resistant. From these cultures, L. acidophilus NCFM-F (sensitive strain) an isolate from predominating type colony originated from growth on MRS alone (without the inhibitor) was selected and used throughout this work. It is worth noting that the plates prepared with MRS agar plus the inhibitory spent broth present yielded very low numbers of colonies, indicating that the resistant strains are present in very low numbers in the parent strain L. acidophilus NCFM.

The resistant strain(s) appear to be the ones responsible for the production of the inhibitory substance present in the spent broth of L. acidophilus NCFM culture grown in MRS broth at pH 6.0.

## Sensitivity of Various Bacteria to the Inhibitory Spent Broth

The disc assay technique was used to compare sensitivity of various cultures to the inhibitory principle present in spent broth from L. acidophilus NCFM-L (TABLE X). The test organisms include species from the Lactobacillaceae, Streptococcaceae, Enterobacteriaceae, Micrococcaceae and one unidentified strain of a gram negative psychrotrophic organism (here identified as Psychrotroph HSK).

Inhibition was limited exclusively to the Lactobacillaceae, namely L. acidophilus 4962; L. acidophilus-1; L. acidophilus HM2; L. acidophilus NCFM; L. acidophilus NCFM-F, L. lactis 39A1 and L. lactis 403E-5. The producer strain L. acidophilus NCFM-L was not sensitive to the inhibitor and neither were the other lactobacilli L. acidophilus RP32; L. acidophilus RAM; L. casei 7; L. casei LN; L. casei 7469; L. salivarius 59 and L. acidophilus NCFM-S. None of the other nonpathogenic and pathogenic organisms utilized in this experiment were sensitive to the inhibitory substance present in the spent broth of L. acidophilus NCFM-L.

## Influence of Catalase, Trypsin and Pepsin on Activity of Inhibitor

The cell-free spent broth was treated with the proteolytic enzymes pepsin and trypsin to determine their affect on the inhibitory principle. Catalase also was included in this experiment to corroborate the ineffectiveness of this enzyme in preventing the inhibitory activity observed during growth of L. acidophilus NCFM in MRS broth at pH 6.0. Data from tube assays with treated and untreated spent broth are

TABLE X

SENSITIVITY OF VARIOUS BACTERIA TO INHIBITOR PRESENT IN SPENT BROTH FROM L. ACIDOPHILUS NCFM-L

| Indicator Organism | Sensitivity to Spent Broth Trial # 1 | 2 | 3 |
|---|---|---|---|
| Enterobacter aerogenes | -[B] | - | - |
| Escherichia coli B44 | - | - | - |
| Escherichia coli 026:K60 | - | - | - |
| Lactobacillus acidophilus NCFM | +[A] | + | + |
| Lactobacillus acidophilus NCFM-F | + | + | + |
| Lactobacillus acidophilus NCFM-L | - | - | - |
| Lactobacillus acidophilus NCFM-S | - | - | - |
| Lactobacillus acidophilus 4962 | + | + | + |
| Lactobacillus acidophilus RP32 | - | - | - |
| Lactobacillus acidophilus RAM | - | - | - |
| Lactobacillus acidophilus 1 | + | + | + |
| Lactobacillus acidophilus HM2 | + | + | + |
| Lactobacillus casei 7 | - | - | - |
| Lactobacillus casei LN | - | - | - |
| Lactobacillus casei 7469 | - | - | - |
| Lactobacillus lactis 39A1 | + | + | + |
| Lactobacillus lactis 403E-15 | + | + | + |
| Lactobacillus salivarius 59 | - | - | - |
| Psychrotroph HSK | - | - | - |
| Salmonella typhimurium | - | - | - |
| Salmonella pullorum | - | - | - |
| Staphylococcus aureus B925 | - | - | - |
| Streptococcus lactis AC2 | - | - | - |

[A] (+) indicates presence of zone of inhibition.

[B] (-) indicates absence of zone of inhibition.

presented in TABLE XXVI (Appendix G) and the average results of the three trials are shown in Figure 9. The inhibitory substance produced by L. acidophilus NCFM when grown in MRS broth in fermentors at pH 6.0 was completely inactivated when treated either with pepsin or trypsin. Growth of L. acidophilus NCFM inoculated into tubes containing untreated spent broth and in tubes containing spent broth treated with catalase, or control tubes for each enzyme was inhibited. Analysis of variances of $A_{620}$ nm values at 6 h. incubation (TABLE XXVII, Appendix G) indicated that catalase and the pH adjustments included in the controls for each enzyme treatment were not significantly different ($p > 0.05$) among themselves but all were significantly lower than growth observed in control tubes (no spent broth added) and in tubes containing added spent broth treated with proteolytic enzymes. The cultures inoculated into tubes containing spent broth treated with the proteolytic enzymes grew as well as the culture in the control tubes, yielding growth curves almost undistinguishable from the control. The amounts of growth in these tubes at 6 h. were not significantly different from that in the control tube ($p > 0.05$).

Results from disc assays to test the inhibitory action of enzyme treated spent broth (TABLE XI) corroborated the results obtained by the tube assay method. The sensitive organism (which show zone of inhibition - L. acidophilus NCFM and L. acidophilus NCFM-F) acted much the same indicating the predominance of the sensitive strain L. acidophilus NCFM-F in the L. acidophilus NCFM culture. The resistant strain (no zone of inhibition - L. acidophilus NCFM-L) was unaffected by the presence of the inhibitory spent broth.

