BATCH CULTURE STUDIES OF *BIFIDOBACTERIUM BIFIDUM*

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A thesis presented in partial fulfilment of the requirements for the degree of Master of Technology in Biotechnology and Bioprocess Engineering

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ABSTRACT

The growth of *Bifidobacterium bifidus* on batch culture was investigated. Growth was studied with regard to growth rates, substrate utilisation rates, biomass yields and product formation rates. The organism was cultured on a variety of substrates and at different pH values. The objective was to determine whether different sugars or different medium pH values had any effect on specific growth rates, substrate utilisation rates, biomass yields and product formation rates.

B. bifidum (Hansen's strain) from a stock culture obtained from New Zealand Dairy Research Institute (DRI) was used for this study. The organism was grown on TPY medium supplemented with L-cysteine-HCl, sodium chloride, potassium chloride, magnesium chloride, potassium di-hydrogen phosphate, di-potassium phosphate and ammonium chloride. The experiments were carried out at uncontrolled and controlled pH.

At controlled pH, maximum growth of this organism was obtained on medium containing mixed sugars (glucose and lactose) at pH 5.5. On single carbohydrate sources, glucose, maltose and raffinose were readily utilised by this organism to give high rates of growth and formation of products. Very high concentrations of lactose were growth limiting. Lactulose and cellobiose were fermented but with low specific growth and product formation rates, while xylose was not fermented at all.

Growth of *B. bifidum* was pH-dependent. The pH below which this organism did not grow was $3.5 \leq pH \prec 4.1$. Optimum growth occurred at $4.9 \geq pH \geq 6.5$. It was demonstrated

that the A: L (acetic acid to lactic acid) ratio of products was different at different pH.

Accumulation of the fermentation products, in particular, acetic acid, caused limitations on growth. It was demonstrated that this inhibition was pH-dependent. Inhibition was higher at lower pH than at higher pH, and this was a result of the degree of dissociation of the acid at the different pH values.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Associate Professor Ian Maddox for giving the opportunity to work with him in this study. His constant guidance, supervision and encouragement was greatly appreciated.

I would also like to thank my co-supervisor, Dr. Noemi Gutierrez, for her guidance and supervision. I am deeply grateful to her assistance.

I would also like to thank Professor R. L. Earle (former Head of Department of Process and Environmental Technology) for allowing me the opportunity to do this work.

I am also grateful to the New Zealand Ministry of Foreign Affairs and Trade for giving me the scholarship during the period of this study.

I would also like to extend my appreciation and gratitude to the all the people who provided the technical and laboratory assistance, namely. Mrs. A-M Jackson, Mr. J Sykes, Mr. M Sahayam, Mr. M. Stevens, Mr. J. Alger, Mr. D. Maclean and Mr. B. Collins.

I would also like to thank my dear friends, Samuel Oppong for his friendship, encouragement and support, Kathy and Sam Hansen for their greatly appreciated friendship and support throughout my stay in New Zealand.

Last but not least, I would like to express my deepest love and gratitude to my parents,whose love, support and encouragement were felt from so many miles away.

TABLE OF CONTENTS **PAGE**

List of Tables

List of figures

CHAPTER 1 INTRODUCTION

Micro-organisms evoke mixed opinions among people. They have an alarming or disgusting aura surrounding them, so much so that any suggestion that the majority of them are trivial or even beneficial is rarely understood. However, this is so.

They play an important part in the cyclical changes that the biological elements undergo on earth. Sewage treatment systems depend on them, but out of control they can be destructive. They ferment our foods, but foul food is dangerous. They aid our digestion, but upset our intestines if they get out of control. This is to say that some are good and some are bad. Of particular importance in this study are the Bifidobacteria.

Bifidobacteria are indigenous gut organisms which are well adapted for metabolism in the gastro-intestinal tract of humans and animals (Biavati and Matarelli, 1991). They constitute over 25% of the intestinal flora in healthy breast-fed infants, while *Lactococci*, *Enterococci*, and coliforms represent less than 1% of the population (Rasic, 1983).

The first organism of the bifidobacteria was isolated from the faeces of breast fed infants by Tissier, who then called it *Lactobacillus bifidus*. Since their discovery by Tissier in 1900, a lot of attention has been directed to the habitat, nutritional and immunological role, biochemistry and taxonomy of these organisms. In the past there was confusion in the taxonomy of bifidobacteria. However recent progress in the definitive speciation of this genus has facilitated

more detailed micro-ecological research into these organisms (Mitsuoka, 1984). At present, bifidobacteria are reputed to play a very important role in nutrition and resistance to infection in breast fed infants. Within the last decade, considerable interest has developed among researchers, in the use of Bifidobacteria in foods, pharmaceutical and feed product application. These organisms play a role in controlling the pH of the contents of the large intestine through the production of lactic and acetic acids, which in turn prohibit the growth of many potential pathogens (Rasic, 1983).

Bullen et al. (1977) found that enteric infections in children can be prevented or minimised when their intestinal tract contains high levels of bifidobacteria. Recent research has focused on establishing these bacteria as major influences on certain normal functions of the intestinal tract and in exploring their role in human health and disease.

Bifidobacteria are used as dietary adjuncts or as starter cultures for yoghurt and other cultured dairy products with the idea that such products may help the promotion of health. The effects of the daily intake of such products are reported as (1) to suppress the putrefactive bacteria as well as putrefaction, so as to prevent constipation and geriatric diseases, including cancer; (2) to prevent and treat antibiotic-associated diarrhoea, and (3) to stimulate the immune response, therefore contributing to a greater resistance to infection (Mitsuoka, 1992). Due to the reputed therapeutic effects of these organisms, the Japanese have taken an active involvement in the development and application of these organisms in foods (Mitsuoka, 1982; Yamakazi et al., 1985). In Japan, successful treatment of diarrhoeal diseases in children is accomplished by

feeding dairy products containing large numbers of bifidobacteria (Mitsuoka, 1982). It has also been found that the feeding of bifidobacteria, in conjunction with antibiotic therapy, can correct intestinal conditions such as upset or irritable stomach (Hotta et al., 1987). Some workers believe these organisms have anti-carcinogenic (Mitsuoka, 1982) as well as anti-cholesterolemic (Hata et al., 1982) properties. Since 1986, the traditional microflora of yoghurt, *Streptococcus salivarius* spp *thermophilus* and *Lactobacillus delbruickii* spp *bulgaricus*, has been enhanced by a third bacterium belonging to the genus *Bifidobacterium*, and sometimes associated with *Lactobacillus acidophilus* (Ballongue, 1993).

Clearly, not only are these organisms beneficial to mankind, but they are also unique. It is for these reasons that they have drawn such an interest in researchers. However, little is known about their physiology.

1.2 OBJECTIVES OF THE STUDY

The objective of this research is to study the growth of batch cultures of a selected strain of Bifidobacteria. Growth will be conducted on a variety of substrates, such as glucose, lactose, oligosaccharides, pectin and starch with regard to growth rates, substrate utilisation rates, product formation rates, biomass yields, and product yields. The objective of using different substrates is to determine if the type of sugar used has any effect on growth rates and product yields.

CHAPTER 2 LITERATURE REVIEW

2.1 DESCRIPTION

2.1.1 Historical background

In 1900, Tissier discovered an organism, in the faeces of infants, that became known as *Bacillus bifidus communis*, (Ballongue, 1993). At about the same time, Moro, in Italy, discovered, under similar conditions, an organism which he recognised as being different to that of Tissier, and which he identified as belonging to the genus *Lactobacillus*. Because of the differences, this new organism was named *Lactobacillus bifidus* (Ballongue, 1993). It was not until 1924 that Orla-Jensen described the genus *Bifidobacterium* and *Bacillus bifidus communis* was transferred into this genus and became known as *Bifidobacterium bifidum* (Mitsuoka, 1982;). Despite this transfer, the name *Lactobacillus bifidus* was reported in the seventh edition of Bergey's Manual (1957). Thus, for many years, bifidobacteria were included in the genus *Lactobacillus* as *Lactobacillus bifidus*, but they are now classified in a separate genus *Bifidobacterium,* as suggested by Orla-Jensen, since the 8th edition of the Bergey's Manual, 1974, (Modler et al, 1990), on the basis of their characteristic morphology, biochemical characters, cell wall constituents, and DNA base composition (Mitsuoka and Kaneuchi, 1977; Scardovi, 1986; Biavati et al., 1991).

In the past, several workers recognised only one species in the genus *Bifidobacterium*

(Mitsuoka et al., 1977), but on the basis of carbohydrate fermentation, several more species and biotypes were identified. Since 1957, when Dehnert recognised the existence of multiple biotypes of bifidobacteria (Mitsuoka, 1984), the taxonomy of these organisms has been a subject of great interest.

Early distinction between species of this genus was based on carbohydrate fermentation patterns. By 1963, Reuter proposed seven members of the genus *Bifidobacterium* and he differentiated these on the basis of fermentation and serological features (Scardovi et al., 1969; Matteuzi et al., 1971; Scardovi and Crociani, 1974; Scardovi and Zani, 1974; Trovatelli et al., 1974; Mitsuoka, 1977). Since 1970, this genus has further expanded, mainly through the use of DNA/DNA hybridisation or polyacrylamide gel electrophoresis of sodium dodecyl sulphate solubilised whole cell protein extracts (SDS-PAGE) (Matteuzi, 1971; Biavati et al., 1982; Mitsuoka, 1990). Through the use of this technique, eleven (11) different species can be described (Ballongue, 1993) and it soon came to light that some species were synonymous, e.g., *B. lactentis*, *B. liberorum* and *B. infantis* have been combined to form a single species under the name, *B. infantis* and *B. breve* and *B. parvulorum* came under a single name of *B. breve* (Scardovi et al., 1971).

In the last few years, more species have been described . Twenty four (24) species are classified under this genus in Bergey's Manual (Scardovi, 1986). However, more species have been added to the genus since then. At present twenty nine (29) species are now validly recognised, of which ten (10) are of human origin, fourteen (14) of warm blooded animal origin, three (3) from

honey bees and two (2) have been isolated from sewage (Ballongue, 1993).

2.1.2 Morphology

Bifidobacteria are Gram positive bacteria of varying morphology. They are rods of various shapes; club shaped or spatulated - single or in chains of many elements, in star-like aggregates or disposed in 'V' or palisade arrangement. They are non-acid-fast, non-sporing and non-motile. These organisms are anaerobic though some species can tolerate oxygen (O_2), but only in the presence of carbon dioxide (CO_2). The colonies are smooth, convex, entire edges, cream to white, glistening and of soft consistency (Scardovi, 1986).

2.1.3 Identification

Methods of identification of species of *Bifidobacterium* are still not completely reliable (Mitsuoka, 1984). Morphology of these organisms provides little help in their identification at genus level. This is because of the similarities they have with *Lactobacillus*. However, on the species level, morphology may be more relevant as a tool of identification, as many species show unusual and unique morphological traits. Such traits include disposition and number of branchings, cell contours, dimensions, and arrangement in groups, and are characteristic for many *Bifidobacterium* species growing in trypticase- peptone-yeast extract (TPY) medium (Biavati et al., 1991).

Cultural characteristics are similar to other bacteria such as *Lactobacillus*, *Corynebacterium*,

and *Actinomyces*. However, *Bifidobacterium* ferments hexose through a special metabolic pathway, the so-called bifid shunt, with fructose 6-phosphate phosphoketolase as the characteristic key enzyme. This enzyme seems to be absent when other anaerobic Gram positive bacteria with bifid-like morphology (*Actinomyces*, *Arthrobacter*, *Corynebacterium* and *Propionibacterium*) are tested with fructose 6-phosphate as the substrate (Scardovi and Trovatelli, 1965). Therefore the presence of this enzyme, fructose 6-phosphate phosphoketolase, in cellular extracts is the most reliable characteristic for identification of the genus, *Bifidobacterium*. The presence of this enzyme is demonstrated by a colour reaction (Scardovi, 1986; Biavati et al., 1991) or by starch gel electrophoresis (Scardovi et al., 1971). The starch gel electrophoresis method allows for the discrimination of three different types of *Bifidobacterium* - the animal, the human, and the honey bee types.

Identification of the fermentation products is an alternative method of primary differentiation of Bifidobacteria from related groups (Biavati et al., 1991). This approach involves the determination of organic acids at the end of fermentation, notably acetic acid and lactic acid. They are normally produced in the ratio 3:2. In addition, species of the genus Bifidobacterium produce the L+ isomer of lactic acid (Ballongue, 1993). However, measurement of fermentation end-products is sometimes difficult to interpret, as side reactions give variable amounts of formic and succinic acids, as well as ethanol, and so give an unexpected pattern (Biavati et al., 1991).

Protein profiles of species of *Bifidobacterium* show the presence of a genus-specific protein

band, which migrates the same distance for all species, except *B. boum*, where the distance of migration is slightly greater from the anode (Biavati et al., 1982). The presence of this band can therefore be used as a clear indication that an unknown organism belongs to this genus.

Analysis of the cell composition with regard to lipids and phospholipids of the cell wall and membrane is a good criterion for differentiating between the genus *Bifidobacterium* and *Lactobacillus*. Both genera contain disphosphatidylglycerol and phosphatidylglycerol. However, only *Bifidobactrium* contains polyglycerolphospholipids and their lyso-derivatives, alanylphosphatidylglyceol, and the lyso-derivatives of diphosphatidylglycerol (Ballongue, 1993).

Species identification is generally accomplished by isozyme patterns in starch gels for transaldolase and 6-phosphogluconate dehydrogenase (Biavati, et al., 1991), supplemented with either a limited number of fermentation tests, or with the electrophoretic migration of 3-phosphoglyceraldehyde dehydrogenase (Mitsuoka and Kaneuchi, 1977; Ballongue, 1993).

Another important parameter in the identification of bifidobacteria is the murein type of the cell wall (Lauer and Kandler, 1983). The type of peptidoglycan allows discrimination of closely related species (Biaviati et al., 1991). DNA/DNA hybridisation is also important in identification of these organisms (Scardovi et al., 1979). However, this method is laborious and cannot be carried out on a routine basis in most laboratories, as it needs a large number of reference DNAs.

Recent studies show that phenotypic characterisation of *Bifidobacterium* strains can help in resolving the taxonomic problems encountered in this genus (Biavati et al., 1991), although they will probably never allow clear-cut discrimination of all present and future species.

2.2 ECOLOGY

Bifidobacteria have been isolated from diverse ecological niches. These include faeces of humans, the rumen of cattle, sewage, the human vagina, dental caries, and the honey bee intestines (Biavati et al., 1991). These organisms are also found in the intestines of various other animals, including chickens, dogs, pigs, mice, rats, hamsters, rabbits, horses, and monkeys (Mitsuoka, 1977). However, the numbers and consistency varies from animal to animal. In the intestinal tract of human adults and animals, bifidobacteria coexist with a large variety of bacteria, most of which are obligate anaerobes (Biavati et al., 1991).

2.2.1 Bifidobacteria in humans

Bifidobacterium constitutes a numerically major part of the faecal flora of various age groups of healthy humans, (Mitsuoka and Kaneuchi, 1977). These investigators found that *Bifidobacterium* appeared on the second to the fifth day of life and continued to be one of the most numerous bacteria amounting to about 10^{10}/g of stools, although in children, adults, and senile men, anaerobic bacteria such as bacteroidaceae, eubacteria, anaerobic lactobacilli and

anaerobic Gram-positive cocci were more numerous and usually outnumbered bifidobacteria. In elderly persons, the occurrence and numbers of bifidobacteria decrease significantly or disappear (Mitsuoka, 1990).

Since these organism were discovered, it was generally believed that they were found exclusively in the faeces of breast-fed infants whereas in bottle-fed infants *Lactobacillus acidophillus* was the most numerous organism. However, studies have shown that the differences in the occurrence and numbers of bifidobacteria between breast fed and bottle fed infants were not significant (Mitsuoka and Kaneuchi, 1977). Bifidobacteria were found present in all of 30 breast fed and 29 of 30 of bottle fed infants, with approximately the same mean counts (Scardovi, 1986). This author found that the principal difference between breast fed and bottle fed infants was that the number of Enterobacteriaceae, streptococci and other anaerobes were present in significantly greater numbers in bottle fed infants than in breast fed infants.

There are definite differences in the *Bifidobacterium* species isolated from humans of different age groups and from animals (Mitsuoka, 1992). These differences are shown in Table 1. The infant type *Bifidobacterium* species are never isolated from children or adults, and animal type *Bifidobacterium* species are never isolated from the intestines of humans (Mitsuoka, 1990). The most common bifidobacteria isolated from infants belong to *B. infantis* and *B. breve*. *Bifidobacterium bifidum*, *B. longum*, and *B. adolescentis* are occasionally isolated from infants. In contrast, *B. adolescentis* and *B. longum* are found in high numbers in the intestines of children, adults and elderly people (Mitsuoka, 1990).

Bifidobacterium longum and *B. adolescentis* biovars A and B are found in monkeys, while *B. adolescentis* biovars C and D and *B. pseudolongum* are found in dogs. Most of the pig and cattle strains are identified as *B. pseudolongum* and *B. thermophilum.* Chickens commonly harbour *B. pseudolongum, B. gallinarum,* and *B thermophilum.* In the intestines of mice and rats, *B. pseudologum* and *B. animalis* are found, while guinea pigs and rabbits harbour *B. animalis* and *B. magnum* (Mitsuoka, 1990).

Bifidobacteria can colonise the intestinal tract (Mitsuoka, 1989). Colonisation varies, depending upon the specific bacterium-to-host affinity that also includes individual differences. Many studies have shown that bifidobacterial colonisation sometimes fails in some infants (Tomkins et al., 1981; Stark and Lee, 1982). However, these authors were uncertain as to why some infants and some nurseries are deficient in faecal bifidobacteria. Studies showing bifidus deficiency do not rule out the possibility that the deficiency is caused by the administration of antibiotics to the mother during and after pregnancy (Mitsuoka and Kaneuchi, 1977).

The greatest density of bacteria is found in the distal end of the small intestine, where most of the lactobacilli are found. Bifidobacteria have been found to be more prevalent in the large intestine, especially in the area of the caecum (Cummings and Macfarlane, 1991).

Table 1: Distribution of *Bifidobacterium* species in the human colon

Population	Predominating species	Minor species
Breast-fed infants	*B. longum* *B. infantis* *B. breve*	
Bottle-fed infants	*B. adolescentis*	*B. bifidum* biovar b
Children	*B. infantis,* *B. breve,* *B. bifidum* biovar b, *B. longum*	
Adults	*B. adolescentis* biovars a and b, *B. longum*	*B. bifidum* biovar a
Old aged	*B. adolescentis* biovar b, *B. longum*	

Source: Ballongue, 1993

2.2.2 Colonisation in humans

It is generally believed that at the time of birth, a foetus is completely surrounded by a sterile environment. After birth, the digestive tract is rapidly colonised by bacteria (Mitsuoka et al., 1974; Bezirtgou, 1985). Most bacteria appear within the first 48 hours after birth, but bifidobacteria appear only between 2 and 5 days (Mitsuoka, 1974) and become dominant barely one week after birth at 10^{10}-10^{11}/g of stools. These organisms appear to reach a level of 99% of the faecal flora, while levels of other bacteria decline sharply by up to 1000-fold (Hoffmann, 1966). Most anaerobes and other putrefying bacteria are greatly reduced or completely disappear.

Studies show that bifidobacteria enter the body of the neonate by the oral route. Mutai and Tanaka (1987) isolated and observed *Bifidobacterium, Propionibacterium, Peptostreptococcus, Fusobacterium, Enterococcus*, *Lactobacillus* and *Enterobacteriaceae* from the mouths of 23 neonates 10 minutes after birth. These neonates were born through the vaginal tract. Of the 9 neonates born by caesarean birth that were investigated, only *Propionibacterium* and *Enterococcus* were isolated in 8 of the 9. Mitsuoka et al. (1974) isolated bifidobacteria in 41% of infants born by the genital route, while Bezirtglou (1985) found them in 21% of infants aged 4 days and born by the caesarean route. However, this author reported an increase to 41% 15 days after birth.

There appears to be several factors, besides the method of delivery, which influence colonisation of neonates by bifidobacteria . These are;

(i) Prematurity

It appears that bifidobacteria find it difficult to implant at this stage, due to a lack of receptors and/or endogenous substrates. On the other hand, enterobacteria and Bacteroides readily colonise the colon (Hoffmann, 1966; Stark and Lee, 1982; Stevenson et al., 1985).

(ii) Method of feeding

Bifidobacteria are believed not to be present in mother's milk (Bezirtglou, 1985). However, Mayer and Moser (1950) isolated *B. bifidum* from the colostrum and milk of a mother just before she breast fed (Ballongue, 1993).

Early studies suggested that bifidobacteria were the dominant intestinal flora of breast fed infants while lactobacilli were dominant in bottle fed babies. However, subsequent research disproved this belief. It has been found that there is no apparent difference in the qualitative distribution of species between these two types of feeding. The difference, instead, lies in the quantitative level of the proportion of *Bifidobacterium* to other species (Braun, 1981; Mitsuoka, 1982; Benno et al., 1984; Moreau et al., 1986). Therefore, the main difference lies in the maintenance of high levels of optional aerobic species, *E. coli* and Streptococci, which initially colonise the digestive tract and thus lead to the establishment of anaerobes, *Bacteroides, Clostridium, Eubacteria, Peptostreptococcaceae, Enterobacteriaceae* and *Streptococcus* (Mitsuoka and Kaneuchi, 1977; Mitsuoka, 1989). The appearance of *Bifidobacterium* comes later than this (Neut et al., 1981).

Changes in the faecal flora are observed at weaning. Due to the change in diet, the numbers of bifidobacteria decrease sharply in some children, though remain stable in some (Neut et al., 1985).

Ballongue (1993) suggested that the discrepancies in these findings are due to the different techniques used in identifying the strains. Some workers, especially the Japanese, used carbohydrate fermentation to identify the strains, while the Italians used DNA/DNA hybridisation.

Human milk contains a high concentration of lactose. It also contains lactoferrin and lactulose, a low concentration of proteins used by putrefying bacteria, and it has a low buffering capacity (Bullen et al., 1977). These factors are essential to development of bifidobacteria in the intestines.

In 1930, Polonowski and Lespagnol isolated oligosaccharides other than lactose from human milk, which they named gynolactose and allolactose [-D-galactopyranosyl-(1,6)-D-glucopyranose] (Ballongue, 1993). When Levesque et al. (1960) administered high quantities of N-acetyl-glucosamine to infants with low levels of *B. bifidum*, they observed that this species then appeared in the stools of the infants and then disappeared when the administration was stopped (Ballongue, 1993). Other workers, Inoue and Nagayama (1970) observed that when children were fed milk containing added porcine and mucin, the pH of the stools dropped and the bifidobacteria levels rose. All this seems to suggest that there are numerous substrates involved in maintaining the equilibrium of bifidobacteria in the infant's intestinal flora.

(iii) Endogenous substrates

These are substrates which exist in the digestive tract without a dietary source. They are produced by the host and may be used by bacteria. Hoskins and Boulding (1976) reported that some strict anaerobic bacteria produce enzymes that are able to degrade blood group antigens and mucin oligosaccharides. Included in these bacteria are *B. bifidum* and *B. infantis*. They are able to remove the N-acetyl-D-galactosamine residues from the blood group A factors and also secrete α-L-fucosidases, sialidases and ß-glycosidases (Neutra et al., 1987).

(iv) Environment

Mitsuoka et al. (1974) isolated mainly *B. infantis* from the stools of Japanese infants but 10 years later, *B. breve* was recognised as the dominant species. It is believed that the country, hospital and even the unit within which a delivery takes place influences the rate of colonisation by *Bifidobacterium* (Mitsuoka, 1992). It is suggested that obstetrical and therapeutic customs play a role in the colonisation of neonates by these organisms. It even appears that very strict hygienic conditions delay the implantation of *Bifidobacterium* (Sihmon et al., 1982; Yoshioka et al., 1984; Lundequist et al., 1985).

2.3 PHYSIOLOGY

2.3.1 Respiration

Bifidobacteria are strictly anaerobic, though there is some degree of oxygen-tolerance depending on the species and culture medium (De Vries and Stouthamer, 1969; Scardovi, 1986).

De Vries and Stouthamer (1969) observed that when growth conditions are switched from anaerobic to aerobic, three types of response are observed. First, aerobic growth is observed, with no accumulation of hydrogen peroxide. Generally, a strain of *B. bifidum,* which is aero-tolerant, forms small quantities of hydrogen peroxide by NADH oxidation. The absence of hydrogen peroxide in aerobic culture is explained by the measure of an unknown peroxidase system which can destroy the hydrogen peroxide. Secondly, there is limited growth, with accumulation of hydrogen peroxide. The accumulation of peroxide is considered to be toxic for the fructose 6-phosphoketolase. Finally, no growth is observed, and no accumulation of hydrogen peroxide occurs.

When carbon dioxide is present, the sensitivity to oxygen varies considerably, depending on the strain. Among the strains that are able to grow in the presence of oxygen, some remain catalase negative, and others become catalase positive. For some strains, the presence of catalase is associated with the presence of hemin in the medium (Scardovi, 1986). A study of the

absorption of oxygen by five strains of bifidobacteria of human origin has shown that the partial pressure of oxygen falls in the medium during the multiplication of these strains (Shimamura, 1989). The endogenous absorption of oxygen is linked to the presence of NADH oxidase. It takes place even in the absence of glucose and appears to depend directly on the quantity of polysaccharides accumulated in the cells. All the five strains accumulated hydrogen peroxide which is subsequently reduced by NADH peroxidase though the activity of the enzyme varied according to the strain investigated. The strain most sensitive to oxygen had low NADH peroxidase activity, resulting in the accumulation of toxic hydrogen peroxide.

When Bifidobacteria are cultivated for industrial application, it is important to prevent the toxic effects of oxygen. Prevention of oxygen toxicity is especially crucial during manufacturing and processing of biomass and ensuring cell viability during storage of products containing these organisms. Shimamura et al. (1990) have reported that *Bifidobacterium* species exhibit oxygen uptake which results from reduced NAD-(NADH)-oxidase activity. In a study to elucidate the mechanisms with which enzymatic activities in *Bifidobacterium* species are correlated with the apparent sensitivity to oxygen, Shimamura et al. (1992), found that these organisms express reduced NAD-oxidase and peroxidase activities. These activities function in a pathway for two-electron reduction of molecular oxygen, thus producing hydrogen peroxide, and subsequently water. They concluded that reduced NAD-oxidase and reduced NAD-peroxidase in these organisms play a role in the prevention of oxygen toxicity (Shimamura et al., 1992).

2.3.2 Temperature

The optimum temperature for growth of the species isolated from humans is 36^0-38^0C, and 41^0-43^0C for the species isolated from animals. Some of the latter may even grow at 46.5^0C. Below 20^0C, there is no growth at all, and the organisms show no thermo-resistance above 46^0C. *B. bifidum* dies at 60^0C (Scardovi, 1986; Biavati et al., 1991).

2.3.3 pH

The optimum initial growth pH is 6.5-7.0 and no growth can occur below pH 5.0 or above pH 8.0 (Scardovi, 1986; Biavati et al., 1991).

2.3.4 Metabolism

(a) Sugar metabolism

Hexoses are degraded exclusively and specifically by the fructose 6-phosphate pathway. Fructose 6-phophoketolase is the key enzyme in this pathway, while glucose 6-phosphate hydrogenase is absent (Scardovi and Trovatelli, 1965; De Vries and Stouthamer, 1967). Generally, the fermentation of two glucose molecules leads to the formation of 3 moles of acetate and 2 moles of L(+) lactate. In reality, pyruvic acid can be broken down via two pathways; (i) reduction of the pyruvate to form L(+) Lactate by L(+) dehydrogenase, whose activity is controlled by fructose 1,6-diphosphate, and (ii) the splitting of the pyruvate by phosphoroclastic enzyme to form formic acid and acetyl phosphate, a portion of which is

subsequently reduced to form ethyl alcohol and so generate NAD. The proportions of the products of fermentation vary considerably from one strain to another and even within the same species. Small quantities of succinic acid are produced by some strains and a small amount of carbon dioxide may be produced during the degradation of gluconate (Scardovi, 1986; Biavati et al., 1992) (Figure 1).

In an experiment to determine the fermentation of glucose, lactose, galactose, mannitol and xylose by *Bifidobacterium* species, DeVries and Stouthamer (1969) found that glucose was always fermented completely. Working out the fermentation balances, the authors found that bifidobacteria converted pyruvate from the fructose 6-phosphate phosphoketolase route by two paths. In the first path, pyruvate is reduced to L(+) lactate by the action of L(+) lactate dehydrogenase and requires fructose-1,6-diphosphate for activity. In the second path, cleavage of pyruvate into acetyl phosphate and formate by the phosphoroclastic enzyme takes place. Some of the acetyl phosphate formed is reduced to ethyl alcohol. These authors also found that the amount of pyruvate converted via either pathway is influenced by the growth substrate.

It appears that there are three factors which determine the choice of pathway taken. The first is the relative amounts of phosphoroclastic enzyme and lactate dehydrogenase. A second factor is the amount of fructose-1,6-diphosphate present in the cell. Finally, a different affinity of lactate dehydrogenase and the phosphoroclastic enzyme toward pyruvate, might explain the differences in the fermentation balances between different substrates (De Vries and Stouthamer, 1969).

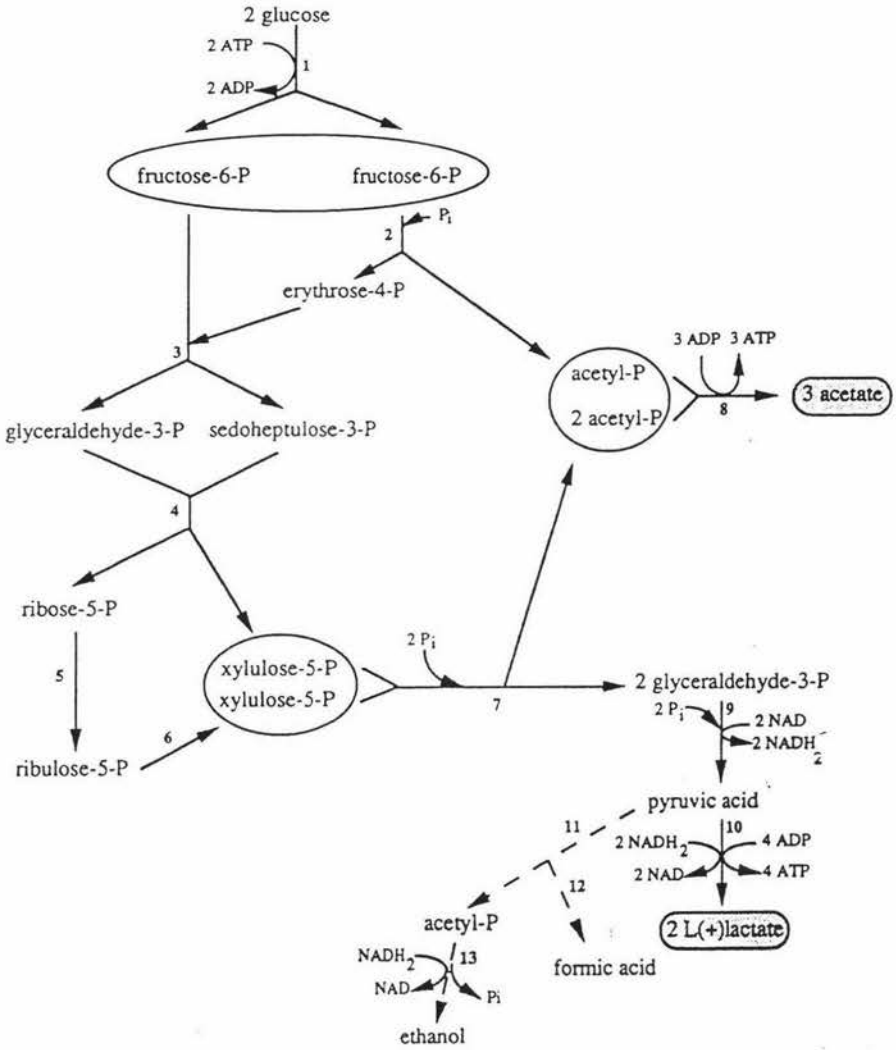

Figure 1: The metabolic pathway of *Bifidobacterium.*

1 = hexokinase and glucose-6-phosphate isomerase; 2 = fructose-6-phosphate phosphoketolase; 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphoketolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase; 11 = phosphoroclastic enzyme; 12 = formate dehydrogenase (EC 1.2.1.2); 13 = alcohol dehydrogenase (EC 1.1.1.1)

Source: Ballongue (1993)

(b) Enzymes

The characteristic enzyme of the sugar metabolism is fructose-6-phosphoketolase (F6PPK) (Scardovi and Trovatelli, 1965; De Vries and Stouthamer, 1967). This enzyme is specific to the genus and is absent from the anaerobic bacteria which could be morphologically confused with the bifidobacteria. According to Biavati et al. (1986), there are three different types of this enzyme, depending on the ecological source of the strain, be it mammalian, bee or man. The animal type has actually been purified (Sgorbati et al., 1976).

In addition, 14 isoenzymes of transaldolase and 29 isoenzymes of 6-phosphogluconate dehydrogenase have been identified (Ballongue, 1993).

(c) Vitamins

Deguchi et al. (1985) have studied and found that bifidobacteria of human origin synthesise six water-soluble vitamins, namely, thiamine(B1); riboflavin (B9); pyridoxine (B6); folic acid (B9); cyanocobalamine (B12) and nicotinic acid (PP) (Table 2). With the exception of riboflavin, all the other five are synthesised by most of the human strains. These authors also observed that the concentration of the vitamin accumulated varied widely among different species or strains. For thiamine, nicotinic and folic acid, the concentration produced was higher in *B. bifidum* and *B. infantis* (higher-accumulators) than in *B. breve*, *B. longum* and *B. adolescentis* (lower-accumulators). In the latter three species, some strains did not accumulate measurable

quantities of the vitamins (non-accumulators).

Table 2: Vitamin production by *Bifidobacterium*

	B. breve	*B. infantis*	*B. longum*	*B. bifidum*	*B. adolescentis*
Thiamin (B1)	+	+++	+	+++	+
Riboflavin (B2)	+	+	+++	++	+
Pyridoxine (B6)	++	++	+++	+	++
Folic acid (B9)	+	+++	+	++	+
Cobalamin (B12)	+	++	+++	+	+
Ascorbic acid (C)	++	++	+++	++	+
Nicotinic acid (PP)	+++	+++	+	+++	+
Biotin (H)	++	+++	++	++	++

Source: Ballongue, 1993

As far as vitamin requirements are concerned, there seems to be no rule for the *Bifidobacterium*. Strains of human origin seem to need B1, B6, B9, B12 and PP for their growth (Deguchi et al., 1985; Teraguchi et al., 1987). It appears added vitamins are required by those strains that are not able to synthesise any measurable quantities of them.

2.3.5 Nutrient requirements

2.3.5.1 Nitrogenous matter

Most species are able to utilise ammonia and ammonium salts as the only source of nitrogen, though there are some strains which develop only in the presence of organic nitrogen.

In vitro, and in the absence of any organic source of nitrogen, bifidobacteria may synthesise large quantities of amino acids (Matteuzi et al., 1978). Tanaka (1987) found that the glutamine synthetase and glutamate dehydrogenase of *Bifidobacterium* may be involved in the assimilation of nitrogenous compounds by these organisms.

2.3.5.2 Trace elements

B. bifidum grows only in the presence of magnesium, manganese, and most important, iron. Iron is assimilated by this species in both oxidation forms, depending on the acidity of the medium (Bezkorovainy et al., 1986; Bezkorovainy and Topouzian, 1981; Ueda et al., 1983). Fe^{2+} is used at pH 5 and its transport depends on a membrane ATPase and its incorporation may be completely inhibited by zinc (Bezkorovainy et al., 1986). Fe^{3+} is used at neutral pH. Through the intermediary of ferroenzymes, iron is involved in the production of acetic acid by *B. bifidum*.

2.3.5.3 Growth factors

Experiments have shown that the species of Bifidobacteria require the presence of growth factors of various types (Poch and Bezkorovainy, 1988). Most species of this genus are unable to grow in a totally synthetic medium and require complex biological substances such as bovine casein digestate, lactoserum of bovine milk, porcine gastric mucin or yeast extract (Poch and Bezkorovainy, 1988). Only *B. adolescentis* and *B. longum* can grow on unsupplemented medium. To date, five growth factors have been identified. These are bifidigenic factors, lactoferrin, lactulose and lactitol, oligoholosides and fructooligosaccharides.

(a) Bifidigenic factors

There are three main groups of these factors and they differ according to species. They are BB, BI and BL factors (Table 3).

The BB factors include BBa and BBb and are characterised as the elements in human milk which do not lose their stimulant activity for *B. bifidum* var. a and var. b respectively after heating or irradiation. BBa factors are found mainly in yeast extracts, liver extracts, lyophilised milks, bovine casein hydrolysate, and porcine mucin (Raynaud, 1959; Bezkorovainy, 1979). BBb factors are found in colostrum, human milk, rat milk (Gyorgy et al., 1954), human casein hydrolysates (Nichols et al., 1974) and porcine mucin (Raynaud, 1959).

There are three groups of natural BB factors. They are;

(i) Gyorgy's bifidus factor 1 or BF1, which is found in milk and colostrum and in the form of gynolactose which is particularly active on variant b. It appears that the presence of an N-acetylglucosamine structure in the oligosaccharide structure is essential but not sufficient to the expression of bifidigenic activity (Seka Assy, 1982). *B. bifidum* var. *pensylvanicus* has N-acetyl-D-glucosaminidase activity which is considerably higher than that found in the other bifidobacteria (Desjardins and Roy, 1990). Native human casein or its trypsin hydrolysate consisting of glycoproteins may be effective for *B. bifidum* var. b (Bezkorovainy et al., 1979; Seka Assy, 1982). Trypsin or chymotrypsin hydrolysis of native human K-casein gives fractions containing 60-70% sugars of galactose, glucosamine and galactosamine, which are themselves active. Mucins (glycoproteins and mucus) are produced and secreted by the mucus cells of the salivary glands, oesophagus, stomach, small intestines and colon (Allen, 1984). They are the major part of mucus (Forstner et al., 1984) and consist of 70-80% sugar (Allen, 1981). Porcine gastro-intestinal mucins and the meconium are an abundant source of BB factors.

(ii) BF2 factors, which are found in a strain of *B. Bifidum* var. a. They appear to consist of non-glycosylated peptides obtained by action of a protease on casein.

(iii) Glycoproteins, which are isolated from human colostrum and milk lactoserum. They appear to be effective for both variants and their activity seems to be related to the sugar fraction (Seka Assy, 1982).

BI and BL Factors

BI factor stimulates growth of *B. infantis* and is destroyed by lyophilisation.