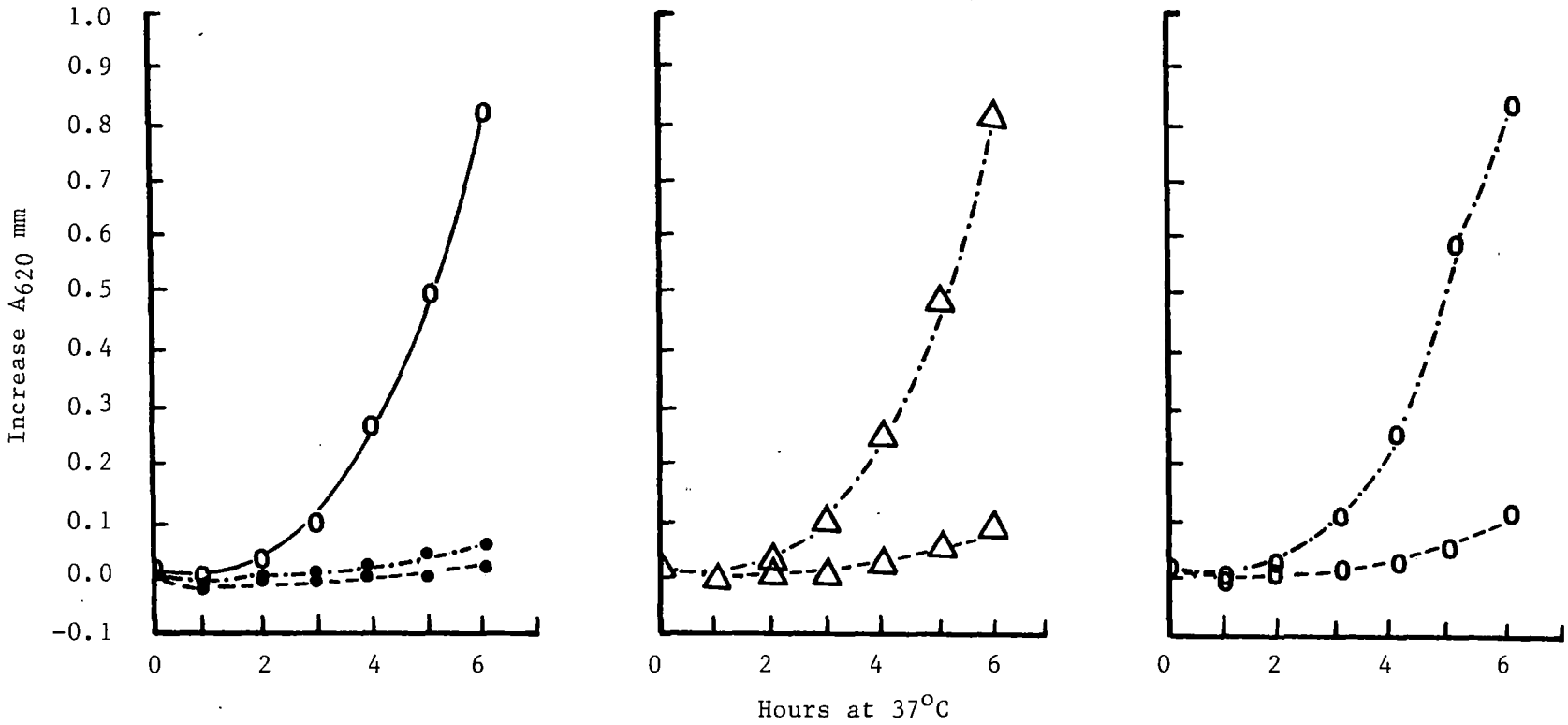


Figure 9. Influence of enzyme treatment on inhibitory action of spent broth: •—— control; •--- catalase control; •-·-· catalase treated; △ --- trypsin control; △ -·-· trypsin treated; o--- pepsin control; o-·-· pepsin treated. Each value is an average of three trials.

TABLE XI

DISC ASSAY FOR INHIBITORY ACTIVITY OF ENZYME TREATED SPENT BROTH ON GROWTH OF L. ACIDOPHILUS NCFM; L. ACIDOPHILUS NCFM-F AND L. ACIDOPHILUS NCFM-L[A]

| Treatment | Test Organism | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | L. acidophilus NCFM Trial # | | | L. acidophilus NCFM-F Trial # | | | L. acidophilus NCFM-L Trial # | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Control[B] | + | + | + | + | + | + | - | - | - |
| pH 6.0 No Catalase | + | + | + | + | + | + | - | - | - |
| pH 6.0 Catalase | + | + | + | + | + | + | - | - | - |
| pH 8.0 No Trypsin | + | + | + | + | + | + | - | - | - |
| pH 8.0 Trypsin | - | - | - | - | - | - | - | - | - |
| pH 3.0 No Pepsin | + | + | + | + | + | + | - | - | - |
| pH 3.0 Pepsin | - | - | - | - | - | - | - | - | - |

[A] Results indicated as (+) presence of zone of inhibition (-) absence of zone of inhibition.

[B] Control contains spent broth without any enzyme treatment; all were adjusted to pH 6.0 prior to disc assay.

# CHAPTER V

## DISCUSSION

L. acidophilus has been used singly or in combination with other species of lactic acid bacteria in the preparation of a variety of fermented and unfermented foods. L. acidophilus NCFM has been used commercially as a dietary adjunct in the production of a nonfermented dairy product. Production of such products depends on the use of frozen concentrated cultures. In the production of concentrated cultures, although it is important to select strains of L. acidophilus which have desirable bioactivites such as bile resistance and ability to displace undesirable organisms (Gilliland and Speck, 1977a), the production of high bacterial population prior to harvesting is important as well.

It is known that the attainable population during growth of many lactic acid bacteria is frequently limited by the accumulation of acid (Roger and Wittier, 1928; Harvey, 1965; Tanfor, 1968; Marquis et al., 1973). This type of inhibition can be prevented either by providing the growth medium with an effective buffering system (Thunnel et al., 1984) or by controlling pH automatically at a level favorable for growth (Bergere, 1968; Pont and Holloway, 1968; Gilliland, 1970). The control of the pH at desirable levels is now a common practice in the production of cells for concentrated cultures. Although the maximum attainable population of some starter cultures is increased when the pH is maintained at pH 6.0 (Pont and Holloway, 1968) for L. acidophilus NCFM this

does not seem to hold true. When growing in some media at pH 6.0 L. acidophilus NCFM appears to enter a "premature" death phase. Mitchell and Gilliland (1983) observed this phenomenon when growing L. acidophilus NCFM in pepsinized whey media containing 2.5%, 5.0% and 7.5% of yeast extract. These authors concluded that an imbalance of nutrients could have been one of the factors responsible for the observed "premature" death phase.

The growth of L. acidophilus NCFM in MRS broth controlled at pH 6.0 resulted in significant death after 12 h. of incubation. This "premature" death phase was not observed when the culture was grown in MRS broth statically or with agitation alone. Thus indicating that the agitation required for dispersion of the neutralizer necessary for pH control was not responsible for the death.

Since many lactic acid bacteria, including L. acidophilus have been shown to produce hydrogen peroxide under aerobic conditions (Dahiya and Speck, 1968; Price and Lee, 1970; Collins and Aramaki, 1980) it was speculated that the major cause of inhibition under the described conditions might have been due to accumulation of the peroxide to auto inhibitory levels. While the lack of death during growth in fermentors with agitation but with no pH control tended to rule out this possibility, it was possible that sufficient hydrogen peroxide may have accumulated in the fermentor being maintained at pH 6.0. However the comparison of growth in fermentors maintained at pH 6.0 with active and inactive catalase ruled out this possibility. Moreover, the treatment of the 12 h. inhibitory spent broth with catalase did not overcome the inhibition. Thus hydrogen peroxide was not the major cause of the "premature" death phase.

Another cause of inhibition could have been the accumulation of lactates. Pont and Holloway (1968) indicated that 2% lactate was present at the end of the log phase of growth of S. lactis growing in a whey-base medium fortified with tryptone and yeast extract. In their experiment, 2% lactate was found to be inhibitory to the test organism. In our work, preliminary studies indicated that lactate neutralized to pH 6.0 at levels detected throughout the 24 h. incubation period were not inhibitory enough to account for the observed results.

The very intense inhibition of L. acidophilus NCFM caused by the spent broth during the early hours of the tube assay followed by what appeared to be maximum growth by the end of the 24 h. incubation period might indicate that the organism acquired resistance to the inhibitory substance or more likely that a resistant strain(s) present in low numbers in the original culture was allowed to predominate. An experiment to isolate component strains of L. acidophilus NCFM resulted in the isolation of a sensitive strain (L. acidophilus NCFM-F) and two resistant strains (L. acidophilus NCFM-L and L. acidophilus NCFM-S). The latter were responsible for the production of the inhibitory substance (active against the L. acidophilus NCFM and L. acidophilus NCFM-F but not active against themselves). Studies with these resistant strains indicated that they were metabolically different from the sensitive strains in that they grew slower in MRS broth. The predominating strain(s) in the original or parent culture was the sensitive type, which grew faster in the absence of the inhibitory spent broth but exhibited no growth in its presence. This evidenced the mixed strain character of the L. acidophilus NCFM and explains, in part, the pattern of growth of the L. acidophilus NCFM in the fermentor maintained at pH 6.0 and in the

tube assay experiments. In the earlier part of growth in the fermentor the faster strain(s) apparently predominated. Although the slower strain(s) were present in low numbers initially they increased in numbers as fermentation proceeded and the inhibitory substance accumulated. The rate of death apparently overrode the rate of growth in the fermentor at 12 h. of growth. Because the resistant strains grew very slowly they could not overcome the phenomenon which culminated with the observed "premature" death phase.

Because the potency of the inhibitory principle present in the spent broth decreased rather than increased with continued incubation lead us to speculate that the "premature" death phase was a result of antagonistic action among the strains of the L. acidophilus NCFM. This antagonistic action among strains of the same species fit the description of bacteriocins (Tagg et al., 1976).

Bacteriocins, the antibiotic-like substances of protein nature differ from the classical antibiotics in their limited action within members of the same or closely related species (DeKlerk, 1967; Tagg et al., 1976). Since these substances have been found among the lactobacilli (Vincent et al., 1959; DeMan et al., 1960; DeKlerk and Smit, 1967; Upreti and Hinsdill, 1973; Barefoot and Klaenhammer, 1983) we considered that we were dealing with a bacteriocin produced by the resistant strains against the sensitive ones which were the predominating cells in a culture of L. acidophilus NCFM.

Attempts made by Vincent et al. (1959) to purify bacteriocin produced by strains of L. acidophilus employing adsorption onto charcoal, calcium tartrate, and ion exchange resins (Amberlite 120 IR-4 and Bio-RAD AG 50X) were either unsuccessful or accompanied by very poor

recovery of activity. DeKlerk and Smit (1967) passed spent broth of L. fermenti 466 which contained bacteriocin through Amberlite IR 410 ($Cl^-$ form) anion and Amberlite IR 120 ($H^+$ form) cation exchange resins. These resins did not retain the bacteriocin. However, reports from Ellison and Kauter (1970) involving large and small type bacteriocins (boticins) indicated that both were strongly adsorbed onto suitably charged surfaces. The large boticin was adsorbed onto anion exchange resin and the small was adsorbed onto filter paper, glass, plastics and cation exchange resins. They also indicated that adsorption was so strong that reversibility was possible only under extreme conditions of pH. This ability that some types of bacteriocins have to adsorb onto some surface was corroborated further by Tagg and Russel (1981) who observed complete loss of streptococcin sal-P. This precluded the application of this technique as a means of studying such preparation. In the present work fractions from ion exchange chromatography or their combination did not exhibit any activity. The reason for our findings could have been twofold. One, the bacteriocin may have been adsorbed onto the cation-exchange resin and could not be eluted from it under the conditions employed. Secondly, it might have been denatured during passage through the acidic ion exchange column (Dowex 50W). Dowex 50W is a polystyrene base, strong acidic resin. Reports in the literature indicate the ability that these resins have in denaturing proteins (Clark and Switzer, 1977).

Studies on the effect of pH on bacteriocins produced by lactobacilli were first published by Vincent et al. (1959). The crude inhibitory extract from L. acidophilus which he named "lactocidin" was most active at neutral pH. Further studies made by DeKlerk and Coetzee

(1961) indicated that bacteriocins from cultures of lactobacilli having a pH in the range of 3.6 to 4.1 were unaffected when adjusted to pH 7.0. Barefoot and Klaenhammer (1983) characterizing lactacin B produced by L. acidophilus N2 indicated that it was active at neutral pH. Despite the numerous reports indicating the stability of the inhibitory action of the bacteriocin and/or bacteriocin-like substances at pH close to neutrality, there is a lack of studies in the literature comparing the activity of these inhibitory substances at various pH levels. In the present study the growth of L. acidophilus NCFM in media adjusted to pH 5.0; 5.5; 6.0; 6.5; and 7.0 in the presence and absence of the inhibitory spent broth was observed and results indicated that the inhibitory substance was active at all pH levels tested. Although the culture grew best at pH 6.0 in the absence of the inhibitory spent broth, it appeared to be most inhibited by the spent broth at this pH. The fact that this bacteriocin-like substance appears to be more active at pH 6.0 might explain, in part, the extent of inhibition of growth in the fermentor which culminates with the "premature" death phase of this culture when growing in MRS broth automatically controlled at pH 6.0.

Since the factors that control activity and inhibition seem to be additive, it is possible that in the fermentor, pH and medium composition enhance the activity of the inhibitory substance which is being produced. This suggestion arises from the fact that for other bacterial cultures, MRS supported larger bacteriocin yields than did simple media (Mayr-Harting et al., 1972). This condition added to the fact that pH 6.0 supported highest growth and still considering that the best conditions for growth are also usually the best conditions for bacteriocin production (Goebel et al., 1956) prompted us to suggest that it might be

possible that at this pH more bacteriocin was produced and it was more active as well.

Heat resistance is a major characteristic of bacteriocins and/or bacteriocin-like substances (Tagg et al., 1976). Variability in the parameters used to evaluate heat stability is evident in the literature. These values vary from 60°C for 30 minutes (Ivanovics and Alfoldi, 1954) to autoclaving for 30 minutes (Ellison and Kauter, 1970). In the present work it was found that heat of sterilization (121°C for 15 minutes) did not affect the activity of the bacteriocin-like substance present in the spent broth corroborating its heat-stability nature. Such heat stability rules out the possibility of the inhibitory action being due to bacteriophage.

As indicated by Mayr-Harting et al. (1972), the earliest evidence as to the nature of bacteriocins was obtained from experiments on the degradation of crude preparations containing bacteriocins by proteolytic enzymes. They indicated further that this method is still useful in initial studies on newly discovered bacteriocins. Several reports have indicated that bacteriocins of different microorganisms vary with respect to their sensitivity to proteolytic enzymes. Bacteriocin from L. fermenti (DeKlerk and Smit, 1962) was reported to be sensitive to trypsin and pepsin as was Lactacin B, a bacteriocin produced by L. acidophilus N2 (Barefoot and Klaenhammer, 1983). Results from the present experiments have shown that the inhibitory substance produced by the strains of L. acidophilus described herein was completely inactivated by either pepsin or trypsin indicating its proteinaceous nature which is a major characteristic of bacteriocins (Nomura, 1967).

Bacteriocins, by definition have little potential for broad

spectrum inhibition. This activity is usually restricted to closely related species and is one of the criteria for their identification (Nomura, 1967; Tagg et al., 1976; Cooper and James, 1985). Although Lactocidin, a bacteriocin produced by strains of L. acidophilus (Vincent et al., 1959), had a broad spectrum of activity against the most common enteric bacteria, the activities of bacteriocins from other lactobacilli have been shown usually to be restricted to the Lactobacillaceae and more specifically to closely related strains (DeKlerk and Coetzee, 1961; Upreti and Hinsdill, 1973; Barefoot and Klaenhammer, 1983). The findings of this work indicated that bacteriocin produced by L. acidophilus NCFM (specifically by the resistant component strains, L. acidophilus NCFM-L and NCFM-S) exhibited a narrow spectrum of activity which was restricted only to some strains of L. acidophilus and some of the other species of lactobacilli. No activity was observed against the pathogenic species (staphylococci and salmonellae) utilized in this work.

Konisky (1978) emphasized the importance that claims for bacteriocinogeny be well documented, including a determination of the protein nature of the antagonistic agent and a demonstration that the producing organism is insensitive or "immune" to its action. The findings in the present work indicate that the inhibitory substance present in the spent broth from L. acidophilus NCFM grown in MRS broth at pH 6.0 is a bacteriocin.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

The "premature" death phase observed for L. acidophilus NCFM growing in MRS broth maintained at pH 6.0 might be explained by: i) maximum production of the bacteriocin occurred at 12 h., ii) from this time on, the rate of growth of the resistant strains did not overcome the rate of death of the sensitive ones within the fermentor culminating in the observed "premature" death.

To explain the dissipation of the bacteriocin action as incubation proceeds after 12 h., one can speculate that the same cells that have been inactivated when one unit of bacteriocin was attached to them are still able to adsorb more units. As these units are being adsorbed onto the cells, the amount of bacteriocin is decreased in the spent broth rendering it less inhibitory. Another reason could be the production of proteolytic enzymes by resistant strains which inactivate the bacteriocins. A third factor might be the ability that sensitive cells have to adsorb bacteriocin even if they are dead, provided the active site(s) is not damaged.