BL factor activates the growth of *B. longum* and is sensitive to heating and irradiation.

Both factors are proteins and are abundant in plant, liver and milk extracts (Beerens et al., 1980). The BI factors from human milk are of two types. One is the thermo- and radiolabile BI and the other is the thermo- and radiostable BI.

Table 3: Characteristics of the main bifidigenic factors

	Bifidigen factor	Species concerned	Source	Resistance			Active structure
				Heat	Ray.	Lyoph.	
BB	BF1	*B. bifidum* var. b	Milk and colostrum Human casein hydrolysate Mucins	+	+		*N*-acetylglucosamine glycoproteins
	BF2	*B. bifidum* var. a	Casein hydrolysate	+	+		Nonglycosyled peptide
	Glycoproteins	*B. bifidum* var. a *B. bifidum* var. b	Human milk and colostrum				Glucidic part
BI		*B. infantis*	Plant extracts Liver extracts Milk	±	±	–	Proteic part
BL		*B. longum*	Plant extracts Liver extracts Milk	–	–		Proteic different from BB factors

Source, Ballongue, 1993

2.3.5.4 Lactoferrin

Lactoferrin and its three metal complexes iron, copper and zinc have a promoting effect on the growth of eight species of Bifidobacteria, five of human origin and three of animal origin, at the beginning of the logarithmic phase of growth. These lactoferrin-metal complexes show an antibacterial activity against *E. coli* and *Staphylococcus aureus* (Shimamura, 1989).

2.3.5.5 Lactulose and Lactitol

Lactulose, a disaccharide (4-O-beta-galactopyranosyl-D-fructose) is a constituent of dairy products which have been subjected to heat treatment and is not present in raw milk.

In vivo, lactulose can increase the growth of *B. bifidum*. However, this factor is not active in vitro and is not present in the free state in the mother's milk. Its action is due to the fact that it is more resistant to degradation by digestive tract lactases than is lactose and can therefore be used massively by Bifidobacteria (Ballongue, 1993). Yazawa et al. (1978) found that lactulose is not used specifically by bifidobacteria and may also be utilised by other intestinal bacteria. Lactitol is considered to be a bifidigenic factor with a less marked effect (Mitsuoka et al., 1987).

2.3.5.6 Oligoholosides and Oligosaccharides

Bifidobacteria utilise raffinose, stachyose, inulin and other oligosaccharides with molecular weight of less than 4500 (Yazawa et al., 1978). Other intestinal bacteria such as *E. coli, L. acidophilus* and *S. faecalis* do not. Oligosaccharides higher than the trisaccharide of inulin and the tri- to penta-saccharides of dextran are also metabolised by species of Bifidobacteria. However, the oligosaccharides of amylose and cellulose are not only metabolised by Bifidobacteria, but may be used by other bacteria.

2.3.5.7 Fructooligosaccharides (FOS)

These are polymers of fructose with a degree of polymerisation between 2 and 35. They have a stimulating effect on growth of bifidobacteria (Hidaka et al., 1986). They are metabolised by bifidobacteria as well as other types of bacteria and are not degraded by human digestive enzymes nor by undesirable micro-organisms of the digestive tract. The most important source of FOS is the Jerusalem artichoke tuber (Mitsuoka et al., 1987). However, similar substances can now be easily prepared by an enzymatic route or by chemical modification of natural products, e. g., lactulose from lactose (Hidaka et al., 1986; 1988; Yamakazi and Dilawri, 1989).

2.3.6 Bile metabolism

The human microflora can biotransform the bile acids, cholic acid and chenodeoxy cholic acid. Bifidobacteria (Ferrari et al., 1980) hydrolyse conjugated bile acids. Although many more genera of the intestine can hydrolyse bile acids, there is variation among species with respect to the presence and extent of this activity (Shimamura, 1990).

Ibrahim and Bezkorovainy (1993) reported that there is a significant decline of growth of bifidobacteria in the presence of primary bile salts, with an obvious dose-dependency. They observed that the higher the bile salt concentration, the lower the growth of the organisms. They also found that as the concentration of the bile salt increased in the medium, the change in the pH values caused by growth grew smaller. They were able to recover all the strains to normal growth when they cultured them in bile salt-free medium.

This study obviously simulates in part, the movement of bifidobacteria from the small intestine, where the bile salt concentration is relatively high (about 0.6 g/L) to the colon, where the bile salt concentration is low. Nevertheless, though the authors demonstrated the survival of *Bifidobacterium*, of human origin, in a bile salt solution, because the study was carried out in vitro, it does not take into account the effects and conditions experienced in vivo.

2.3.7 Mucin metabolism

Mucin is an intestinal mucus glycoprotein. It has been reported that faecal mucinase activity is absent in infants during the first few months of life (Cooperstock and Zedd, 1983). It is unknown whether this mechanism is a nutrient-recycling process in either infants or adults, or not. Poch and Bezkorovainy (1988) reported that a limited number of bifidobacteria grew optimally in synthetic medium to which biologically complex materials, including mucin had been added as a growth-promoting supplements. They used the most commonly occurring human bifidobacterial species, some of which were the adult species and some the infant types.

2.4 APPLICATIONS

2.4.1 Medical and health benefits

The intestinal flora is composed of a variety of organisms comprising hundreds of species, which contain a variety of enzymes that perform extremely varied types of metabolism in the intestines. These bacteria are implicated in the conversion of various substances, resulting in the production of both beneficial and deleterious products to the host. In addition, bacterial toxins and cell components produced by some bacterial species modify the host's immune responses, enhancing or inhibiting immune function. The beneficial intestinal flora protects the intestinal tract from proliferation or infection by harmful bacteria, while the deleterious bacteria

manifest pathogenicity when the host's resistance is decreased (Mitsuoka, 1982).

Recent research has focused on bifidobacteria to establish their influence on certain normal functions of the intestinal tract and in exploring their role in human health and diseases. Bifidobacteria are used as dietary supplements or as starter cultures for yoghurt and other milk products with the thought that such products may help the promotion of health (Mitsuoka, 1982). The daily intake of such products is reported to have the following effects;

(i) to suppress the putrefactive bacteria as well as intestinal putrefaction, so as to prevent constipation and geriatric diseases, including cancer;

(ii) to prevent and treat antibiotic-associated diarrhoea, and

(iii) to stimulate the immune response, thus contributing to a greater resistance to infection (Mitsuoka, 1982).

Most *Bifidobacterium* species metabolise a wide range of indigestible oligosaccharides to acetic and lactic acids and subsequently act as effective scavengers in the large intestine when these indigestible oligosaccharides are ingested. Studies show that improvement of intestinal flora as well as intestinal environment were observed after oral administration of various oligosaccharides, including fructo-oligosaccharides, isomalto-oligosaccharides, galacto-oligosaccharides, palatinose condensate, raffinose, and soy bean oligosaccharides. Most of the oligosaccharides stimulated the growth of bifidobacteria in vitro and in vivo, and caused a reduction im the levels of faecal pH, ß-glucuronidase, azoreductase and indole, and serum

cholesterol and triglyceride levels as well as the blood pressure of elderly patients with hyperlipaemia (Tanaka et al., 1983; Mitsuoka et al., 1987; Kohmoto et al., 1988). These investigators concluded that oligosaccharides enhanced the intestinal bifidobacteria and improved the intestinal flora, stool consistency, and lipid metabolism.

Tanaka et al. (1983) observed that feeding 200 ml of Bifidus milk supplemented with 10^{10}-10^{11} of *B. breve* per ml per day to healthy subjects with otherwise normal diet resulted in an increase of indigenous bifidobacteria, reduction of the counts of bacteroidaceae, clostridia and enterobacteraceae in the stools, and a decrease of faecal ammonia.

Tohyama et al. (1982) studied the effects of the administration of *B. breve* on bacterial metabolic activities in the intestines. They observed that the activities of bacterial enzymes such as ß-glucuronidase, tryptophanase and lysine decarboxylase in faeces were significantly reduced, and bacterial metabolites such as phenols, ammonia and cadaverine in urine were also reduced.

Mitsuoka (1982) studied the effects of oral administration of freeze-dried *Bifidobacterium longum* to five healthy 25-35 year old volunteers. The subjects were administered 3 x 10^9 freeze-dried *B. longum* per day for five weeks. During the administration, it was observed that the counts of bifidobacteria in the stools increased, while the counts of clostridia decreased significantly. A decrease of ammonia concentration and ß-glucuronidase activity in both faeces and serum were observed.

When Hartley male rabbits were fed a 0.25% cholesterol diet supplemented with 10^{10} cells of *B. longum* per day for 13 weeks, it was observed that in 2 of 3 rabbits, *B. longum* remarkably suppressed an increase in cholesterol level in serum. Homma (1988), reported that the presence of *Bifidobacterium* restrained the neoformation of LDL receptors of human activated T-cells. In rats, *Bifidobacterium* reduced serum cholesterol level by affecting HMG CoA reductase activity.

Orally administered bifidobacteria preparations have been used in the treatment of various intestinal disorders (Mitsouka, 1992). Hotta et al. (1987) investigated the effects of administration of *Bifidobacterium* preparations for the treatment of infantile intractable diarrhoea. Fifteen patients, ranging in age from 1 month to 15 years, were receiving antibiotic therapy for the treatment of septicaemia and respiratory tract infections. In most cases, a disturbed intestinal flora was observed, where there was a marked decrease of anaerobes, especially bifidobacteria. Pathogens or toxins responsible for causing diarrhoea were not detected during this period. When these patients were administered a bifidobacteria preparation, the frequency of the stools as well as appearance, were improved after 3-7 days. The faecal flora of all subjects studied also became normal with predominance of resident bifidobacteria, and the balance of intestinal flora improved to normal levels.

The relationship between liver tumourigenesis and bifidobacteria was studied in mice. Gnotobiotic C3H/He male mice monoassociated and polyassociated with intestinal bacteria were used in the study. The incidence of liver tumours was higher in most of the gnotobiotes

and conventional mice than in the germ-free mice (Mizutani and Mitsuoka, 1980). These workers observed live tumours in 100% of mice associated with a bacterial combination of *E. coli*, *S. faecalis*, and *C. paraputrificum*, in contrast to 30% of germ-free mice and 75% of conventional mice. This tumour-promoting effect by intestinal bacteria was found to be suppressed 46% by the addition of *B. longum* to the promoting combination, and 65% by *L. acidophilus*. It has been suggested that the mechanism of the suppressive effect of bifidobacteria on liver tumours might be related to their ability to detoxify carcinogens.

2.4.2 Bifidobacteria in food

Bifidobacteria are the most recent organisms to be recognised as dietary adjuncts. Some of the benefits that have been listed as a result of consumption of foods containing bifidobacteria are 1) improved flavour and nutritional quality of some foods such as yoghurt, 2) improved nutritional value of baby formulae, 3) control of intestinal infection in the very young and the very old, 4) anti-carcinogenic activity, and 5) improved lactose utilisation, especially for people who are lactose intolerant (Laroia and Martin, 1990).

Recent research has indicated that maintaining a proper balance among the various normal micro-organisms in the intestinal tract is needed for optimum health. Obviously, there are many factors that affect the balance among the intestinal microflora. Diet, infections and antibiotics can alter this balance. It is therefore important that if an organism is to be used as a dietary adjunct it must have certain characteristics. First, such an organism must be part of the natural

flora of the intestinal tract of a healthy person. To reach the intestines, this organism must be able to survive extreme conditions of the upper digestive tract. Thirdly, if it does survive these conditions, it must be able to establish and grow in the intestine. Once established, it must produce the beneficial effects that are expected of it. Finally, and perhaps most importantly, to have any effect, it must be viable in the food to which it is added at the time of consumption.

The use of bifidobacteria as dietary adjuncts helps maintain a proper balance of the intestinal flora (Laiora and Martin, 1990). Consumption of fermented foods containing these organisms allows their continuous passage through the gut (Modler et al,. 1990). In this way, they compete for essential nutrients and attachment sites on the epithelium with other gut organisms, thus minimising or inhibiting colonisation of the intestinal tract by invading pathogens (Tamura, 1983).

It has been reported that the composition of the intestinal flora is influenced by the type of diet. Benno et al., (1986) studied the influence of diet on the intestinal flora of nine rural healthy Japanese and eight healthy Canadians. The two population groups ate typical Japanese and western diets, respectively. The numbers and occurrence of bifidobacteria were higher in the Japanese population than in the Canadians. In addition, these investigators found that the incidences of *B. adolescentis* biovars a and b, and *B. bifidum* biovar b, were significantly higher in the Japanese than in the Canadians.

When the faecal flora of vegetarian and non-vegetarian Seventh Day Adventists were examined,

no significant differences in bifidobacterial numbers were observed (Mitsuoka, et al., 1977). Hentges et al. (1977), compared the faecal counts of volunteers on high and low beef diets. They found that bifidobacteria counts were $1.05 \pm 0.49 \times 10^{10}$ and $1.98 \pm 0.76 \times 10^{10}$, respectively. Furthermore, Reddy et al. (1975), observed a lower anaerobe count in faecal samples from persons consuming a non-meat diet, compared with those on a high-meat diet. Bifidobacterial counts were reported as $7.94 \pm 0.08 \times 10^{10}$ and $2.63 \pm 0.10 \times 10^{10}$ per gram of faeces, respectively.

On the basis of the above information, it appears there is no general agreement in terms of whether or not the bifidobacterial flora of individuals on high-meat diets differ from those individuals on low-meat diets. However, Mitsuoka (1984) believes that a Japanese-style diet, is superior to a western-style diet, with respect to bifidobacterial counts in the intestinal flora.

Another type of diet that has been shown to have an effect on the numbers of intestinal flora, especially bifidobacteria, is one containing fructo-oligosaccharides. These compounds are found in plant materials such as onions, asparagus, wheat, etc. Human digestive enzymes are not able to hydrolyse these compounds, but bifidobacteria can (Mitsuoka, 1982). When a population of elderly people were orally administered 8 g of fructo-oligosaccharides per day for two weeks, it was observed that the numbers of bifidobacteria in faeces increased about 10 times compared with levels before administration. The average pH of the stools decreased, about 0.3 units lower, than before administration (Mitsuoka et al., 1987).

Kohmoto and others (1988) administered 13.5g of isomalto-oligosaccharides (found in fermented foods), per day for two weeks to a group of healthy adults. At the end of the administration period, they found that bifidobacteria counts had remarkably increased.

Due to the health promoting effects associated with bifidobacteria, various countries are taking a keen interest in incorporating these organisms into fermented milk products. However, to manufacture fermented milk products containing viable cells of these organisms, the organisms must be able to survive in the products, during manufacture and storage in acidic milk. In the intestines, growth of these organisms is associated with the so-called bifidus factors (Modler, 1990). Klaver et al. (1993) found that growth of bifidobacterial species in unsupplemented milk was generally poor. Of 17 strains they used in their experiment, only 3 displayed any growth, albeit poor, while the rest did not grow at all. When the milk was supplemented with casitone, all but 3 strains grew fairly well, and when they supplemented the milk with a mixture of free amino acids, all strains grew fairly well except one. It appears that different bifidobacterial strains have different nitrogen requirements.

In fermented products, Klaver et al. (1993) found that 3 of 17 strains did not show any significant reduction in counts during storage while the rest showed a significant reduction by a factor of more than 10^3 in 2 weeks of storage. In this case, it appears that changes in pH were responsible for the reduction in counts.

2.4.2.1 Bifidobacteria in dairy products

Cultured milk products are more easily digested by human beings than the milk from which they are produced. They are also reported to provide other beneficial effects in the diets of certain groups of individuals (Laroia and Martin, 1990).

Most strains of bifidobacteria are sensitive to pH values below 4.6. Therefore in practical application, the pH value of the final product must be maintained above 4.6, otherwise the bifidobacteria population will decrease rapidly (Tamine and Robinson, 1988; Modler et al., 1990; Laroia and Martin, 1991). The most often used species of bifidobacteria, for dietary adjuncts are *B. bifidum, B. breve, B. infantis, B. longum* and *B. adolescentis* (Table 4). Japan produces the most products with Bifidobacteria cultures (Modler et al., 1990; Puhan, 1990). Most of these products are of dairy origin and include cultured milk, beverages, cheese products, powdered milk, cookies, ice cream and items sold as 'health foods' (Table 4).

Table 4: Fermented Milk Products containing bifidobacteria

Microorganisms involved
B. bifidum or *B. longum*
B. bifidum, S. thermophilus
B. bifidum, L. acidophilus, S. thermophilus
B. bifidum, L. acidophilus, P. acidilactici
B. bifidum, (B. longum), L. acidophilus, S. thermophilus, L. delbrueckii subsp. bulgaricus
B. bifidum, L. acidophilus
B. bifidum, L. acidophilus
B. bifidum, B. breve, L. acidophilus
S. lactis subsp. diacetylactis, S. lactis subsp. cremoris, L. acidophilus and/or *B. bifidum*

Source: Laoira and Martin, 1990

2.4.2.2 Selection of Bifidobacteria for use as dietary adjuncts

Micro-organisms with the best chance of passing through the stomach and small intestine and colonising the gastrointestinal tract are those endogenous to the species consuming the fermented product. It is for this reason that research has been focused on the genus *Bifidobacterium*, which, unlike the bacteria of yoghurt, is isolated from animals and humans.

The therapeutic properties of this genus of bacteria led the Japanese to introduce it to their diet (Mitsuoka, 1982; Yamakazi et al., 1985).

The proportions of the final fermentation products vary from strain to strain, even within species (De Vries and Stouthamer, 1968; Lauer and Kandler, 1976). Small amounts of carbon dioxide have been/may be produced by the degradation of gluconate (Scardovi, 1986).

The nutritional quality of food depends on the nutrients available, digestible and assimilable (Welch, 1987). Many of the food nutrients such as proteins and carbohydrates are pre-digested in fermented foods, making them highly nutritious and easy to digest.

Protein is one of the constituents that is pre-digested in fermented foods. There are two main reasons for its improved digestibility. First, some of the milk protein is broken down into peptides and free amino acids during fermentation. It is easier to absorb free amino acids than intact milk protein. Lactic acid also improves absorption (Gilliland, 1979). Secondly, the protein is dispersed in a coagulated form because of the acid produced (lactic acid). This provides a larger surface area upon which digestive enzymes can act (Renner, 1986). Goodenough and Kleyn (1976) have shown that incorporation of *B. bifidum* with yoghurt culture in the ratio 2:1 resulted in greater changes in nitrogen compounds than with yoghurt culture alone. This was attributed to the proteolytic activity of *B. bifidum* in milk.

In addition, the lactose content of fermented milk is lower than that of the original milk because of partial fermentation of the lactose. Lactose is fermented to lactic acid by lactic acid bacteria while Bifidobacteria produce L(+)-lactic acid and acetic acid. This is very important for infants since they can totally metabolise L(+)-lactic acid in the synthesis of glucose or glycogen. The L(+)-lactic acid is also used in respiratory processes. D(-)-lactic acid is only partially metabolised and at a lower rate (Rasic and Kurmann, 1983).

2.5 CONCLUSIONS

Due to the obviously huge health benefits of bifidobacteria to health, recent research has focussed on their use in probiotic foods. In this study, growth of these organisms will be studied on different carbohydrate sources and at different pH values, with an effort to establish the best growing conditions.

CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Organism

The species of *Bifidobacterium* used in this study was *Bifidobacterium bifidum* (Hansen's strain), obtained from the New Zealand Dairy Research Institute, Palmerston North, New Zealand.