Classification of the inhibitory material as a bacteriocin is based on the following factors: i) catalase experiments indicated that the "premature" death phase of L. acidophilus NCFM growing at pH 6.0 was not due to hydrogen peroxide accumulation, ii) lactate determinations prove quite conclusively that the "premature" death observed in fermentors was

not due to the accumulation of lactate per se. Samples from a static culture with similar levels of lactate were not as inhibitory. iii) The inhibitor was unaffected by heating at 121°C for 15 minutes indicating the heat stable nature of this proteinaceous material. iv) The inhibitory substance has a narrow spectrum of activity being active only against other lactobacilli. v) Activity of the inhibitory spent broth was eliminated by treatment with 25 mg of pepsin or 25 mg of trypsin for 30 minutes at 37°C. Preparations without the enzymes retained full activity. vi) The fact that inhibition decreases towards the end of incubation period corroborates the findings of Goebel (1956) who observed the same phenomenon in broths containing colicin K.

There is a need for studies with pure component cultures isolated from L. acidophilus NCFM. Such studies could focus on the mechanism of action and inactivation of bacteriocins and could contribute to a better understanding of interactions in mixed cultures. Since the literature indicates the possibility of bacteriocins to be plasmid linked due to its unstable character, there is a need to verify whether or not the production of bacteriocins by the resistant strains of L. acidophilus NCFM is plasmid linked or not. If this is the case, transfer of plasmid to cells unable to produce bacteriocins could result in improved strains for use as dietary adjuncts. The ability to transfer such desirable genetic traits is just beginning to be developed for the lactobacilli.

To conclude, there is a need to purify, to characterize and to study the factors that control the bacteriocin produced by the resistant strains of L. acidophilus NCFM growing in MRS broth maintained at pH 6.0.

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# APPENDIXES

# APPENDIX A

## GROWTH OF L. ACIDOPHILUS NCFM IN MRS BROTH MAINTAINED AT PH 6.0; WITH AGITATION ONLY AND UNDER STATIC CONDITIONS

TABLE XII

GROWTH OF L. ACIDOPHILUS NCFM IN MRS BROTH IN FERMENTORS MAINTAINED UNDER DIFFERENT CONDITIONS

| Growth Conditions | Sampling Time (h) | $\log_{10}$ CFU ml $ml^{-1}$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | MEAN |
| pH 6.0 | 0 | 6.95 | 6.83 | 6.97 | 6.92 | 6.91 | 6.92 |
| | 3 | 7.18 | 7.40 | 7.34 | 7.32 | 7.23 | 7.29 |
| | 6 | 7.84 | 8.23 | 7.90 | 7.94 | 7.84 | 7.95 |
| | 9 | 8.04 | 8.90 | 8.94 | 8.65 | 8.41 | 8.59 |
| | 12 | 7.92 | 8.75 | 9.00 | 8.60 | 8.91 | 8.64 |
| | 15 | 7.23 | 7.45 | 8.32 | 7.75 | 9.15 | 7.98 |
| | 18 | 6.60 | 6.23 | 7.43 | 6.32 | 7.92 | 6.90 |
| | 21 | 6.18 | 5.87 | 7.64 | 5.66 | 7.71 | 6.61 |
| | 24 | 5.16 | 5.87 | 6.08 | 5.33 | 7.51 | 6.15 |
| Agitation only | 0 | 6.93 | 6.85 | 6.97 | 6.88 | 6.99 | 6.92 |
| | 3 | 7.15 | 7.45 | 7.38 | 7.26 | 7.38 | 7.32 |
| | 6 | 7.80 | 8.23 | 7.97 | 7.95 | 8.04 | 8.00 |
| | 9 | 8.11 | 8.62 | 8.76 | 8.64 | 8.72 | 8.57 |
| | 12 | 8.35 | 8.66 | 8.87 | 8.53 | 8.51 | 8.68 |
| | 15 | 8.08 | 8.70 | 9.26 | 8.70 | 8.70 | 8.69 |
| | 18 | 7.79 | 8.81 | 8.54 | 8.81 | 8.53 | 8.50 |
| | 21 | 8.30 | 8.79 | 8.56 | 8.73 | 8.58 | 8.59 |
| | 24 | 8.00 | 8.73 | 8.38 | 8.51 | 8.57 | 8.44 |
| Static | 0 | 6.94 | 6.83 | 7.00 | 6.90 | 6.93 | 6.92 |
| | 3 | 7.30 | 7.51 | 7.46 | 7.34 | 7.46 | 7.41 |
| | 6 | 8.60 | 8.62 | 8.63 | 8.64 | 8.60 | 8.62 |
| | 9 | 8.90 | 8.94 | 8.66 | 8.90 | 8.93 | 8.87 |
| | 12 | 8.04 | 9.00 | 9.18 | 8.90 | 8.94 | 8.81 |
| | 15 | 8.91 | 8.98 | 8.94 | 8.80 | 8.94 | 8.91 |
| | 18 | 8.86 | 9.04 | 9.08 | 9.00 | 9.08 | 8.01 |
| | 21 | 8.88 | 8.92 | 9.08 | 7.94 | 8.94 | 8.75 |
| | 24 | 8.89 | 8.98 | 9.04 | 8.99 | 9.00 | 8.98 |

TABLE XIII

ANALYSIS OF VARIANCE OF GROWTH IN THE FERMENTORS UNDER THE THREE CONDITIONS AT 12 H.

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (5% level) |
|---|---|---|---|---|---|
| Total | 1.89 | 14 | ---- | ---- | ---- |
| Among treatments | 0.08 | 2 | 0.04 | 0.27 | 5.10 |
| Error | 1.81 | 12 | 0.15 | ---- | ---- |

TABLE XIV

ANALYSIS OF VARIANCE OF GROWTH IN THE FERMENTORS UNDER THE THREE CONDITIONS AT 24 H.

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (5% level) |
|---|---|---|---|---|---|
| Total | 25.36 | 14 | ----- | ----- | ---- |
| Among treatments | 22.57 | 2 | 11.29 | 49.08[A] | 5.10 |
| Error | 2.79 | 12 | 0.23 | ----- | ---- |

[A] Significant at $p < 0.05$; LSD required for differences among means at 5% and 0.1% levels are 0.6609 and 1.3097, respectively.