3.1.2 Bacteriological media

The bacteriological media used in this study were peptone water, tryptone and yeast extract obtained from GIBCO BRL (Life Technologies, Ltd, Paisley, UK). MRS agar was obtained from OXOID (Unipath Ltd, Basingstoke, Hampshire, England).

3.1.3 Chemical reagents

All chemicals used were of analytical grade. The chemicals were;

Sodium chloride, sodium hydroxide, potassium chloride, potassium di-hydrogen phosphate, magnesium chloride 6-hydrate, and L-cysteine-HCl obtained from BDH Laboratory Supplies (Poole, England). Formic acid-88%, butan-2-ol, ortho-phosphoric acid-80%, and acetic acid

were supplied by Ajax Chemicals (Auburn, Australia);

L(+) lactic acid , Tween 80, nicotinamide-adenine dinucleotide (NAD) were supplied by SIGMA Chemical Co., (St Louis, Missouri, USA). Anaerobic system (hydrogen and carbon dioxide generator) Gas Pak envelopes were obtained from BBL (Cockeysville, Maryland, USA), and oxygen - free Nitrogen was supplied by New Zealand Industrial Gases, Palmerston North, New Zealand.

3.1.4 Growth substrates

Glucose, lactose and starch were obtained from Ajax Chemicals. Maltose, raffinose, lactulose, cellobiose, xylose and pectin were obtained from SIGMA chemicals.

3.1.5 Enzymes

Lactate dehydrogenase and formate dehydrogenase, were obtained from SIGMA Chemicals.

3.1.6 Equipment

The equipment used in the study were;

PHM-82 standard pH meter from Radiometer, Copenhagen, Denmark; Phoenix pH electrode supplied by Scientific Supplies Ltd, Auckland, New Zealand; Horizon pH controller (Horizon

Ecology Co., Chicago, Illinois, USA); YSI Glucose analyser (Yellow Springs Instrument Co., Yellow Springs, Colorado, USA); U-2000 Spectrophotometer (Hitachi Ltd, Tokyo, Japan); ALC/GPC 244 High Performance Liquid Chromatography (HPLC) with a model 6000 A solvent delivery system and a U6K septumless injector (Waters Associates Inc., Millipore Corporation, Milford, Massachusetts, USA) connected to a Waters model 740 data module recorder; BHG HERMLE Z 320 bench top centrifuge (John Morris Scientific, Pty Ltd, Gosheim, Germany); Model 6000 Vega Series 2 GC (Carlo Erba Instruments,) connected to a D-2500 Chromato integrator (Hitachi Ltd); and CH Microscope (Olympus Optical Co., Tokyo, Japan).

3.2 METHODS

3.2.1 Cultivation

A stock culture of the organism was cultivated on slants consisting of MRS agar, supplemented with 0.5 gL^{-1} L-cysteine-HCl, 0.75 gL^{-1} Bacto-agar, 0.2 gL^{-1} $Na_2S_2O_3$ and 0.1gL^{-1} $CaCl_2.2H_2O$. The slants were incubated anaerobically in an anaerobic jar, at 37^{0}C for 48h. After growth the slants were stored at ~4^{0}C.

The working culture was cultivated in tryptone-peptone-yeast extract (TPY) medium. This was a medium containing 10.0 gL^{-1} Tryptone, 5.0 gL^{-1} Peptone water, 5.0 gL^{-1} Yeast extract, 0.5 gL^{-1} Tween 80, 4.5 gL^{-1} NaCl, 0.5 gL^{-1} KCl, 0.15 gL^{-1} $MgCl_2.6H_2O$, 0.4 gL^{-1} KH_2PO_4, 1.0 gL^{-1}

NH_4Cl, 1.2 gL^{-1} L-cysteine-HCl, and 10gL^{-1} carbohydrate (depending on which substrate was under study at the time).

Media and fermentation equipment were sterilised by autoclaving at 121^{0}C for 15 minutes. The pH probe used in situ was sterilised by immersing in a 50% solution of ethanol in water for 6 hours, then washing with sterile distilled water. In addition, the pH probe was inserted into the fermentation jar when the medium was still hot.

3.2.2 100-ml scale fermentation

The starting medium pH was adjusted to about pH 6.0 before sterilisation. The pH then decreased naturally to about pH 5.5 during sterilisation at 121^{0}C for 15 minutes. After sterilisation and cooling, the organism was inoculated and incubated overnight at 37^{0}C. The growing culture (~12 h), was then inoculated into bottles containing approximately 100 mL of medium. The bottles were then incubated at 37^{0}C in an anaerobic jar (Gas Pak). Samples of about 10 mL were withdrawn at 0 h (at time of inoculation) and at 12 hour intervals for a total of 60 hours.

Upon withdrawal, the sample pH, and biomass concentration were measured. In determining biomass concentration, necessary dilutions were made so that all readings fell within the sensitivity of the spectrophotometer. The remainder of the sample was centrifuged at 4100 rpm for about 15 minutes and the supernatant solution was stored frozen to await further analysis,

that is, measurements for ethanol, acetic acid, lactic acid, formic acid, and substrate utilisation.

3.2.3 1.2-L scale fermentation

Batch fermentation experiments were carried out using apparatus as pictured in Figure 2, and described below. A growing culture was inoculated into a 2L fermentation glass jar, with a working volume of approximately 1.2 L. The vessel was provided with a polyethylene-polypropylene (PP) head with insertion holes for the pH probe, a nitrogen gas delivery tube and outlet, alkali delivery tube, and sample collection point. In these fermentations, the pH was measured in situ and controlled during the fermentation. A pH electrode was inserted into the medium in the jar, which was in turn connected to a pH controller. The pH controller was connected to a timer, which was in turn connected to a Master flex peristaltic pump, with a size 14 pump head (Cole Parmer Instrument Co., Chicago, Illinois, USA). The latter was connected to a reservoir of 4M NaOH. When the culture pH value dropped below the set value, a signal was sent to the pump, which started dosing alkali to the culture. The timer was used to control the amount of alkali that went into the culture medium and to avoid any chance of overshooting the pH. Mixing of the alkali with the medium was achieved by use of a magnetic stirrer and a magnetic flea. Oxygen-free nitogen gas was used to keep the system anaerobic at a flow rate of 50 mL/min until the organism started growing. When the organism was growing, ther rate of flow of nitrogen gas was reduced to 20 mL/minute.

Fig. 2: A picture of the fermentation apparatus used in the 1.2-L scale

Upon inoculation, a sample was withdrawn to mark zero time, then every 4 hours for the first 12 hours, after which samples were withdrawn at 12 hour intervals until no further changes in optical density were observed. The samples were withdrawn through the sample withdrawal point on the PP head using a sterilised pipette. The collected samples were analysed immediately upon withdrawal for pH, using an independent pH meter, in order to check the reading observed on the pH meter used in the experiment and any discrepancies were immediately corrected, and optical density for biomass concentration. The sample was then centrifuged using a benchtop laboratory centrifuge at 4100 rpm for approximately 15 minutes.

The supernatant liquid was stored frozen, to be analysed further for lactic acid, acetic acid, formic acid and ethanol and residual substrate.

3.3 ANALYSES

3.3.1 pH

The pH of the samples was measured using a PHM-82 standard pH meter. The pH meter was calibrated using pH 4.0 and 7.0 buffer solutions.

3.3.2 Biomass

3.3.2.1 Biomass calibration curve

The calibration curve for biomass concentration was constructed as follows; 5, 10, 15 and 20 mL aliquots of a 60 h old culture were made up to 20 mL volume with water. For each of the dilutions, the optical density was measured at 600 nm. The cells were then separated from the broth by centrifuging at 4100 rpm using a benchtop centrifuge for 15 minutes. The supernatant solution was decanted and the cells were washed twice with distilled water. The cells were then resuspended in water and filtered through a pre-weighed 0.45 μm Millipore membrane. The

filtered cells were then dried to a constant weight at 105^{0}C in an oven. After drying, the cells were weighed and the biomass concentration was determined by difference. A plot of biomass concentration (in dry weight) was constructed against optical density (Absorbance) which was used as the standard curve.

3.3.2.2 Biomass concentration

The biomass concentration was measured using optical density. The cell optical density was measured at 600nm on a U-2000 Spectrophotometer. The readings were then converted to dry cell weight per litre of broth, using a calibration curve.

3.3.3 Substrate concentration

For part of this study, glucose was analysed by use of the YSI glucose analyser. The samples were measured against a standard of known glucose concentration. Later during the study, all the substrates, that is, glucose, lactose, maltose, raffinose, lactulose, and cellobiose were analysed by high performance liquid chromatography (HPLC), using a Sugar Pak column. The analyses were carried out at 90^{0}C. The solvent system was distilled water, to which 50μg/L EDTA had been added and filtered. The solvent flow rate was 0.500 mL/min. Samples of 50 μL were injected into the chromatograph using a septumless Hamilton syringe (Reno, Nevada, USA). The intergator was calibrated using standard solutions of 25 gL^{-1} and 50 gL^{-1} of substrate.

3.3.4 Ethanol and Acetic acid

Ethanol and acetic acid were determined simultaneously, by gas chromatography (GC) using a Vega Series 2 GC 2000 chromatograph equipped with a flame ionisation detector. The carrier gas used was nitrogen at a flow rate of 60 mL/min. The column was maintained at 200^{0}C, and the injector and detector temperature at 220^{0}C. Samples were injected at 2 μL aliquots, and concentrations determined by the "internal stardard" method. The internal standard was 10 g sec-butanol in 1 L of 80% othophosphoric acid; 0.1 ml of this solution was added to 1.0 ml of sample prior to injection. For calibration, the standard solution contained 1.0 gL^{-1} ethanol and 1.0 gL^{-1} acetic acid, to which was added 0.1 ml of internal standard solution.

The GC was connected to a Hitachi D-2500 chromatogram integrator which calculated a response factor which was then used in calculating the concentrations of ethanol and acetic acid in the sample.

3.3.5 Lactic acid

The method for analysing lactic acid was taken from Bergmeyer et al., (1984). Initially, both the L- and the D- form of lactate were analysed for, and it was found that only the L-form was produced by the organism.

The acid was determined as lactate following the principle that;

$$\text{L(+)Lactate} + NAD^+ \xrightarrow{\text{L(+) LDH}} \text{Pyruvate} + NADH + H^+$$

The formation of NADH is measured by increase in Absorbance at 340nm. The equilibrium lies far to the left, therefore the reaction products must be removed. Thus,

$$\text{L(+)Lactate} + NAD^+ + \text{Hydrazine} \xrightarrow[\text{pH 9.0}]{\text{L(+) LDH}} \text{Pyruvate hydrazone} + NADH + H_3O^+$$

3.3.5.1 Solution Preparation

A. Hydrazine (0.4M)/glycine (0.5M) buffer. pH 9.0

11.4g glycine was dissolved in ~200ml H_2O, 25ml of 24% hydrazine hydrate was then added to the solution. The pH was checked and adjusted to 9.0. The solution was then made up to 300ml with water.

B. NAD (40mM)

0.030g of β-NAD in 1ml H_2O. This solution was made up fresh every time needed.

C. L(+) Lactate dehydrogenase (L(+) LDH)

~5000 units/mL, used undiluted.

D. L(+) Lactate standard

0.40mg of L(+) Lactate was dissolved in 1ml water. 3/10 dilutions were made and 1 mL volumes of the diluted standard were stored at ~-20^{0}C

3.3.5.2 Reaction mixture

A. Buffer 0.795 mL,

B. NAD 0.100 mL,

C. L(+) LDH 0.005 mL

Total volume of the reaction mixture was determined by the number of samples and standards.

3.3.5.3 Sample preparation

Samples were diluted so as to contain 4-20 μg of lactate/100 μL

3.3.5.4 Standard curve

0, 20, 40, 60, 80 and 100 µL of standard were dispensed into labelled tubes (in duplicate) and made up to 100 µL with water. The volumes were dispensed using a Hamilton syringe. A curve of lactate concentration was plotted against absorbance, and this produced a straight line.

3.3.5.5 Assay

100 µL of sample (in duplicate) or standard was placed in a small tube. To that, 0.9 ml of the reaction mixture was added, and the solution was vortexed. The mixture was then incubated at 25^{0}C for 1 hour. After the hour had elapsed, the absorbance of the solution was read against water at 340 nm.

The lactate concentration in the sample was calculated using the following equation

$$\text{mM L(+) Lactate} = (A_{sample} - A_{blank}) \times 1.77 \times \text{Dilution factor}$$

3.3.6 Formic acid

The method of formate analysis in this study was taken from Bergmeyer et al., (1984), and followed the principle that;

$$HCOOH + NAD+ \leftrightarrows CO_2 + NADH + H^+$$

that is, oxidation of formic acid to carbon dioxide by nicotinamide-adenine dinucleotide (NAD) in the presence of formate dehydrogenase (FDH). The amount of NADH formed is equivalent to the quantity of formic acid. NADH is determined by its absorbance at 339 nm. Measurement is carried out at pH values between 7.0 and 7.5 at room temperature for optimum conditions.

3.3.6.1 Reagents and Solutions

Preparation of solutions

All solutions were prepared in sterilised water. To prevent microbial contamination, all containers were sterilised.

1. Phosphate buffer (0.15 mol/l, pH 7.5):

   2.04 g KH_2PO_4, anhydrous was dissolved in 100 ml water (ssolution A) and 1.99 g of K_2HPO_4, anhydrous was dissolved in 100 ml water (solution B). 14 ml of solution A was then mixed with 100 ml of solution B.

2. Nicotinamide-adenine dinucleotide solution (β-NAD, approximately 42 mmol/L):

   330 mg NAD-Li.2h_2O was dissolved in 11 ml water.

3. Formate dehydrogenase, (FDH):

   10 mg of FDH was dissolved in 1 ml water

4. Formic acid standard (1 g/L):

   1 g formic acid was dissolved in 999 ml water. Stock solution was then diluted with water to 0.5, 0.2, and 0.1 g/L.

3.3.6.2 Procedure

Calibration curve

1.0 ml of the formic acid standard was added to 9ml of water, in order to obtain samples with concentrations between 0 and 100 mg/L. These standards were then treated like samples. The standard curve was obtained by a linear regression to the origin. The curve is linear up to a conentration of 200 mg/L (4.35 mmol/L).

Sample prepparation

Samples were diluted by an appropriate factor so as to contain up to 100 mg/L of formate. A blank was prepared using water instead of sample.

Assay conditions

Wavelength (λ) 339 nm; light path 10 mm; final volume 3.05 ml; meaurement aginst air.

3.3.6.3 Measurement

Pipetted successfully into cuvette:		Concentration in assay mixture
sample (or standard)	0.1 ml	formic acid : up to 143mmol/L
NAD solution	0.5 ml	NAD: 6.88 mmol/L
buffer	1.0 ml	phosphate: 49.2 mmol/L
water	1.4 ml	
mix thoroughly and read absorbance A_1		
FDH solution	0.05 ml	FDH: 328 U/L
mix, incubate for 30 minutes at room temperature, read absorbance A_2		

Calculation

$A_2 - A_1 = \Delta A$

Formate concentration in the sample was determined by reading the value corresponding to ΔA from the standard curve and multiplying by the dilution factor.

CHAPTER 4 RESULTS

4.1 MORPHOLOGY

The colonies observed on the MRS slants were cream in colour, shiny and had a soft consistency. These colonies were generally variable in shape. However, in some cases the 'amphora-like' grouping of the cells (Scardovi, 1986) was noticed.

The organisms were Gram positive, non-motile rods with irregular shape but mostly connected together to form a very wide 'Y'.

4.2 GROWTH OF *Bifidobacterium bifidus*

Growth of *Bifidobacterium bifidus* is described by a sigmoidal or logistic curve. This finding is in accordance with the findings of Desjardins et al. (1990). Experimental results of biomass, substrate consumption, and product formation were determined by linear regression analysis using time derivatives. These results were obtained by use of a data graphics program known as Fig P (The Scientific Fig. Processor). Temporal variations of biomass, substrate, and products facilitated the calculation of the fermentation kinetics in this study.

4.3 INITIAL GROWTH AT DIFFERENT pH VALUES

The objective of this experiment was to determine the pH value below which no growth took place and to determine the pH values to be used in the study. This experiment was carried out on the 100-ml scale using TPY broth, as described in section 3.2.2. The substrate used was glucose at a concentration of 10 g/L. The pH values were adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 before sterilisation using sodium hydroxide or hydrochloric acid as appropriate. After sterilisation the pH values had naturally decreased to 3.5, 4.1, 4.6, 5.0, 5.4, 5.8, 6.1 and 6.6, in respective order. To keep the bottles anaerobic, small discs of metal (about 3 mm in diameter) were removed from the cap, but not the septum, to allow for sample withdrawal with little oxygen entry. The sample was then withdrawn by a sterile syringe through the septum, without having to remove the bottle caps. The organism was incubated anaerobically at 37^0C. Growth was investigated by measure of optical density every 2 hours for 12 hours. The organism was then left to grow for a further 36 hours after which samples were withdrawn and analysed for amount of products at each initial pH value.

A graph of biomass (dry weight) against time was plotted (Fig. 3) to show growth at the different initial pH values. Also plotted was the amounts of products formed after 36 hours of incubation against initial pH values (Fig. 4).

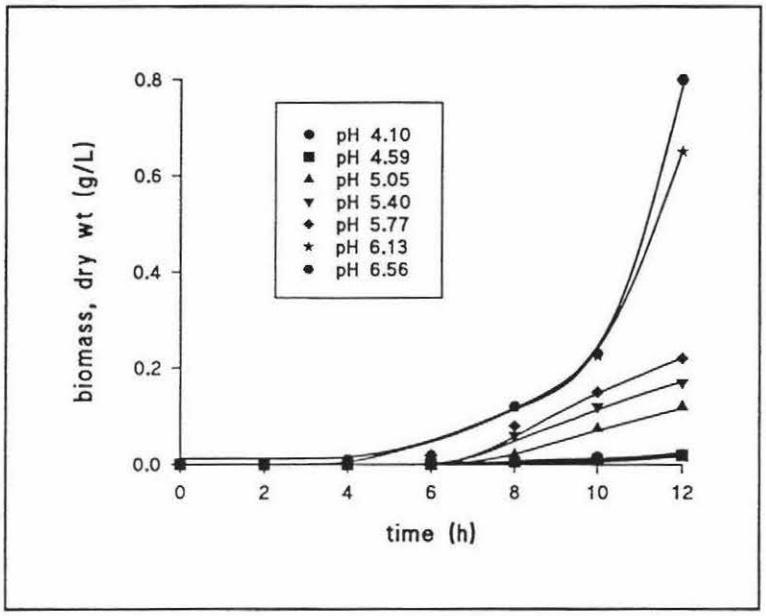

Fig. 3: Plot of biomass produced versus time at different initial pH values

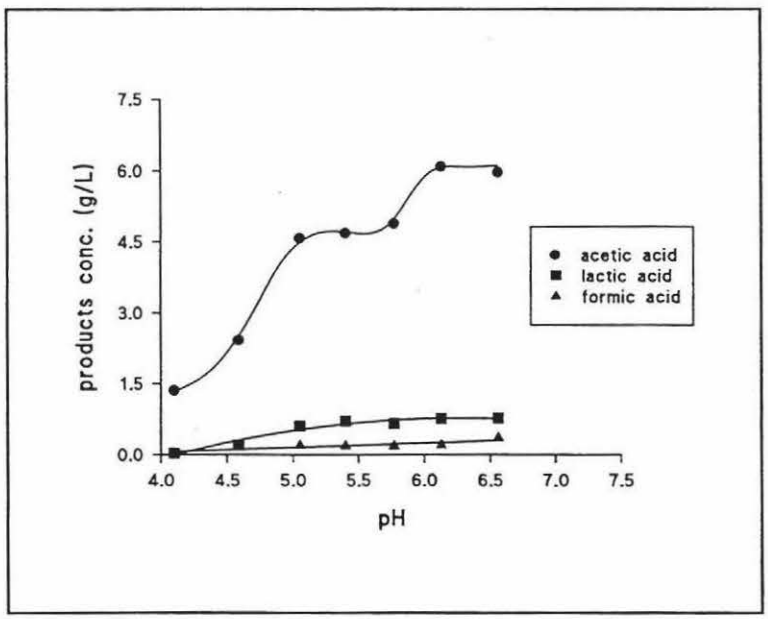

Fig. 4: Plot of products at t_{36} versus pH

The results of this experiment indicated that the growth of *B. bifidum* at pH 4.1 to pH 5.8 (Fig. 3) was more or less the same. Though it appears that there were some differences in the amount of biomass produced, they were only very slight. At these pH values, the amount of biomass produced after 12 hours ranged from 0.05 - 0.20 g/L. However, at pH 6.1 and pH 6.6, there was a marked increase in the amount of biomass produced, 0.64 and 0.80 g/L. No growth occurred at pH 3.5.