# APPENDIX B

## GROWTH OF L. ACIDOPHILUS NCFM IN MRS BROTH WITH ACTIVE AND INACTIVE CATALASE

TABLE XV

GROWTH OF L. ACIDOPHILUS NCFM IN MRS BROTH WITH ACTIVE AND INACTIVE CATALASE IN FERMENTOR WITH PH 6.0

| Growth Conditions | Sampling Time (h) | $\log_{10}$ CFU $ml^{-1}$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | Mean |
| pH 6.0 Active Catalase | 0 | 6.97 | 6.63 | 6.83 | 6.85 | 6.86 | 6.82 |
| | 3 | 7.23 | 6.86 | 7.20 | 7.23 | 7.36 | 7.18 |
| | 6 | 7.95 | 7.96 | 7.79 | 7.95 | 7.70 | 7.87 |
| | 9 | 8.04 | 7.95 | 7.98 | 8.23 | 8.26 | 8.09 |
| | 12 | 7.45 | 7.62 | 8.00 | 8.04 | 8.52 | 7.93 |
| | 15 | 6.08 | 7.81 | 7.82 | 7.82 | 8.53 | 7.61 |
| | 18 | 5.70 | 7.74 | 8.18 | 8.00 | 8.18 | 7.56 |
| | 21 | 6.10 | 7.90 | 8.20 | 8.20 | 8.08 | 7.70 |
| | 24 | 6.28 | 8.08 | 8.04 | 7.79 | 7.95 | 7.63 |
| pH 6.0 Inactive Catalase | 0 | 6.84 | 6.65 | 6.81 | 6.83 | 6.92 | 6.81 |
| | 3 | 7.26 | 6.87 | 7.15 | 7.15 | 7.38 | 7.16 |
| | 6 | 8.15 | 7.51 | 7.76 | 8.00 | 7.91 | 7.87 |
| | 9 | 8.87 | 8.32 | 8.18 | 8.70 | 8.61 | 8.54 |
| | 12 | 8.43 | 8.86 | 8.79 | 8.00 | 8.97 | 8.61 |
| | 15 | 8.45 | 7.68 | 8.45 | 7.30 | 8.23 | 8.02 |
| | 18 | 7.92 | 5.60 | 8.26 | 7.00 | 7.73 | 7.30 |
| | 21 | 7.65 | 5.28 | 7.94 | 7.00 | 7.45 | 7.06 |
| | 24 | 7.32 | 4.90 | 7.96 | 6.92 | 7.23 | 6.87 |

TABLE XVI

ANALYSIS OF VARIANCE OF AMOUNT OF GROWTH OF L. ACIDOPHILUS NCFM AT 12 H., IN MRS BROTH WITH ACTIVE AND INACTIVE CATALASE IN FERMENTOR MAINTAINED AT PH 6.0

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (5% level) |
|---|---|---|---|---|---|
| Total | 2.49 | 9 | ---- | ---- | ---- |
| Among treatments | 1.17 | 1 | 1.17 | 7.15 | 7.57 |
| Error | 1.32 | 8 | 0.16 | ---- | ---- |

TABLE XVII

ANALYSIS OF VARIANCE OF AMOUNT OF GROWTH OF L. ACIDOPHILUS NCFM AT 24 H., IN MRS BROTH WITH ACTIVE AND INACTIVE CATALASE IN FERMENTOR WITH PH 6.0

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (5% level) |
|---|---|---|---|---|---|
| Total | 9.18 | 9 | ---- | ---- | ---- |
| Between treatments | 1.46 | 1 | 1.45 | 1.50 | 7.57 |
| Error | 7.72 | 8 | 0.97 | ---- | ---- |

# APPENDIX C

## ASSAY FOR INHIBITORY ACTION OF SPENT BROTH

TABLE XVIII

ASSAY FOR INHIBITORY ACTION OF SPENT BROTH OF *L. ACIDOPHILUS* NCFM GROWING IN MRS IN FERMENTORS WITH DIFFERENT CONDITIONS
INCREASE IN $A_{620}$ nm IN ASSAY TUBES