In terms of the products of fermentation, there was little difference in the amounts of lactic acid and formic acid produced at different pH values (Fig. 4). Acetic acid production, on the other hand, increased with initial pH, the highest production being observed at initial pH 6.6.

4.4 GROWTH AT UNCONTROLLED pH

The aim of these experiments was to determine differences, if any, of growth rate, product formation and the yield of the organism when different carbohydrate sources were used. These experiments were carried out at 100-ml scale. Different substrates were used, namely, glucose, lactose, pectin and starch. For each substrate, two concentrations were used, that is, 10 g/L and 50 g/L, except for starch and pectin, which were used at concentrations of 2 g/L due to their low solubility. The initial pH of the medium in each of these experiments was adjusted to about pH 6.0 before sterilisation. The pH then decreased naturally to about pH 5.5 during sterilisation.

A growing culture (about 12 h) of the organism was inoculated into the medium. The organism was incubated anaerobically in a Gas Pak anaerobic system at 37^0C. Samples were withdrawn every 12 hours for a total of 60 hours.

The 10 g/L and 50 g/L glucose experiments were run in duplicate.

4.4.1 Specific rate of growth

To determine the specific rate of growth (Table 5) of the organism, the logarithm (base 10) of the biomass concentration was plotted against time (figures 5 - 10), and

$$\text{Specific rate} = \mu = 2.303 \times \text{Slope},$$

where the slope was measured during the initial stage of growth ($t \leq 24$ h).

The doubling time (Table 5) of the organism;

$$t_d = (2.303 \times \text{Log } 2)/\mu$$

$$= 0.693/\mu$$

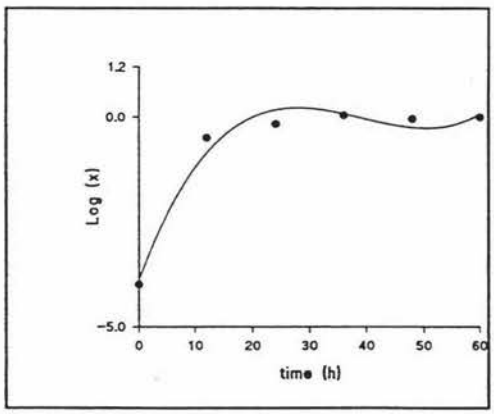

Fig. 5: Log (x) versus time at 10 g/L glucose

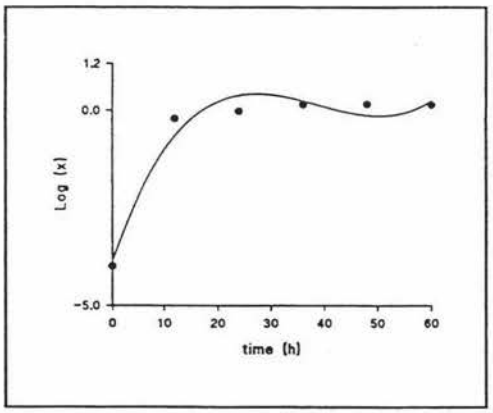

Fig. 6: Log (x) versus time at 10 g/L glucose

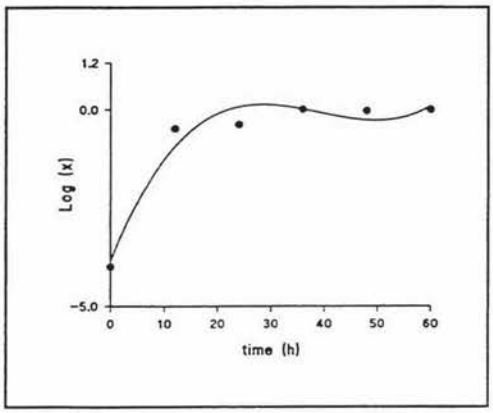

Fig. 7: Log (x) versus time at 50 g/L glucose

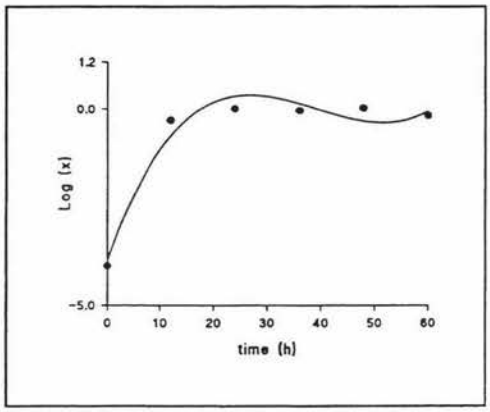

Fig. 8: Log (x) versus time at 50 g/L glucose

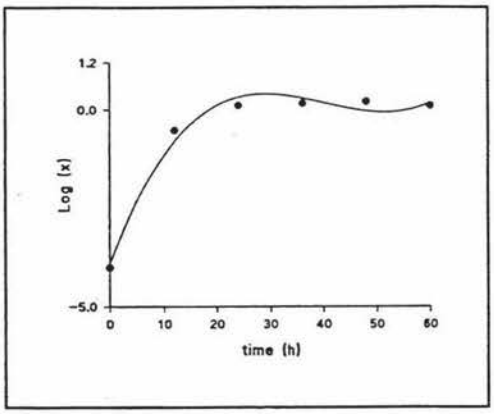

Fig. 9: Log (x) versus time at 10 g/L lactose

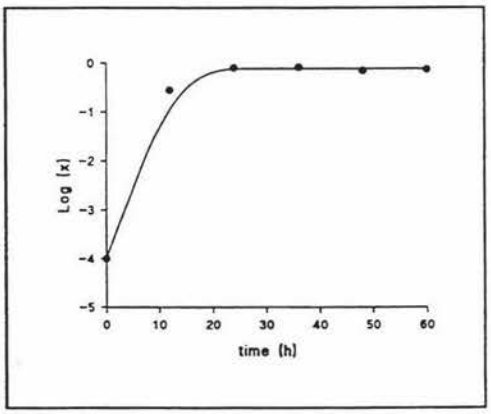

Fig. 10: Log (x) versus time at 50 g/L lactose

Table 5: Rates of growth of *B. bifidum* at uncontolled pH

Substrate	Maximum biomass concentration in dry weight (g/L)	Specific rate of growth (h^{-1})	Doubling time (h)
Control	0	-	-
2 g/L Pectin	0	-	-
2 g/L starch	0	-	-
10 g/L Glucose	1.10	0.386	1.80
10 g/L Glucose	1.42	0.382	1.81
50 g/L Glucose	1.01	0.348	1.99
50 g/L Glucose	1.06	0.385	1.80
10 g/L lactose	1.27	0.394	1.76
50 g/L Lactose	0.82	0.375	1.85

The results of this experiment showed that *B. bifidum* ferments glucose and lactose (Figures 5 - 10). No growth was observed in the control experiment, that is, when no carbon substrate was present, or when pectin or starch were used as the substrate (Table 5). The measured rates of growth range from 0.348 - 0.394 /h and doubling times range from 1.76 - 1.99 hours when glucose and lactose were used as substrates (Table 5). There appeared to be no differences when these substrates were used at concentrations of 10 or 50 g/L. The amount of biomass produced was approximately the same, ranging from 1.02 to 1.42 g/L. However, the biomass produced at 50 g/L lactose was lower than the others (0.82 g/L).

4.4.2 Substrate utilisation

To determine the rate of substrate utilisation (Table 6), substrate concentration was plotted against time (figures 11 - 16), and

$$\text{Rate} = -(\text{Slope})$$

where slope was measured at t = 24 h.

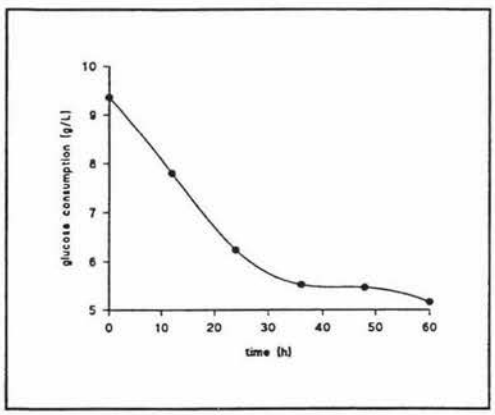

Fig. 11: Substrate concentration versus time at 10 g/L glucose

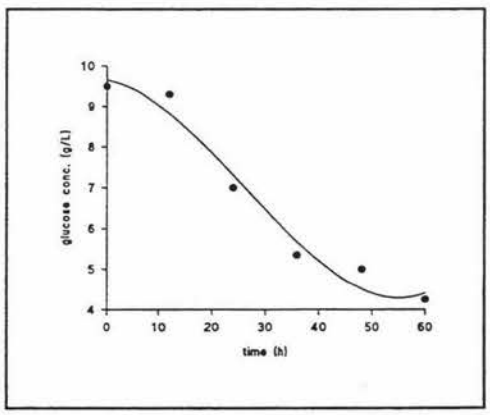

Fig. 12: Substrate concentration versus time at 10 g/L glucose

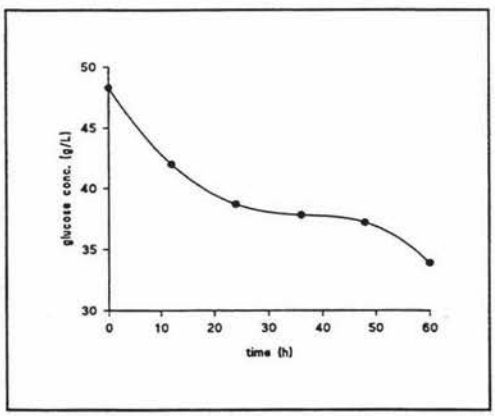

Fig. 13: Substrate concentration versus time at 50 g/L glucose

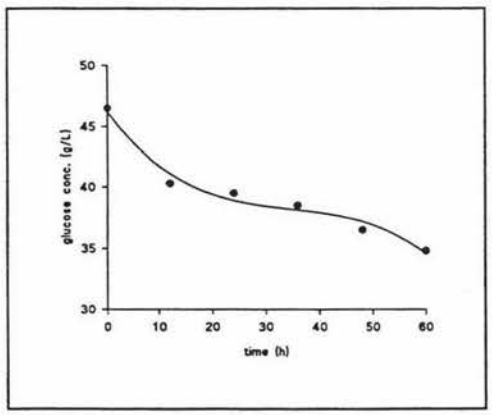

Fig. 14: Substrate concentration versus time at 50 g/L glucose

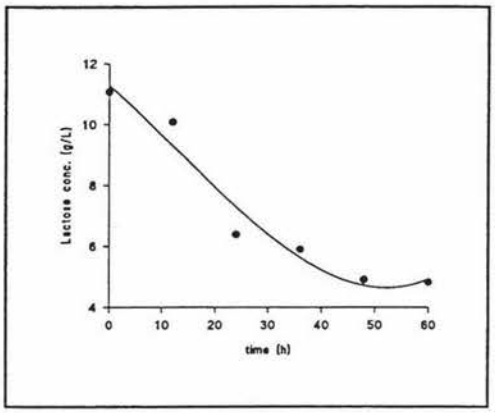

Fig. 15: Substrate concentration versus time at 10 g/L lactose

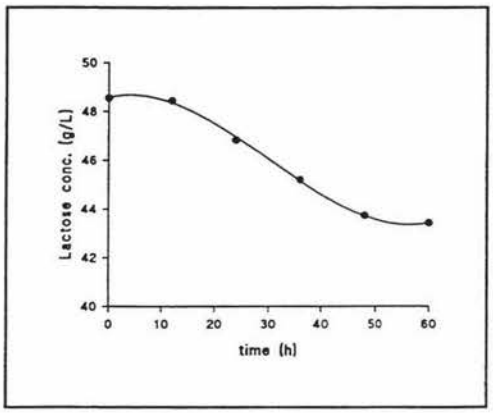

Fig. 16: Substrate concentration versus time at 50 g/L lactose

Specific rate of substrate utilisation

The specific rate of substrate utilisation (Table 6) was calculated as follows;

Specific rate of substrate utilisation = volumetric rate / biomass

where, volumetric rate of utilisation = amount of substrate used/ time,

and biomass concentration was taken at t = 24 h.

Biomass growth yield, $Y_{x/s}$

To calculate the biomass growth yield, $Y_{x/s}$ (Table 6), the biomass concentration was plotted against the amount of substrate consumed (figures 17 - 22). From this plot,

Y = Slope,

where slope was calculated at t ≈ 18 h (see figures 11 - 16).

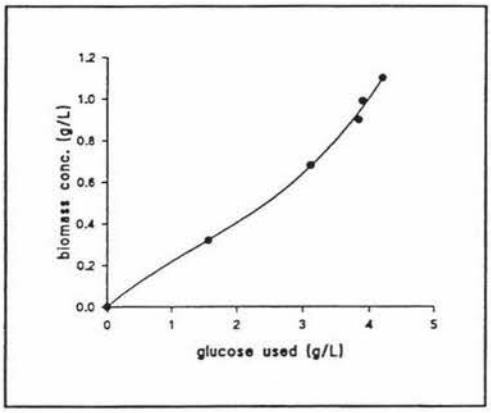

Fig. 17: Substrate concentration versus biomass at 10 g/L glucose

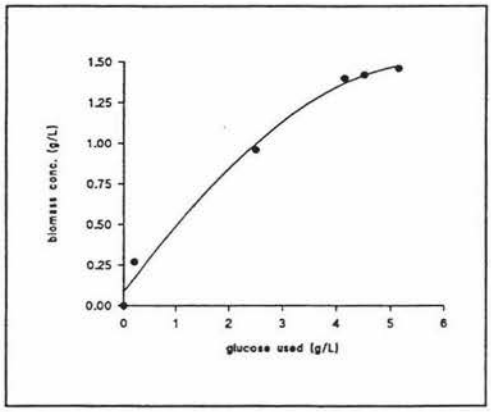

Fig. 18: Substrate concentration versus biomass at 10 g/L glucose

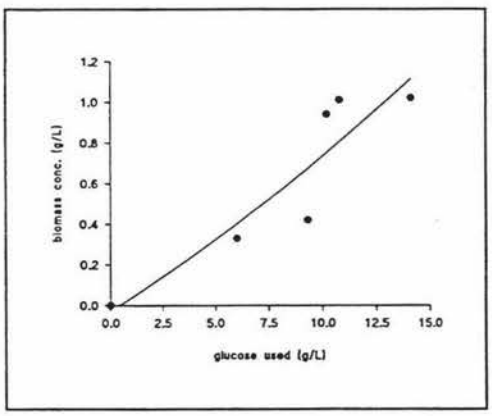

Fig. 19: Substrate concentration versus biomass at 50 g/L glucose

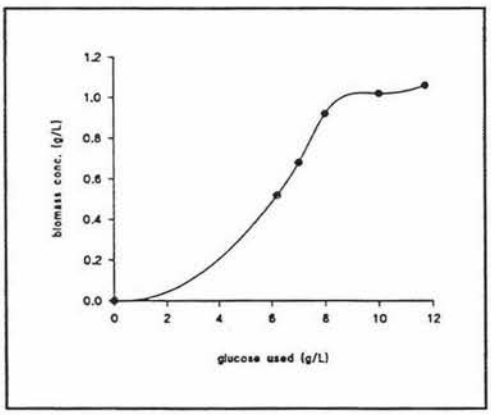

Fig. 20: Substrate concentration versus biomass 50 g/L glucose

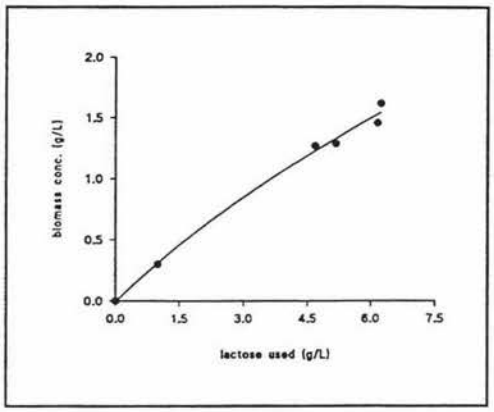

Fig. 21: Substrate concentration versus biomass at 10 g/L lactose

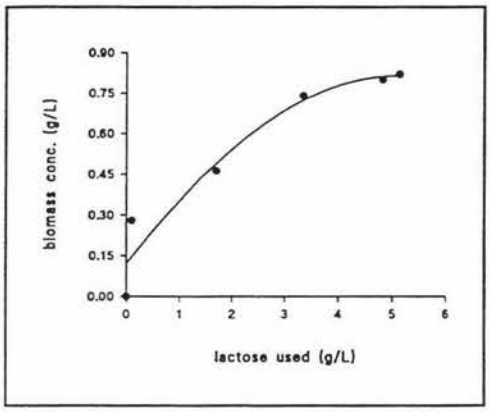

Fig.22: Substrate concentration versus biomass at 50 g/L lactose

Table 6: Rate of substrate utilisation

Substrate (g/L)	Volumetric rate of substrate utilisation (g/L.h)	Specific rate of substrate utilisation ($g/g_{biomass}.h$)	Growth yield, $Y_{x/s}$
10 g/L glucose	0.109	0.160	0.23
10 g/L glucose	0.123	0.128	0.28
50 g/L glucose	0.208	0.167	0.10
50 g/L glucose	0.169	0.131	0.15
10 g/L lactose	0.195	0.151	0.25
50 g/L lactose	0.099	0.124	0.14

The average volumetric rate of substrate utilisation is 0.116 g/L.h at 10 g/L glucose concentration and 0.189 g/L.h at 50 g/L glucose concentration. However, the specific rates of substrate utilisation are 0.144 $g/g_{biomass}$.h at 10 g/L glucose, and 0.149 $g/g_{biomass}$.h at 50 g/L. These values are approximately the same. It is not clear why the volumetric rate of substrate utilisation is higher at 50 g/L glucose than at 10 g/L glucose. For lactose, the volumetric rate of substrate utilisation is 0.195 g/L.h at 10 g/L lactose concentration and 0.099 g/L.h at 50 g/L lactose concentration. The specific rates are 0.151 and 0.124 $g/g_{biomass}$.h respectively. The biomass growth yields (Figures 17 - 22; Table 6) are 0.26 and 0.25 for glucose and lactose at 10 g/L. At 50 g/L, the biomass growth yields are 0.13 and 0.14 for glucose and lactose, respectively. These results indicate that a lot more substrate is used to produce biomass at 50 g/L substrate concentration than is required at 10 g/L. Probably this is because at the higher concentration, more substrate is being converted to products rather than biomass.

4.4.3 Product formation

The fermentation products determined in the experiments were acetic acid, L(+) lactic acid and small amounts of formic acid. D-lactic acid was analysed for, but was found absent. Ethanol was produced in very small ($\leq$ 0.1 g/L) amounts. To determine product formation rate, the concentration of the product was plotted against time (figures 23 - 40; Table 7), and

$$\text{Rate of product formation} = \text{Slope}$$

where the slope is calculated at t = 24 h.

The specific rates of product of formation were calculated as follows;

Specific rate of product formation = volumetric rate/biomass concentration

where, volumetric rate = concentration of product/time, and

biomass concentration was taken at t = 24 h.

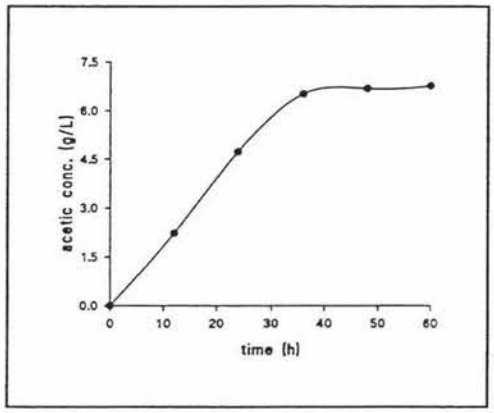

Fig. 23: Acetic acid concentration at 10 g/L glucose

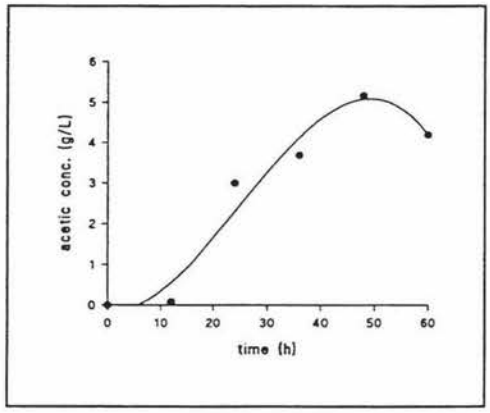

Fig. 24: Acetic acid concentration at 10 g/L glucose

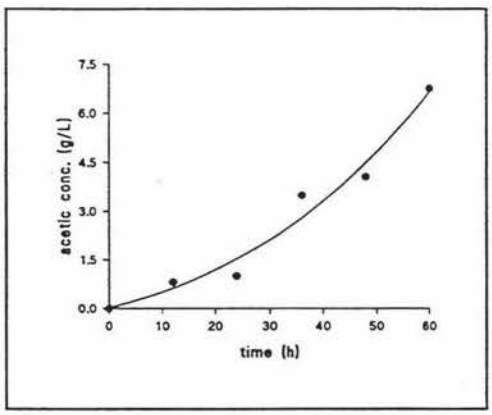

Fig. 25: Acetic acid concentration at 50 g/L glucose

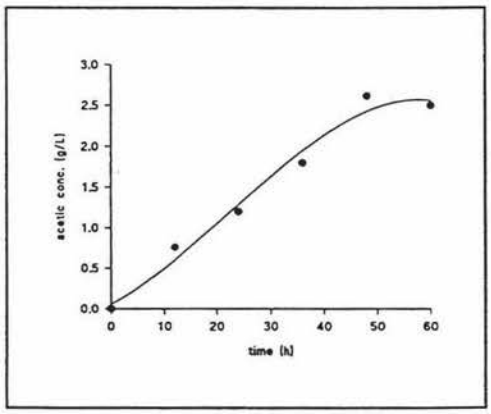

Fig. 26: Acetic acid concentration at 50 g/L glucose

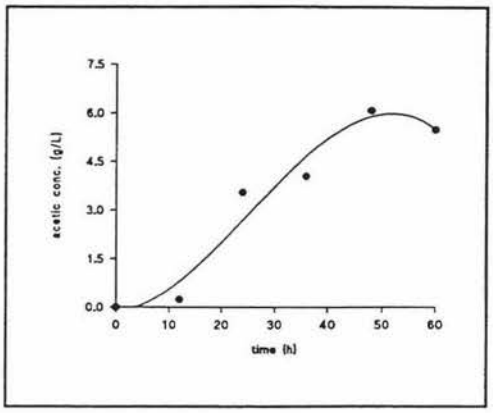

Fig. 27: Acetic acid concentration at 10 g/L lactose

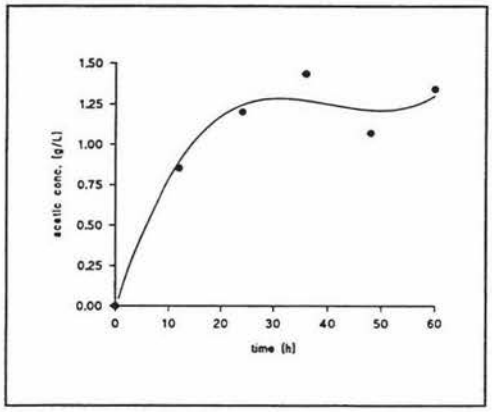

Fig. 28: Acetic concentration at 50 g/L lactose

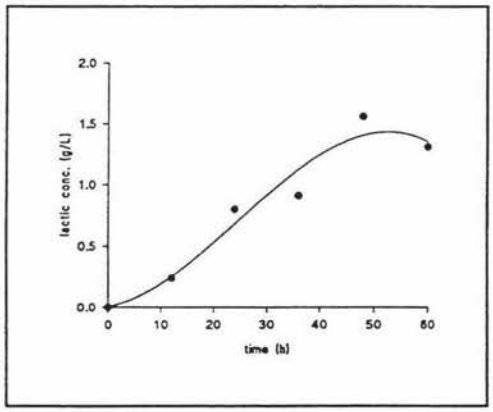

Fig. 29: Lactic acid concentration at 10 g/L glucose

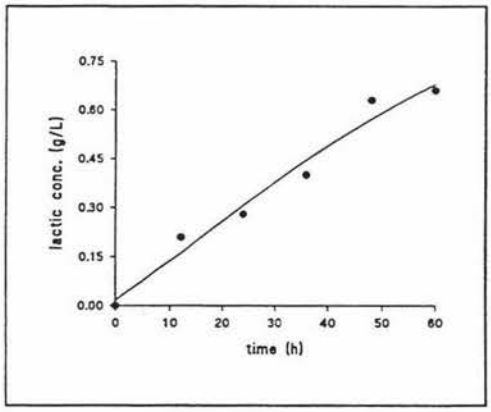

Fig. 30: Lactic acid concentration at 10 g/L glucose

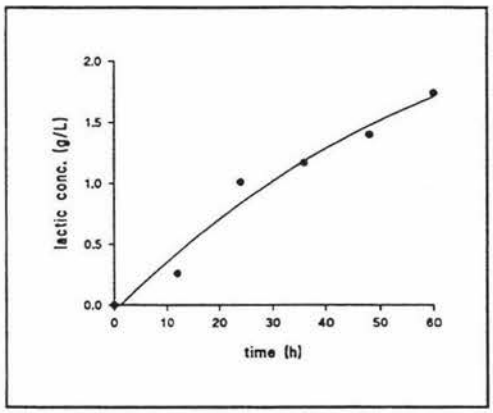

Fig. 31: Lactic acid concentration at 50 g/L glucose

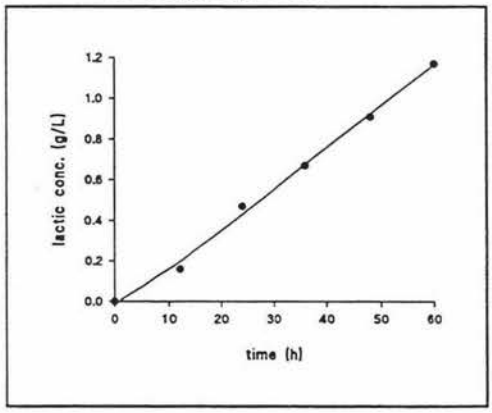

Fig. 32: Lactic acid concentration at 50 g/L glucose

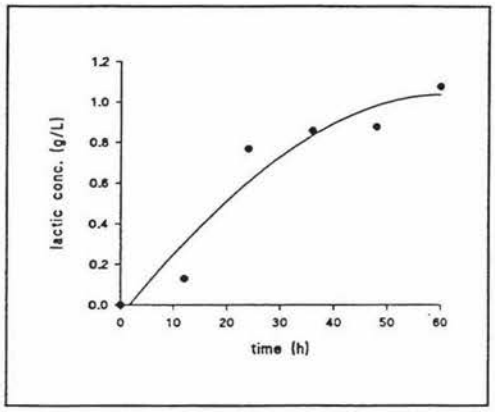

Fig. 33: Lactic acid concentration at 10 g/L lactose

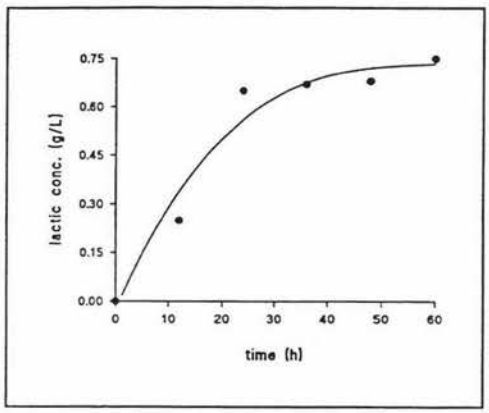

Fig.34: Lactic acid concentration at 50 g/L lactose

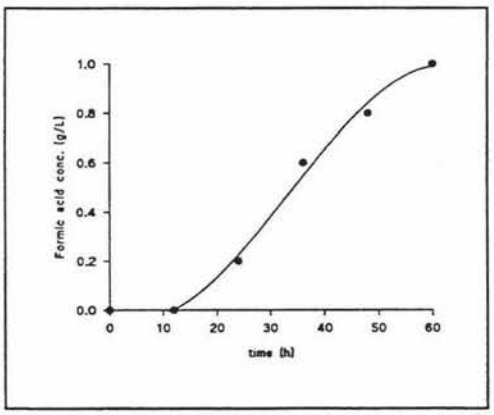

Fig. 35: Formic acid concentration 10 g/L glucose

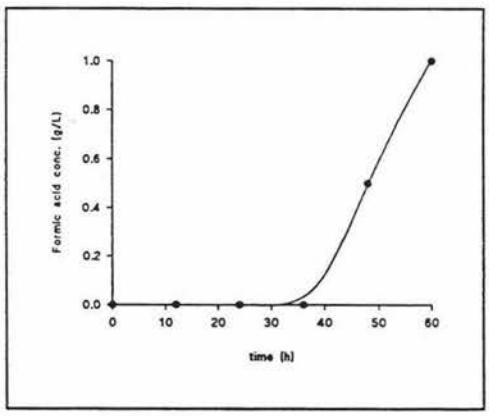

Fig. 36: Formic acid concentration at 10 g/L glucose

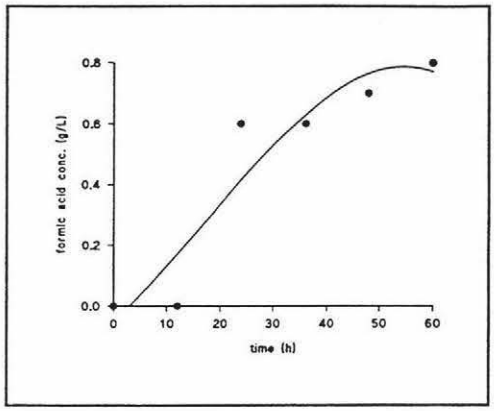

Fig. 37: Formic acid concentration at 50 g/L glucose

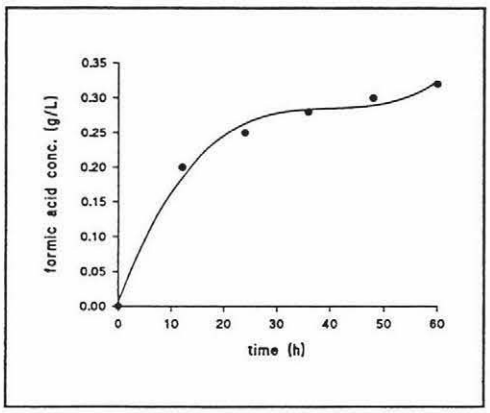

Fig.38: Formic acid concentration at 50 g/L glucose

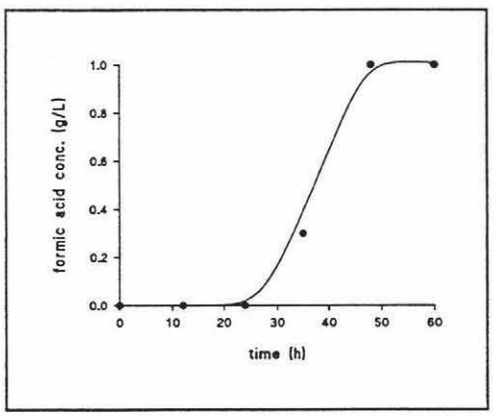

Fig. 39: Formic acid concentration at 10 g/L lactose

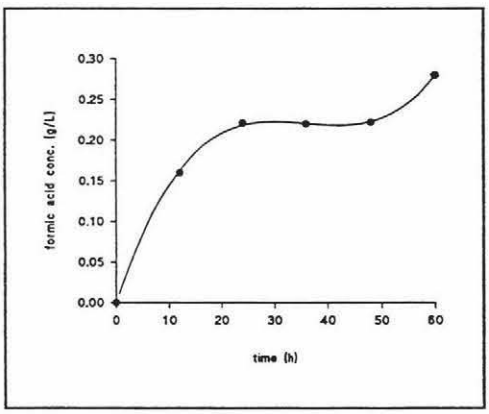

Fig. 40: Formic acid concentration at 50 g/L lactose

Table 7: Volumetric rates of product formation

Substrate	Acetic acid production (g/L.h)	Lactic acid production (g/L.h)	Formic acid production (g/L.h)
10 g/L glucose	0.117	0.033	0.019
10 g/L glucose	0.088	0.025	0.015
50 g/L glucose	0.092	0.042	0.015
50 g/L glucose	0.050	0.020	0.019
10 g/L lactose	0.109	0.018	0.020
50 g/L lactose	0.050	0.027	0.009

Table 8: Specific rates of product formation

Substrate	Acetic acid production ($g/g_{biomass}.h$)	Lactic acid production ($g/g_{biomass}.h$)	Formic acid production ($g/g_{biomass}.h$)
10 g/L glucose	0.172	0.049	0.023
10 g/L glucose	0.130	0.036	0.016
50 g/L glucose	0.065	0.030	0.013
50 g/L glucose	0.049	0.020	0.015
10 g/L lactose	0.115	0.014	0.013
50 g/L lactose	0.063	0.034	0.011

The fermentation balances were calculated at t = 24 h. The amount of substrate used, acetic acid, lactic acid and formic acid produced at this time were measured and the obtained values used to calculate the balances in moles and the carbon recovery in percentages.

Table 9: Fermentation balances in moles/mole of substrate used and carbon recovery for *B. bifidum*

Substrate	Acetic acid (mol.)	Lactic acid (mol.)	Formic acid (mol.)	A: L ratio	%C - recovery
10 g/L glucose	2.00	0.45	0.20	4.4: 1	92.5
10 g/L glucose	2.18	0.35	0.50	6.2: 1	95.2
50 g/L glucose	2.20	0.24	0.22	9.2: 1	89.0
50 g/L glucose	2.09	0.25	0.13	8.4: 1	76.7
10 g/L lactose	4.55	0.69	0.46	6.6: 1	93.3
50 g/L lactose	4.00	0.55	0.81	7.3: 1	87.2

The volumetric and specific rates of product formation for acetic acid, lactic acid and formic acid show a lot of variation at both substrates and at the different concentrations. The volumetric rates range from 0.050 to 0.117 g/L.h for acetate, 0.018 to 0.042 g/L.h for lactate and 0.009 to 0.020 g/L.h for formate. At 50 g/L glucose and 50 g/L lactose concentrations, the rates are much lower than at 10 g/L glucose and lactose concentrations (Table 7 and 8). The same observation was made with the volumetric rate of substrate utilisation.

There is variation in the fermentation balances of the products. The calculated balances indicate that more acetic acid (in moles) is produced during the fermentation of one mole of lactose than during the fermentation of one mole of glucose (Table 9). This is to be expected since lactose is a disaccharide. Overall, the fermentation balance obtained in this

study are different from the theoretical;

2 moles hexose → 3 moles acetate : 2 moles lactate, (De Vrries and Stouthamer, 1968; Scardovi, 1991). DeVries and Stouthamer (1968) however, observed that this ratio to be true and that *Bifidobacterium* fermented glucose via the fructose-6-phosphate phosphoketolase route. These differences can be attributed to pH variations during the fermentation, as well as to species and strain differences. Moreover, the theoretical ratio of 1.5 moles of actetate to 1.0 mole of lactate per mole of hexose is scarcely ever found in growing cultures of bifidobacteria (Scardovi, 1991). Lauer and Kandler, 1976, observed varying ratios of acetate to lactate when studying the fermentation of different species of *Bifidobacterium.* One strain of *B. pseudolongum* gave a ratio of about 14.5 when grown on glucose at pH 5.38. The reason for such variations is that the phosphoroclastic cleavage of some pyruvate to formic acid and acetyl phosphate and reduction of acetyl phosphate to ethanol often alters the fermentation balance. In this study, it was observed that the acetic acid to lactic acid ratio was much higher when glucose was used at 50 g/L than at 10 g/L. It is not understood why this was so.

Carbon recovery values range from 76.7 - 95.2%. The highest carbon recovery values were obtained at 10 g/L glucose (93.9%) and 10 g/L lactose (93.3%). The 6 - 7% difference needed to make complete recovery (100%), has gone to biomass production and experimental error. In general, all the carbon put into the fermentation was recovered.

4.4.4 Changes in pH during fermentation

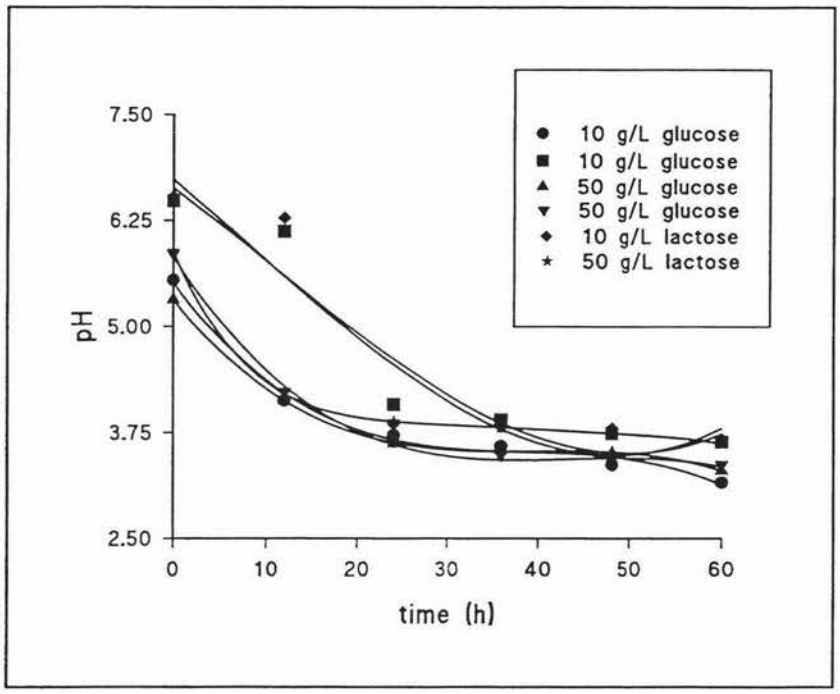

Fig. 41: Plot of pH versus time

The organism stopped growing at about 24 - 36 hours. At this point, there was an appreciable drop in the pH of the growth medium (Fig. 41). The pH dropped at least 2 units in all the experiments. The organism stopped growing, even though there was still substrate left in the medium. This could mean that the organism could not survive and grow at high acidity, or that there was some other factor such as product inhibition that stops the organism from growing.