| Growth Conditions | Sampling Time (h) | Increase in $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| Control | ---- | 0.05 | 0.16 | 0.44 | 0.68 | 1.05 | 1.16 | 1.38 |
| | | 0.01 | 0.03 | 0.07 | 0.15 | 0.32 | 0.81 | 1.28 |
| | | 0.04 | 0.11 | 0.26 | 0.49 | 0.76 | 0.97 | 1.39 |
| | | 0.04 | 0.13 | 0.31 | 0.63 | 0.91 | 1.08 | 1.36 |
| Average | | 0.03 | 0.11 | 0.27 | 0.49 | 0.76 | 1.00 | 1.36 |
| pH 6.0 | 0 | 0.05 | 0.16 | 0.46 | 0.68 | 1.05 | 1.26 | 1.38 |
| | | 0.01 | 0.03 | 0.07 | 0.15 | 0.34 | 0.65 | 1.28 |
| | | 0.04 | 0.11 | 0.27 | 0.49 | 0.76 | 0.97 | 1.39 |
| Average | | 0.03 | 0.10 | 0.27 | 0.44 | 0.72 | 0.96 | 1.35 |
| | 3 | 0.04 | 0.15 | 0.45 | 0.70 | 1.03 | 1.24 | 1.37 |
| | | 0.01 | 0.03 | 0.07 | 0.16 | 0.34 | 0.65 | 1.28 |
| | | 0.04 | 0.14 | 0.23 | 0.49 | 0.76 | 0.97 | 1.39 |
| Average | | 0.03 | 0.11 | 0.25 | 0.45 | 0.71 | 0.95 | 1.35 |
| pH 6.0 | 6 | 0.05 | 0.16 | 0.46 | 0.67 | 1.03 | 1.19 | 1.37 |
| | | 0.01 | 0.03 | 0.07 | 0.16 | 0.35 | 0.71 | 1.28 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase in $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| | | 0.04 | 0.12 | 0.25 | 0.52 | 0.75 | 0.96 | 1.39 |
| Average | | 0.03 | 0.10 | 0.26 | 0.45 | 0.71 | 0.95 | 1.35 |
| | 9 | 0.04 | 0.17 | 0.38 | 0.62 | 0.95 | 1.15 | 1.37 |
| | | 0.01 | 0.03 | 0.07 | 0.16 | 0.35 | 0.66 | 1.28 |
| | | 0.04 | 0.12 | 0.22 | 0.42 | 0.67 | 0.87 | 1.40 |
| Average | | 0.45 | 0.11 | 0.22 | 0.40 | 0.66 | 0.89 | 1.35 |
| | 12 | 0.01 | 0.02 | 0.03 | 0.04 | 0.07 | 0.09 | 1.38 |
| | | -0.002 | 0.003 | 0.01 | 0.014 | 0.03 | 0.045 | 1.28 |
| | | -0.006 | 0 | 0.006 | 0.02 | 0.04 | 0.07 | 1.39 |
| | | -0.018 | -0.018 | -0.015 | -0.009 | -0.003 | 0 | 1.26 |
| Average | | -0.004 | 0.001 | 0.008 | 0.016 | 0.03 | 0.05 | 1.33 |
| | 15 | -0.006 | -0.006 | -0.003 | -0.003 | 0.00 | 0.00 | 1.37 |
| | | -0.006 | -0.003 | 0.00 | 0.00 | 0.000 | 0.01 | 1.28 |
| | | -0.006 | 0.003 | 0.01 | 0.03 | 0.06 | 0.11 | 1.39 |
| Average | | -0.006 | -0.002 | 0.002 | 0.009 | 0.02 | 0.04 | 1.35 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase in $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| pH 6.0 | 18 | -0.006 | -0.006 | -0.006 | 0.00 | 0.00 | 0.00 | 1.37 |
| | | 0.00 | 0.00 | 0.006 | 0.018 | 0.023 | 0.05 | 1.28 |
| | | 0.00 | 0.02 | 0.03 | 0.06 | 0.11 | 0.19 | 1.39 |
| Average | | -0.002 | 0.005 | 0.01 | 0.03 | 0.04 | 0.08 | 1.35 |
| | 21 | 0.006 | 0.01 | 0.018 | 0.018 | 0.02 | 0.03 | 1.38 |
| | | 0.012 | 0.03 | 0.068 | 0.140 | 0.30 | 0.54 | 1.28 |
| | | -0.003 | 0.003 | 0.015 | 0.03 | 0.07 | 0.09 | 1.40 |
| Average | | 0.005 | 0.01 | 0.03 | 0.063 | 0.13 | 0.22 | 1.35 |
| | 24 | 0.02 | 0.04 | 0.09 | 0.13 | 0.24 | 0.39 | 1.38 |
| | | 0.12 | 0.024 | 0.07 | 0.149 | 0.32 | 0.58 | 1.28 |
| | | 0.04 | 0.11 | 0.22 | 0.47 | 0.67 | 0.82 | 1.39 |
| Average | | 0.06 | 0.06 | 0.13 | 0.25 | 0.41 | 0.60 | 1.35 |
| Agitation | 0 | 0.04 | 0.18 | 0.49 | 0.71 | 1.07 | 1.20 | 1.20 |
| | | 0.02 | 0.03 | 0.08 | 0.17 | 0.35 | 0.68 | 1.28 |
| | | 0.05 | 0.13 | 0.26 | 0.53 | 0.76 | 0.97 | 1.39 |
| Average | | 0.04 | 0.11 | 0.30 | 0.47 | 0.73 | 0.95 | 1.29 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase in $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| Agitation | 3 | 0.04 | 0.17 | 0.47 | 0.70 | 1.07 | 1.15 | 1.37 |
| | | 0.01 | 0.04 | 0.08 | 0.19 | 0.36 | 0.68 | 1.28 |
| | | 0.04 | 0.13 | 0.26 | 0.53 | 0.76 | 0.97 | 1.40 |
| Average | | 0.03 | 0.11 | 0.27 | 0.47 | 0.73 | 0.93 | 1.35 |
| | 6 | 0.06 | 0.19 | 0.50 | 0.71 | 1.04 | 1.20 | 1.38 |
| | | 0.01 | 0.024 | 0.07 | 0.18 | 0.34 | 0.70 | 1.27 |
| | | 0.05 | 0.12 | 0.26 | 0.57 | 0.83 | 1.02 | 1.39 |
| Average | | 0.04 | 0.11 | 0.28 | 0.49 | 0.74 | 0.97 | 1.35 |
| | 9 | 0.05 | 0.19 | 0.50 | 0.71 | 1.04 | 1.16 | 1.38 |
| | | 0.04 | 0.06 | 0.07 | 0.18 | 0.38 | 0.67 | 1.27 |
| | | 0.04 | 0.13 | 0.27 | 0.60 | 0.80 | 0.97 | 1.39 |
| Average | | 0.04 | 0.13 | 0.28 | 0.50 | 0.74 | 0.93 | 1.35 |
| | 12 | 0.05 | 0.18 | 0.47 | 0.68 | 1.04 | 1.14 | 1.37 |
| | | 0.02 | 0.04 | 0.08 | 0.18 | 0.38 | 0.74 | 1.28 |
| | | 0.05 | 0.13 | 0.25 | 0.51 | 0.76 | 0.97 | 1.39 |
| | | 0.04 | 0.15 | 0.34 | 0.65 | 0.95 | 1.16 | 1.39 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase in $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| Average | | 0.04 | 0.12 | 0.29 | 0.51 | 0.78 | 1.00 | 1.36 |
| Agitation | 15 | 0.05 | 0.18 | 0.49 | 0.70 | 1.07 | 1.15 | 1.37 |
| | | 0.01 | 0.04 | 0.08 | 0.18 | 0.35 | 0.70 | 1.28 |
| | | 0.06 | 0.13 | 0.25 | 0.49 | 0.73 | 0.95 | 1.40 |
| Average | | 0.04 | 0.12 | 0.27 | 0.46 | 0.72 | 0.93 | 1.35 |
| | 18 | 0.05 | 0.20 | 0.48 | 0.71 | 1.01 | 1.20 | 1.38 |
| | | 0.01 | 0.03 | 0.07 | 0.18 | 0.38 | 0.68 | 1.28 |
| | | 0.06 | 0.13 | 0.27 | 0.49 | 0.73 | 0.97 | 1.40 |
| Average | | 0.04 | 0.12 | 0.27 | 0.46 | 0.71 | 0.95 | 1.35 |
| | 21 | 0.05 | 0.20 | 0.47 | 0.71 | 1.01 | 1.20 | 1.37 |
| | | 0.01 | 0.04 | 0.08 | 0.17 | 0.38 | 0.68 | 1.28 |
| | | 0.04 | 0.12 | 0.25 | 0.47 | 0.69 | 0.91 | 1.39 |
| Average | | 0.03 | 0.12 | 0.27 | 0.45 | 0.69 | 0.93 | 1.35 |
| | 24 | 0.06 | 0.18 | 0.47 | 0.71 | 1.04 | 1.16 | 1.38 |
| | | 0.01 | 0.03 | 0.07 | 0.17 | 0.38 | 0.65 | 1.28 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| | | 0.04 | 0.11 | 0.24 | 0.47 | 0.70 | 0.32 | 1.39 |
| Average | | 0.04 | 0.11 | 0.26 | 0.45 | 0.71 | 0.88 | 1.35 |
| Static | 0 | 0.05 | 0.19 | 0.52 | 0.76 | 1.08 | 1.20 | 1.38 |
| | | 0.00 | 0.02 | 0.07 | 0.17 | 0.32 | 0.66 | 1.27 |
| | | 0.06 | 0.13 | 0.25 | 0.54 | 0.81 | 0.72 | 1.40 |
| Average | | 0.04 | 0.11 | 0.28 | 0.49 | 0.74 | 0.86 | 1.35 |
| | 3 | 0.05 | 0.19 | 0.51 | 0.74 | 1.07 | 1.15 | 1.37 |
| | | 0.01 | 0.04 | 0.07 | 0.16 | 0.36 | 0.68 | 1.28 |
| | | 0.05 | 0.14 | 0.25 | 0.53 | 0.80 | 0.97 | 1.40 |
| Average | | 0.11 | 0.12 | 0.28 | 0.48 | 0.74 | 0.93 | 1.35 |
| | 6 | 0.06 | 0.20 | 0.51 | 0.74 | 1.07 | 1.25 | 1.37 |
| | | 0.01 | 0.02 | 0.07 | 0.17 | 0.36 | 0.67 | 1.28 |
| | | 0.05 | 0.14 | 0.28 | 0.53 | 0.80 | 0.71 | 1.39 |
| Average | | 0.04 | 0.12 | 0.29 | 0.48 | 0.74 | 0.88 | 1.35 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| | 9 | 0.06 | 0.18 | 0.50 | 0.73 | 1.03 | 1.19 | 1.37 |
| | | 0.01 | 0.03 | 0.08 | 0.17 | 0.35 | 0.68 | 1.28 |
| | | 0.06 | 0.13 | 0.25 | 0.47 | 0.72 | 0.97 | 1.39 |
| Average | | 0.04 | 0.11 | 0.28 | 0.46 | 0.70 | 0.95 | 1.35 |
| Static | 12 | 0.06 | 0.26 | 0.50 | 0.74 | 1.08 | 1.20 | 1.38 |
| | | 0.02 | 0.04 | 0.08 | 0.18 | 0.36 | 0.68 | 1.28 |
| | | 0.05 | 0.14 | 0.27 | 0.53 | 0.76 | 0.97 | 1.40 |
| | | 0.05 | 0.14 | 0.35 | 0.62 | 0.95 | 1.11 | 1.37 |
| Average | | 0.03 | 0.11 | 0.30 | 0.52 | 0.79 | 0.99 | 1.36 |
| | 15 | 0.06 | 0.20 | 0.51 | 0.71 | 1.04 | 1.16 | 1.38 |
| | | 0.02 | 0.04 | 0.08 | 0.17 | 0.36 | 0.68 | 1.28 |
| | | 0.06 | 0.12 | 0.23 | 0.48 | 0.70 | 0.97 | 1.40 |
| Average | | 0.05 | 0.12 | 0.27 | 0.45 | 0.70 | 0.94 | 1.35 |
| | 18 | 0.06 | 0.20 | 0.50 | 0.74 | 1.04 | 1.20 | 1.38 |
| | | 0.02 | 0.03 | 0.08 | 0.17 | 0.37 | 0.70 | 1.28 |
| | | 0.04 | 0.13 | 0.23 | 0.51 | 0.72 | 0.97 | 1.39 |

TABLE XVIII (CONTINUED)

| Growth Conditions | Sampling Time (h) | Increase $A_{620}$ nm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2h | 3h | 4h | 5h | 6h | 7h | 24h |
| Average | | 0.04 | 0.12 | 0.27 | 0.47 | 0.71 | 0.96 | 1.35 |
| | 21 | 0.06 | 0.21 | 0.54 | 0.75 | 1.14 | 1.26 | 1.38 |
| | | 0.01 | 0.03 | 0.08 | 0.17 | 0.36 | 0.65 | 1.28 |
| | | 0.04 | 0.14 | 0.26 | 0.47 | 0.70 | 0.97 | 1.39 |
| Average | | 0.04 | 0.13 | 0.29 | 0.46 | 0.73 | 0.96 | 1.35 |
| Static | 24 | 0.06 | 0.19 | 0.50 | 0.71 | 1.012 | 1.20 | 1.38 |
| | | 0.01 | 0.03 | 0.08 | 0.15 | 0.37 | 0.67 | 1.27 |
| | | 0.02 | 0.06 | 0.12 | 0.25 | 0.39 | 0.59 | 1.39 |
| Average | | 0.03 | 0.09 | 0.23 | 0.37 | 0.59 | 0.82 | 1.35 |

TABLE XIX

ANALYSIS OF VARIANCES OF ASSAY FOR INHIBITORY ACTION OF SPENT BROTHS FROM L. ACIDOPHILUS NCFM FOLLOWING 12 H. OF GROWTH UNDER DIFFERENT CONDITIONS: INCREASE $A_{620}$ NM OF 6 H. INCUBATION (ASSAY TUBES) DATA FROM TABLE XVIII

| Source of Variance | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (1% level) |
|---|---|---|---|---|---|
| Total | 4.8202 | 15 | ---- | ---- | ---- |
| Among Treatments | 3.9645 | 3 | 1.3215 | 18.53 | 11.75 |
| Error | 0.8557 | 12 | 0.0713 | ---- | ---- |

Significant at $P < 0.01$; LSD required for differences among means at 5% and 1% levels are 0.4113 and 0.5768 respectively.

# APPENDIX D

## TOTAL AND L(+) LACTATE CONCENTRATIONS IN SPENT BROTH FROM *L. ACIDOPHILUS* NCFM GROWING IN MRS IN FERMENTORS MAINTAINED UNDER DIFFERENT CONDITIONS

TABLE XX

TOTAL AND L(+) LACTATE AT 12 H. INCUBATION IN FERMENTORS MAINTAINED UNDER DIFFERENT CONDITIONS

| Fermentor Conditions | Total Lactate $\mu g\ ml^{-1}$ Trial I | II | III | Mean | L(+) Lactate $\mu g\ ml^{-1}$ Trial I | II | III | Mean |
|---|---|---|---|---|---|---|---|---|
| pH 6.0 | 18,750 | 17,000 | 16,625 | 17,450 | 14,562 | 15,750 | 16,000 | 15,437 |
| Agitated | 9,875 | 10,000 | 10,000 | 9,958 | 7,312 | 7,250 | 6,750 | 7,104 |
| Static | 10,875 | 11,500 | 11,500 | 11,291 | 8,625 | 9,000 | 8,750 | 8,792 |

TABLE XXI

ANALYSIS OF VARIANCES OF TOTAL LACTATE CONCENTRATIONS IN 12 H. SPENT BROTH FROM L. ACIDOPHILUS NCFM GROWING IN MRS IN FERMENTORS MAINTAINED AT DIFFERENT CONDITIONS

| Source of Variance | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (1% level) |
|---|---|---|---|---|---|
| Total | 98,899,305 | 8 | ---- | ---- | ---- |
| Among Treatments | 96,055,555 | 2 | 48,027,777.50 | 101.33 | 14.54 |
| Error | 2,843,750 | 6 | 473,958.33 | ---- | ---- |

Significant at $P < 0.01$; LSD required for differences among means at 5% level equal 1,375.

TABLE XXII

ANALYSIS OF VARIANCES OF L(+) LACTATE CONCENTRATIONS IN 12 H. SPENT BROTH FROM L. ACIDOPHILUS NCFM GROWING IN MRS IN FERMENTORS MAINTAINED AT DIFFERENT CONDITIONS

| Source of Variance | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (1% level) |
|---|---|---|---|---|---|
| Total | 117,900,924 | 8 | ---- | ---- | ---- |
| Among Treatments | 116,457,548 | 2 | 58,226,774 | 242.02 | 14.54 |
| Error | 1,443,376 | 6 | 240,586 | ---- | ---- |

Significant at $P < 0.01$; LSD required for differences among means at 5% level equal 980.

# APPENDIX E

## EFFECT OF PH ON INHIBITORY ACTIVITY OF SPENT BROTH--TUBE ASSAYS

TABLE XXIII

INFLUENCE OF PH ON INHIBITORY ACTIVITY OF 12 H. SPENT BROTH FROM L. ACIDOPHILUS NCFM GROWN IN MRS BROTH AT PH 6.0

| | | pH | | | | |
|---|---|---|---|---|---|---|
| Time | Sample | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 |
| 1 | Control | 0.0105 | -0.0015 | 0.0065 | -0.0585 | -0.0030 |
| | S.B[A] | -0.0015 | -0.0035 | 0.0030 | -0.0030 | 0.0060 |
| 2 | Control | 0.3900 | 0.0310 | 0.0645 | -0.0585 | -0.003 |
| | S.B | 0.0065 | 0.0030 | -0.0005 | -0.0030 | 0.0060 |
| 3 | Control | 0.0790 | 0.0890 | 0.1705 | 0.5175 | 0.1630 |
| | S.B | 0.0210 | 0.0125 | -0.0005 | 0.0150 | 0.0360 |
| 4 | Control | 0.1415 | 0.1940 | 0.3755 | 0.4035 | 0.3610 |
| | S.B | 0.0385 | 0.0265 | 0.0095 | 0.0340 | 0.0460 |
| 5 | Control | 0.2480 | 0.3475 | 0.6555 | 0.6650 | 0.6400 |
| | S.B | 0.0720 | 0.0520 | 0.0155 | 0.0675 | 0.0550 |
| 6 | Control | 0.3875 | 0.5960 | 0.9460 | 0.9075 | 0.8645 |
| | S.B | 0.1150 | 0.1075 | 0.0355 | 0.1570 | 0.0770 |
| 7 | Control | 0.5800 | 0.8385 | 1.1070 | 1.0940 | 1.0510 |
| | S.B | 0.1940 | 0.2015 | 0.0750 | 0.2430 | 0.1075 |
| 8 | Control | 0.7585 | 0.9905 | 1.1985 | 1.2045 | 1.1790 |
| | S.B | 0.3160 | 0.3385 | 0.1415 | 0.3750 | 0.1655 |
| 9 | Control | 0.9440 | 1.1330 | 1.2735 | 1.2590 | 1.2015 |
| | S.B | 0.4680 | 0.5305 | 0.2655 | 0.5080 | 0.2640 |

TABLE XXIII (CONTINUED)

| | | pH | | | | |
|---|---|---|---|---|---|---|
| Time | Sample | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 |
| 10 | Control | 1.1075 | 1.2525 | 1.2735 | 1.2885 | 1.2305 |
| | S.B | 0.6310 | 0.7390 | 0.4215 | 0.5480 | 0.3935 |

[A]S.B stands for Spent Broth additions.
Results are average of two trials.