In an effort to explain why growth ceased after only a few hours of fermentation, the experiments were carried out at controlled pH. This would eliminate the vagaries of pH

changes during the fermentation and perhaps remove the differences between duplicate experiments.

4.5 GROWTH AT CONTROLLED pH

The objective of this experiment was to eliminate the vagaries of pH changes during fermentation of *B. bifidum*. It was previously found (section 4.2) that the organism ceases to grow after about 24 hours of incubation. However, it could not be determined whether cessation of growth was a result of changes in culture pH or product inhibition or a combination of both.

Different substrates were used, under the same pH conditions, to determine if there were any differences in the growth of the organism when different carbohydrates sources were used. The substrates used were glucose, lactose, maltose, raffinose, cellobiose, lactulose and xylose. The substrate concentration used was 50 g/L for glucose and lactose, and 40 g/L for maltose, raffinose, cellobiose, lactulose and xylose.

These experiments were carried out on the 1.2-L scale. The initial pH of the medium was adjusted to pH 5.5 and maintained at that value throughout the fermentation by dosing with 4M NaOH, when it dropped below the set value.

4.5.1 Different substrates at pH 5.5

4.5.1.1 Rate of growth

To calculate the specific rates of growth of *B. bifidum* (Table 9), Log_{10} of biomass was plotted against time for each substrate (Fig. 42) and the rate calculated as,

$$\text{Specific rate of growth} = \mu = 2.303 \times \text{Slope},$$

where the slope was measured during initial growth ($t \leq 12$ h).

and the doubling time, t_d as,

$$t_d = (2.303 \times \text{Log } 2)/\mu$$

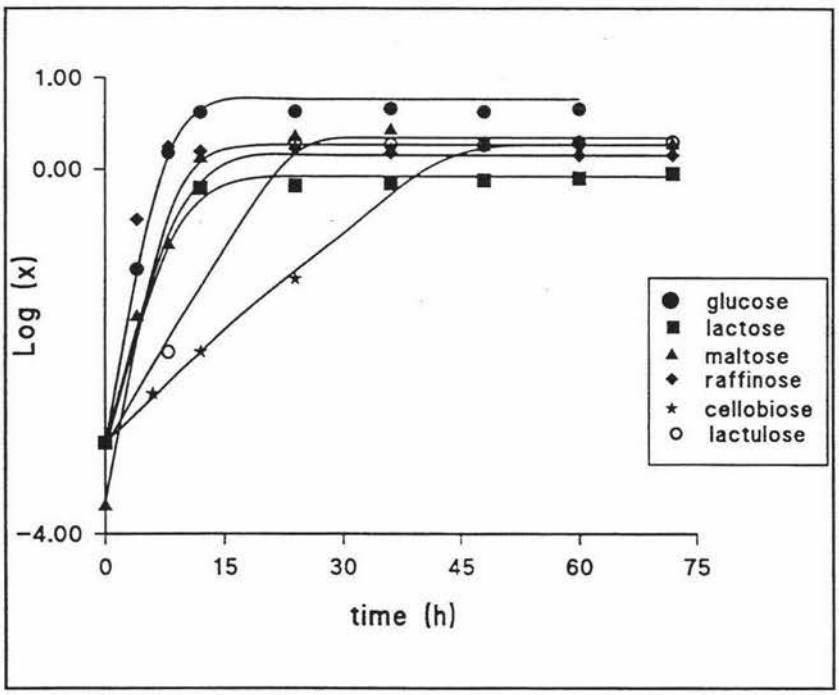

Fig. 42: Log_{10} (x) versus time at controlled pH and different sugars

Table 10: Specific rates of growth of *B. bifidum* at pH 5.5

Substrate	Maximum biomass concentration (g/L)	Specific rate of growth (/h)	Doubling time, t_d (min)
Glucose	4.58	0.699	59.5
Lactose	0.90	0.536	77.6
Maltose	2.64	0.705	59
Raffinose	1.68	0.597	69.7
Cellobiose	1.99	0.199	209
Lactulose	2.00	0.227	183.2
Xylose	No growth	0	-

The specific rate of growth of *B. bifidum* at pH 5.5 ranged from 0.199 to 0.705 (Table 10). These values indicate that *B. bifidum* grows fastest on glucose and maltose. The specific rate of growth for these substrates was approximately the same, 0.699 and 0.705 h^{-1}. The specific rates of growth, on raffinose and lactose were next, at 0.597 and 0.536 h^{-1} respectively. Lactulose and cellobiose supported poor growth, with rates of 0.227 h^{-1} and 0.199 h^{-1}, respectively.

The organism did not ferment xylose. This is contrary to the findings of De Vries and Stouthamer (1968), who found that *B. bifidum* fermented xylose to acetate, lactate, formate and ethyl alcohol.

In this experiment, more biomass was produced, the specific rates were higher and the doubling times were reduced approximately 2-fold compared to fermentation at uncontrolled

pH (Tables 5 and 9) . From these results, it can be said with certainty that low pH has a detrimental effect on the growth of *B. bifidum.* Therefore, in order to achieve maximum production of biomass from this organism, it is important that pH be controlled, in addition to other applicable growth conditions.

4.5.1.2 Substrate utilisation

To calculate the volumetric rate of substrate utilisation (Table 11), a graph of substrate concentration was plotted against time (Fig. 43), and the rate calculated as;

Rate of substrate utilisation = - Slope

where slope was measured at $t \leq 24$ h.

The specific rate of substrate utilisation (Table 11), was calculated as follows,

Specific rate of substrate utilisation = volumetric rate/biomass concentration

where volumetric rate = amount of substrate used/time

and biomass concentration = biomass at $t \approx 18$ h.

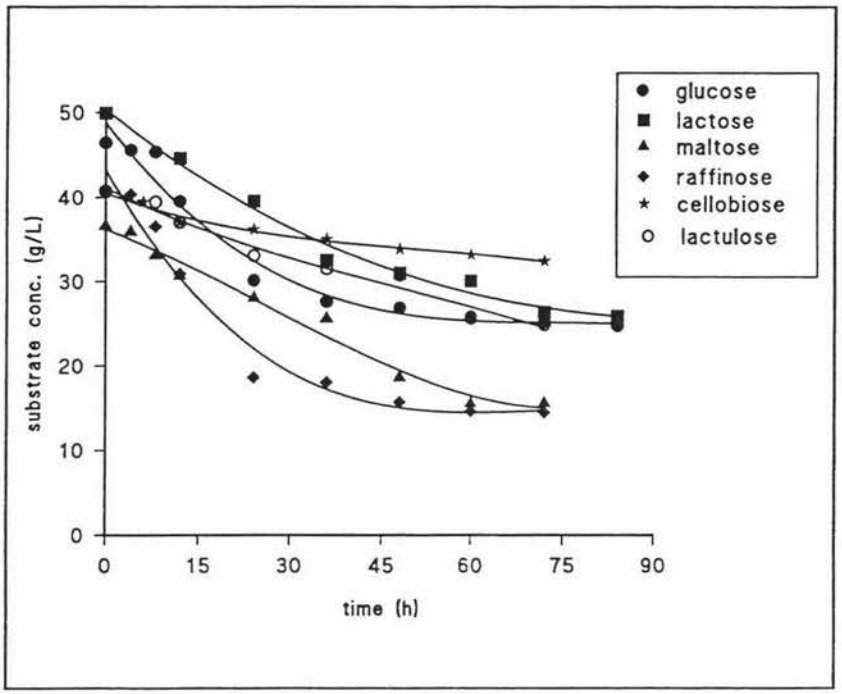

Fig. 43: Substrate concentration versus time at controlled pH

To calculate the yield, $Y_{x/s}$, (Table 11), biomass concentration was plotted against substrate consumed (Fig. 44), and,

$$Y_{x/s} = \text{Slope}$$

where slope was measured at $t \leq 24$ h.

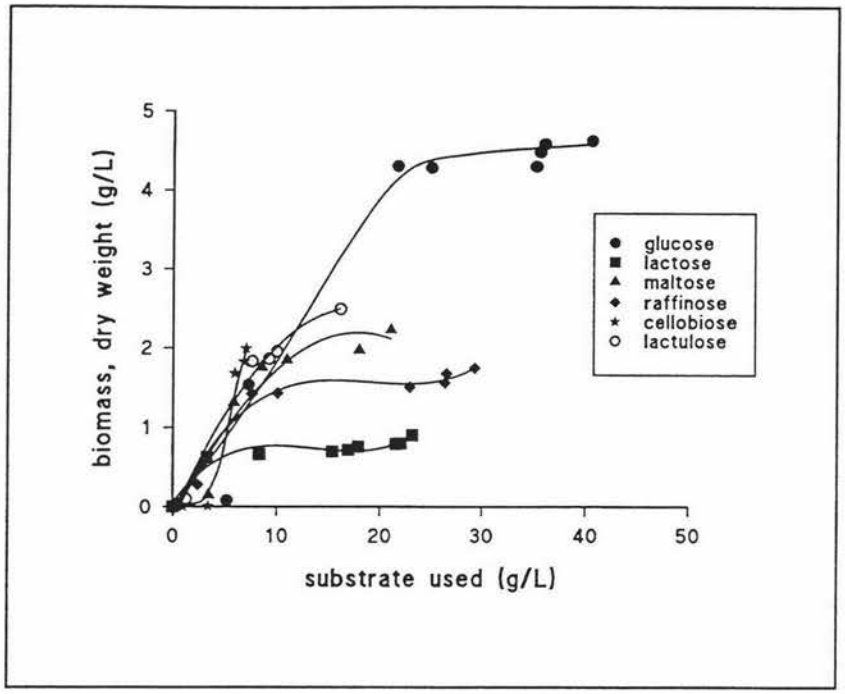

Fig. 44: Biomass produced versus substrate used

Table 10: Rates of substrate utilisation and biomass yield

Substrate	Volumetric rate of substrate utilisation (g/L.h)	Specific rate of substrate utilisation ($g/g_{biomass}.h$)	Biomass Yield
Glucose	0.722	0.314	0.226
Lactose	0.432	0.480	0.037
Maltose	0.514	0.221	0.219
Raffinose	0.728	0.434	0.144
Cellobiose	0.286	0.171	0.281
Lactulose	0.288	0.144	0.176

The volumetric rates of substrate utilisation ranged from 0.286 g/L.h for cellobiose to 0.728 g/L.h for maltose (Table 10). The specific rates of substrate utilisation ranged from 0.171 $g/g_{biomass}.h$ to 0.480 $g/g_{biomass}.h$. Lactose and raffinose showed high specific substrate utilisation rates at 0.480 and 0.434 $g/g_{biomass}.h$. respectively. It is not understood why this is so. However, similar results were observed for the volumetric rate of lactose utilisation (at 10 g/L) at uncontrolled pH experiments. Lactulose and cellobiose showed the lowest specific rates of substrate utilisation, at 0.144 and 0.171 $g/g_{biomass}.h$, respectively. Maltose seemed to behave as glucose, though the calculated rates were somewhat lower than those for glucose, with specific rates of 0.314 $g/g_{biomass}.h$ for the former and 0.221 $g/g_{biomass}.h$ for the latter. The volumetric rates were 0.514 and 0.722 g/L.h, respectively.

The highest biomass yield was obtained when glucose, maltose and cellobiose were used as substrate (0.226, 0.219 and 0.281), (Table 10). However, though the biomass yield for cellobiose is high, the organism went through a long lag period (~ 5 hour), even though it had been pre-cultured on the same substrate. Once growth took place, biomass production was relatively rapid, with relatively low consumption of substrate.

As already indicated, the low biomass yield shown by lactose is notclearly understood. But since the yield was low for lactose (50 g/L) at uncontrolled pH experiments as well, it is strongly possibile that at high concentrations of this substrate substrate is used for products formation instead of being used solely for biomass production.

4.5.1.3 Product formation

The initial volumetric rates of product formation were calculated from a plot of concentration of substrate against time (Fig. 45 - 47; Table 12) and,

Rate of product formation = Slope

The rates were calculated for each product, that is, acetic, lactic and formic acids at $t \leq 24$ h.

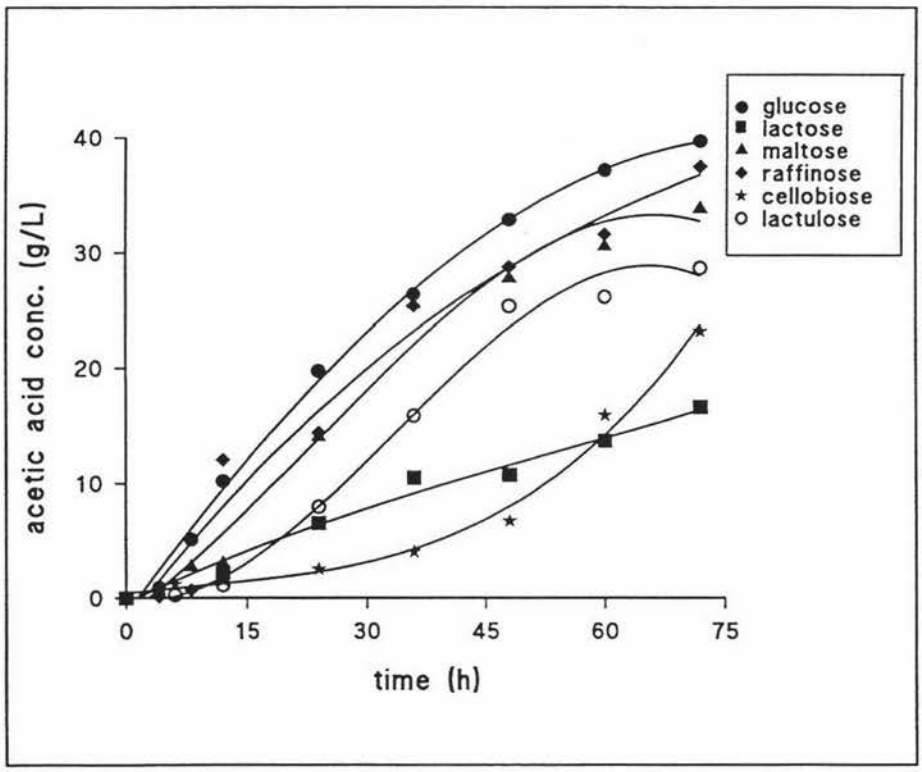

Fig 45: Acetic acid concentration versus time

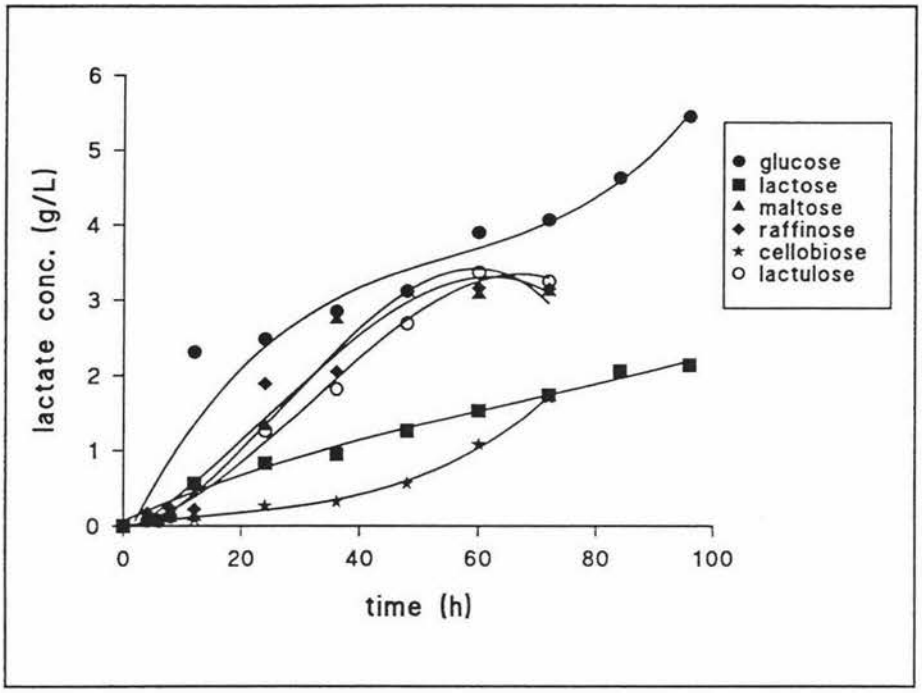

Fig. 46: Lactic acid concentration versus time

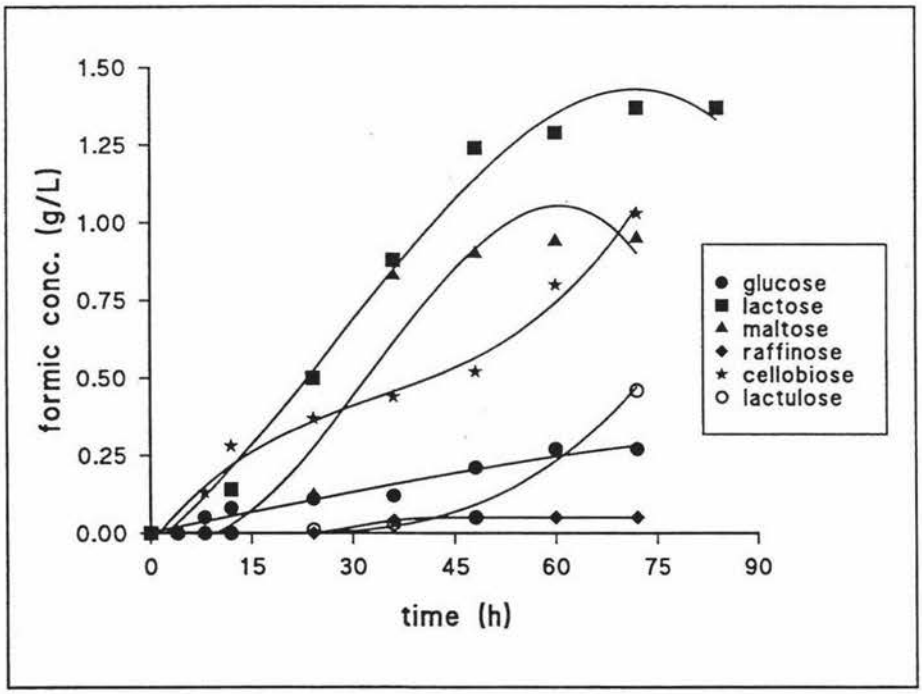

Fig. 47: Formic acid concentration versus time

Table 11: Volumetric rates of product formation

Substrate	Rate of acetic acid production (g/L.h)	Rate of lactic acid production (g/L.h)	Rate of formic acid production (g/L.h)
Glucose	0.871	0.120	0.005
Lactose	0.274	0.035	0.025
Maltose	0.594	0.055	0.020
Raffinose	0.692	0.078	0.001
Cellobiose	0.099	0.011	0.012
Lactulose	0.344	0.056	0.001

Specific rates of production formation (Table 12) were calculated as follows,

Specific rate of product formation = Volumetric rate of formation/biomass concentration

where, volumetric rate of product formation = product concentration/time

and biomass concentration taken where the slope was measured ($t \leq 18$ h).