# APPENDIX F

## EFFECT OF HEAT ON INHIBITORY SUBSTANCE IN 12 H. SPENT BROTH

TABLE XXIV

EFFECT OF HEAT ON INHIBITORY ACTIVITY OF BROTH FROM L. ACIDOPHILUS NCFM GROWN IN MRS BROTH AT PH 6.0 INCREASE IN $A_{620}$ NM AT 6 H. IN TUBE ASSAY

| | Control | | | | Untreated Spent Broth | | | | Heated Spent Broth [A] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | I | II | III | Average | I | II | III | Average | I | II | III | Average |
| 2 | 0.026 | 0.012 | 0.025 | 0.021 | -0.016 | -0.012 | -0.009 | -0.012 | -0.016 | -0.015 | -0.015 | -0.015 |
| 3 | 0.083 | 0.060 | 0.104 | 0.082 | -0.016 | -0.009 | -0.009 | -0.011 | -0.009 | -0.006 | -0.009 | -0.008 |
| 4 | 0.237 | 0.164 | 0.253 | 0.218 | -0.009 | -0.009 | -0.009 | -0.009 | -0.006 | -0.006 | -0.006 | -0.006 |
| 5 | 0.496 | 0.358 | 0.518 | 0.457 | -0.009 | -0.009 | -0.009 | -0.009 | -0.006 | -0.006 | -0.012 | -0.008 |
| 6 | 0.760 | 0.660 | 0.906 | 0.775 | -0.009 | -0.006 | -0.009 | -0.008 | -0.006 | -0.003 | -0.012 | -0.007 |
| 7 | 0.909 | 0.910 | 1.047 | 0.960 | -0.009 | -0.006 | -0.009 | -0.008 | -0.003 | -0.00002 | -0.009 | -0.004 |
| 8 | 0.994 | 0.960 | 1.1613 | 1.040 | -0.006 | -0.006 | -0.009 | -0.007 | 0.000 | 0.000 | -0.006 | -0.002 |
| 24 | 1.186 | 1.210 | 1.316 | 1.237 | 1.058 | 1.164 | 1.123 | 1.115 | 1.115 | 1.120 | 1.158 | 1.139 |

[A] Heated 15 minutes at 121°C

TABLE XXV

ANALYSIS OF VARIANCE OF 6 H. READING IN TUBE ASSAYS
OF DATA FROM TABLE XXIV

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Square | $F_{ratio}$ | Critical F (1% level) |
|---|---|---|---|---|---|
| Total | 1.25357 | 8 | ---- | ---- | ---- |
| Among Treatments | 1.22281 | 2 | 0.61140 | 119.18 | 14.54 |
| Error | 0.03076 | 6 | 0.00513 | ---- | ---- |

LSD ($P < 0.001$) indicated significant differences between control (no inhibitory spent broth added) and untreated (inhibitory spent broth not heated) and treated (inhibitory spent broth heated) samples. However no significant differences were found between treated and untreated samples ($P > 0.05$).

# APPENDIX G

## EFFECT OF ENZYME TREATMENT ON INHIBITORY ACTION OF SPENT BROTH

TABLE XXVI

GROWTH OF L. ACIDOPHILUS NCFM IN THE PRESENCE OF ENZYME TREATED OR UNTREATED SPENT BROTH

| Sample Treatment | Trial | Increase in $A_{620}$ nm[A] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1h | 2h | 3h | 4h | 5h | 6h |
| Control[B] | 1 | 0.000 | 0.024 | 0.091 | 0.251 | 0.537 | 0.828 |
| | 2 | 0.006 | 0.030 | 0.105 | 0.280 | 0.535 | 0.857 |
| | 3 | 0.000 | 0.030 | 0.084 | 0.249 | 0.471 | 0.809 |
| Average | | 0.002 | 0.028 | 0.093 | 0.260 | 0.514 | 0.831 |
| No Catalase | 1 | -0.009 | -0.005 | -0.009 | -0.003 | -0.003 | -0.003 |
| | 2 | 0.000 | 0.003 | 0.009 | 0.018 | 0.034 | 0.062 |
| | 3 | 0.000 | 0.000 | 0.000 | 0.009 | 0.019 | 0.038 |
| Average | | -0.003 | -0.001 | 0.000 | 0.009 | 0.017 | 0.032 |
| Catalase | 1 | -0.015 | -0.014 | -0.009 | -0.010 | -0.010 | -0.015 |
| | 2 | -0.003 | 0.003 | 0.003 | 0.012 | 0.023 | 0.042 |
| | 3 | 0.000 | 0.000 | 0.000 | 0.003 | 0.006 | 0.016 |
| Average | | -0.006 | -0.004 | -0.002 | 0.002 | 0.008 | 0.014 |
| No Trypsin | 1 | -0.012 | -0.010 | -0.010 | -0.010 | -0.010 | -0.010 |
| | 2 | 0.000 | 0.006 | 0.022 | 0.049 | 0.095 | 0.208 |
| | 3 | 0.000 | 0.000 | 0.006 | 0.025 | 0.045 | 0.079 |
| Average | | -0.004 | -0.001 | 0.006 | 0.025 | 0.043 | 0.092 |
| Trypsin | 1 | -0.010 | 0.020 | 0.085 | 0.225 | 0.515 | 0.822 |
| | 2 | 0.003 | 0.030 | 0.097 | 0.255 | 0.514 | 0.857 |

TABLE XXVI (CONTINUED)

| Sample Treatment | Trial | Increase in $A_{620}$ nm | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1h | 2h | 3h | 4h | 5h | 6h |
| | 3 | 0.003 | 0.022 | 0.092 | 0.220 | 0.433 | 0.787 |
| Average | | -0.001 | 0.024 | 0.091 | 0.233 | 0.487 | 0.822 |
| No Pepsin | 1 | -0.012 | -0.015 | -0.010 | -0.012 | -0.012 | -0.010 |
| | 2 | 0.000 | 0.009 | 0.012 | 0.051 | 0.100 | 0.189 |
| | 3 | -0.003 | 0.000 | 0.006 | 0.025 | 0.055 | 0.103 |
| Average | | -0.005 | -0.002 | 0.003 | 0.021 | 0.048 | 0.094 |
| Pepsin | 1 | -0.010 | 0.020 | 0.092 | 0.260 | 0.540 | 0.860 |
| | 2 | 0.000 | 0.041 | 0.126 | 0.269 | 0.553 | 0.854 |
| | 3 | 0.000 | 0.025 | 0.099 | 0.249 | 0.453 | 0.772 |
| Average | | -0.003 | 0.029 | 0.106 | 0.259 | 0.515 | 0.829 |

[A] Results from three trials.

[B] Control contain no spent broth.

TABLE XXVII

ANALYSIS OF VARIANCES OF INCREASE $A_{620}$ NM AT 6 H. GROWTH OF L. ACIDOPHILUS NCFM GROWING IN SPENT BROTH TREATED AND UNTREATED WITH ENZYMES IN ASSAY TUBES--DATA FROM TABLE XXIX

| Source of Variation | Sum of Squares | Degrees of Freedom | Mean Squares | $F_{ratio}$ | Critical F (1% level) |
|---|---|---|---|---|---|
| Total | 3.117751 | 20 | ---- | ---- | ---- |
| Among Treatments | 3.059822 | 2 | 1.529911 | 475.4229 | 7.21 |
| Error | 0.057929 | 18 | 0.003218 | ---- | ---- |

Significant at $P < 0.01$; LSD required for differences among means at 5% and 1% levels are 0.0973 and 0.1333 respectively.

# VITA

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