Table 12: Specific rates of product formation

Substrate	Specific rate of acetic acid production ($g/g_{biomass}.h$)	Specific rate of lactic acid production ($g/g_{biomass}.h$)	Specific rate of formic acid production ($g/g_{biomass}.h$)
Glucose	0.256	0.035	0.001
Lactose	0.303	0.039	0.028
Maltose	0.226	0.025	0.009
Raffinose	0.412	0.046	0.001
Cellobiose	0.157	0.017	0.020
Lactulose	0.173	0.028	0.001

The volumetric rate of acetic and lactic acids formation is highest for glucose, 0.871 and 0.120 g/L.h, respectively, followed by raffinose, then maltose and lactulose (Table 11). When cellobiose was used as the substrate, the lowest rates of product formation were obtained, 0.099 g/L.h for acetic acid and 0.011 g/L.h for lactic acid. The rate of production of formic acid is very low for all six substrates.

In terms of specific rate of acetic formation, raffinose had the highest rate, 0.412 $g/g_{biomass}.h$, followed by lactose, glucose and maltose at 0.303, 0.256, and 0.226 $g/g_{biomass}.h$. Lactulose and cellobiose had the lowest specific rates of acetic acid production at 0.173 and 0.157 $g/g_{biomass}.h$, respectively. More or less the same trend was observed in the specific rates of formation of lactic acid and formic acid.

Unlike in the controlled pH experiments, it was observed that the specific rates of products formation are higher when lactose was the substrate than they were when glucose was used. It would be expected that glucose, maltose, lactulose and cellobiose would have the highest rates of production (in that order) since they had the highest biomass concentrations. However, this is not so. A possible explanation for this occurrence is that since initial rates were measured, product formation ceased at some point further during the course of fermentation, due to inhibition of some form, possibly product.

Table 14: Fermentation balances per mole of substrate and carbon recovery

Substrate	Acetic acid (mol)	Lactic acid (mol)	Formic acid (mol)	A: L ratio	% Carbon recovery
Glucose	2.16	0.33	0.27	6.5: 1	93.0
Lactose	4.06	0.35	0.44	11.6: 1	84.5
Maltose	4.70	0.30	0.16	15.6: 1	88.8
Raffinose	6.50	0.56	0.03	11.6: 1	81.7
Cellobiose	4.33	0.23	0.70	18.8: 1	83.8
Lactulose	3.94	0.37	0.11	10.6:1	75.8

A = acetic acid L = lactic acid

The fermentation balances were calculated at t = 24 h, and were calculated per mole of substrate. From these balances, carbon recovery percentages were calculated. The ratios of acetic acid produced to lactic acid were different for all the substrate, ranging from 6.5: 1 to 18.8: 1 (Table 13). The lowest ratio was obtained when glucose was used while the highest ratio was obtained when cellobiose was used. It appears that these variations are significant, although there might have been some experimental error. However, the diffrences of the ratios obtained for sugars could be a result of experimental error. For example the differences between the A: L ratios for lactose, raffinose and lactulose were probably a result of experimental error. At uncontrolled pH experiments, it was also found that these ratios (A: L), varied a lot (Table 9), within the same substrate, and even within duplicate experiments. It was previously shown that pH has an effect on biomass production and therefore product formation. From the A: L ratios in this experiment, it can be suggested that substrate also affects the rate of products formation. It was not clear how this was so because no particular trend of the A: L ratios was observed.

The amounts of carbon recovered also varied (Table 13). Carbon recovery was highest on glucose, at 93.0%, followed by maltose, lactose, cellobiose and raffinose at 88.8, 84.5, 83.8 and 81.7%, successfully. The carbon recovery from lactulose was 75.8. It should be noted, however, that the carbon recovery percentage was calculated in terms of acetic, lactic and formic acids production. This value does not take into account the biomass produced during the fermentation. Even though inclusion of biomass in calculating the carbon recoveries on the other substrates would not make much of a difference, on glucose, together with experimental error, complete carbon recovery would probably be obtained. On the other substrates used, it not understood where the unrecovered carbon went, unless there might have been formation of another product that was not analysed for. When De Vries and Stouthamer, (1968) studied the fermentation of glucose, lactose, galactose, mannitol and xylose by bifidobacteria, they too did not recover all the carbon. They obtained carbon recovery values ranging from 81 to 97%.

4.6 GROWTH ON MIXED SUBSTRATES

The objective of this experiment was to determine the pattern of growth and substrate utilisation when *B. bifidum* was grown on mixed substrates. Often, sequential utilisation of substrates is observed in mixed substrate batch cultures. The phenomenon is known as diauxie and is characterised by the more readily available substrate being used first, followed by the next available one until all substrates in the mixture have been used. However, in practice this is not always so, since there are other factors which determine how micro-organisms behave.

This experiment was carried out on the 1.2-L scale and the pH culture was controlled at pH 5.5. A mixture of two substrates was used, namely, 15 g/L glucose and 15 g/L lactose.

The growth parameters were then determined as described in previous sections.

4.6.1 Specific rate of growth

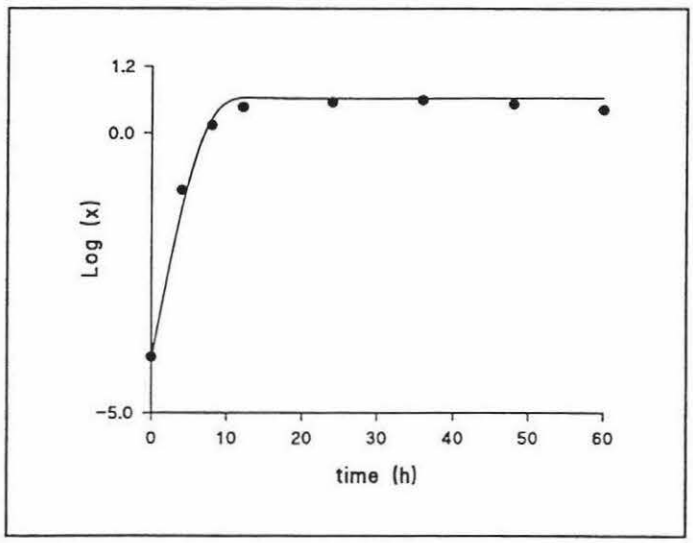

Fig.48: Log_{10} (x) versus time on mixed substrates

4.6.2 Substrate utilisation

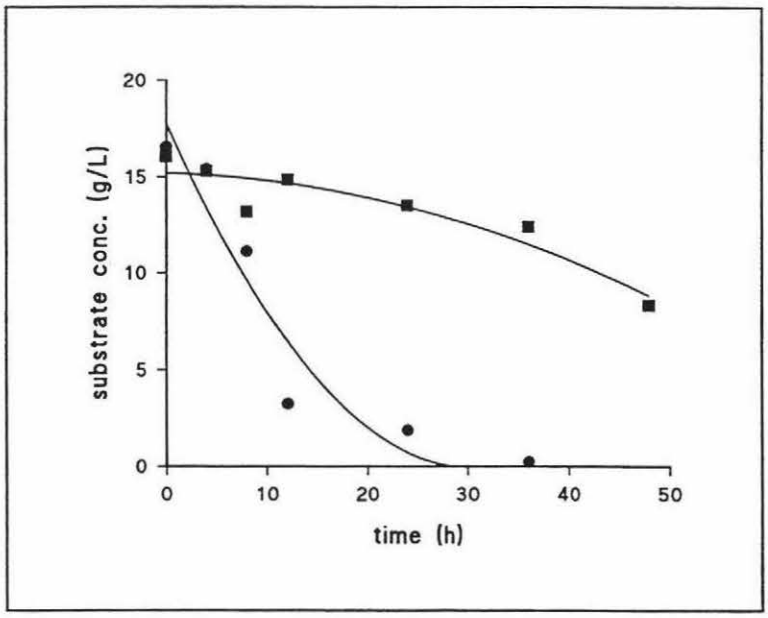

Fig. 49: Substrate concentration versus time on mixed substrates

4.6.3 Product formation

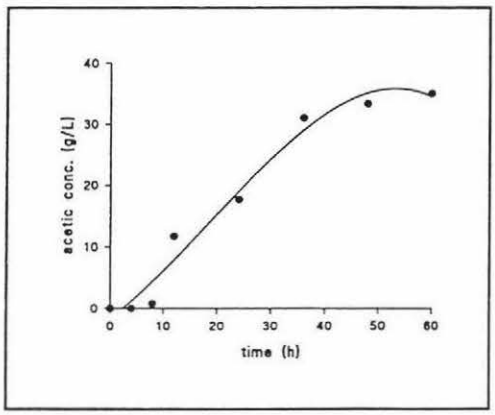

Fig. 50: Acetic acid concentration versus time on mixed substrates

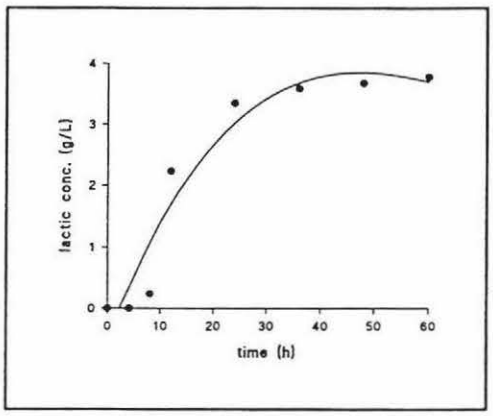

Fig. 51: Lactic acid concentration versus time mixed substrates

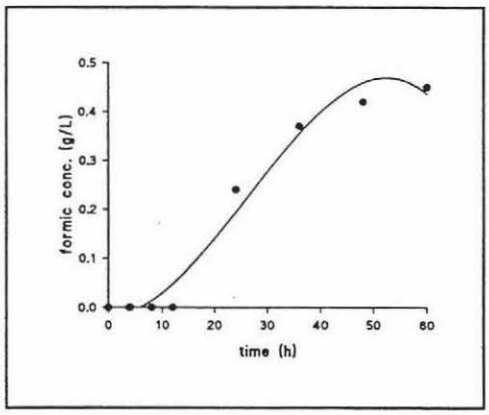

Fig. 52: Formic acid concentration versus time

Table 15: Growth parameters of *B. bifidum* growing in a mixture of glucose and lactose

Maximum biomass concentration (g/L)	3.93 g/L
Specific rate of growth, μ	0.84 h^{-1}
Doubling time, $t_{1/2}$	49.5 min.
Volumetric rate of glucose utilisation	0.672 g/L.h
Specific rate of glucose utilisation	0.187 $g/g_{biomass}.h$
Volumetric rate of lactose utilisation	0.131 g/L.h
Specific rate of lactose utilisation	0.04 $g/g_{biomass}.h$
Yield, $Y_{x/s}$ (overall)	0.21
Acetic acid production rate	0.830 g/L.h
Specific rate of acetic acid production	0.231 $g/g_{biomass}.h$
Lactic acid production rate	0.157 g/L.h
Specific rate of lactic acid production	0.044 $g/g_{biomass}.h$
Formic acid production rate	0.011 g/L.h
Specific rate of formic acid production	0.003 $g/g_{biomass}.h$

When equal amounts of glucose and lactose were mixed, the organism used both substrates simultaneously, (Fig 48; Table 15). However, glucose was used faster than lactose , and was completely utilised, while lactose was not. The specific rate of growth was the highest found in this study. The rates of product formation for acetic acid and lactic acid also varied (Figs. 50, 51 and 52; Table 15). It appears the substrates supplement each other and so the specific rates on individual substrates are cumulative.

The volumetric glucose utilisation rate is approximately equal to the one obtained on controlled pH experiments (Table 10 and 14), whereas the specific rate is lower than at controlled pH experiments. Lactose utilisation is much lower than when the sugar was used individually, at controlled experiments. There was not much difference to the biomass yield compared to when glucose was used individually at controlled pH. Biomass yield was calculated for the overall experiment rather than for the individual sugars since it was not possible to separate the biomass concentration into that produced by glucose and that produced by lactose.

Even though both substrates got used up simultaneously, it seems the organism had a preference for glucose over lactose. In this experiment also, growth stopped even though there was still substrate remaining. This seemed to confirm earlier suspicions that there was product inhibition taking place.

4.7 EFFECT OF pH VALUE ON GROWTH OF *B. bifidum*

The objective of this experiment was to compare the growth of *B. bifidum* at different pH values. The pH was controlled at pH 4.9, 5.5 and 6.5. The experiments were carried out the 1.2-L scale (section 3.2.3). Glucose (50 g/L) was used as the substrate at all three pH values, while lactose (g/L) was used at pH 5.5 and 6.5.

The specific rate of growth, rates if sunstrate utilisation, rates of product formation and biomass yield were calculated as in previous experiments.

4.7.1 Specific rate of growth

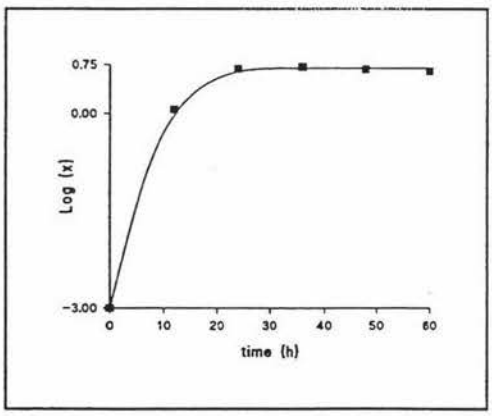

Fig. 53: Plot of Log_{10} (x) versus time at pH 4.9

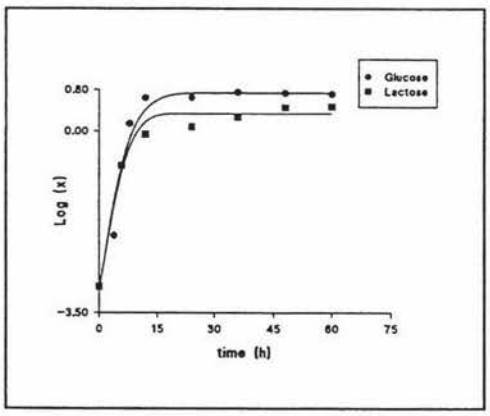

Fig. 54: Plot of Log_{10} (x) versus time at pH 6.5

Table 16: Specific rates of growth at different pH values

pH	Rate of growth, μ (/h)	
	Glucose	Lactose
4.9	0.587	ND
5.5	0.699	0.536
6.5	0.751	0.564

ND = Not done

The measured values for specific rate of growth at different pH were all different. The μ-values increased with increasing pH (Table 15; Fig. 55). This observation occurred when glucose was used as the substrate as well as when lactose was used. However, the increase was slight when lactose was the substrate. Since there are only two values to compare for lactose, much cannot be said at this stage.

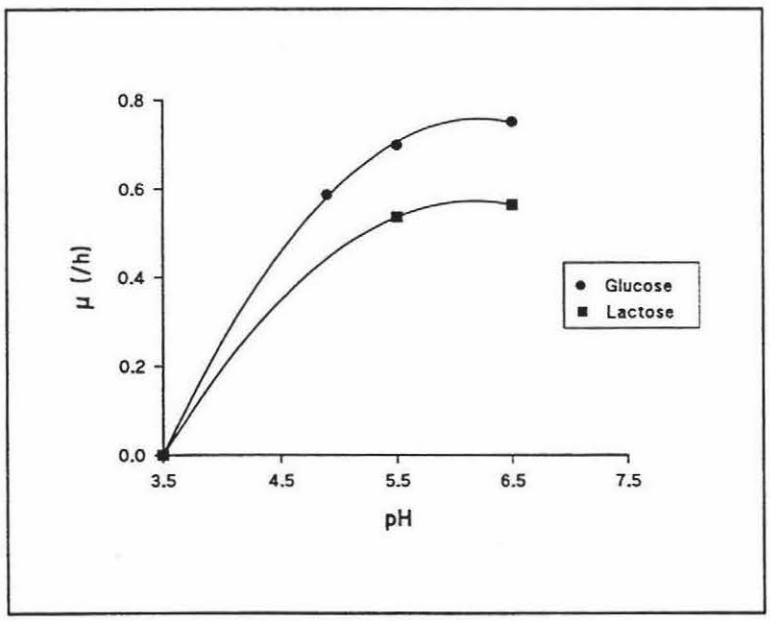

Fig 55: Plot of μ against pH

It should be noted that pH 3.5 is recorded as zero for both μ and Y (Fig. 55) This is because it had previously been determined that no growth occurred at this pH (section 4.1).

4.7.2 Substrate utilisation

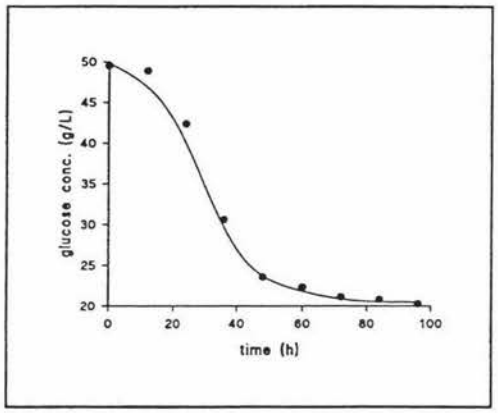

Fig. 56: Substrate concentration versus time at pH 4.9

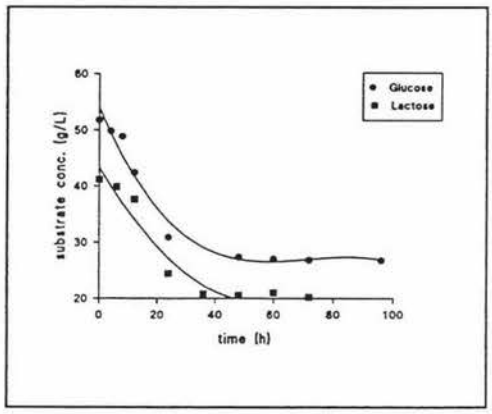

Fig. 57: Substrate concentration versus time at pH 6.5

Biomass Yield

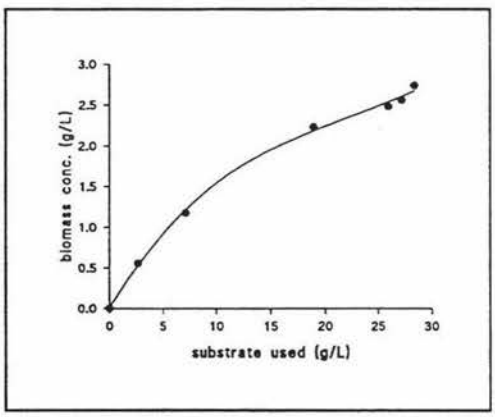

Fig. 58: Biomass concentration versus versus substrate used at pH 4.9

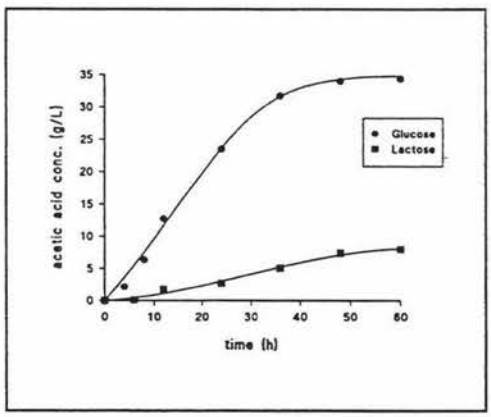

Fig. 59: Biomass concentrattion substrate used at pH 6.5

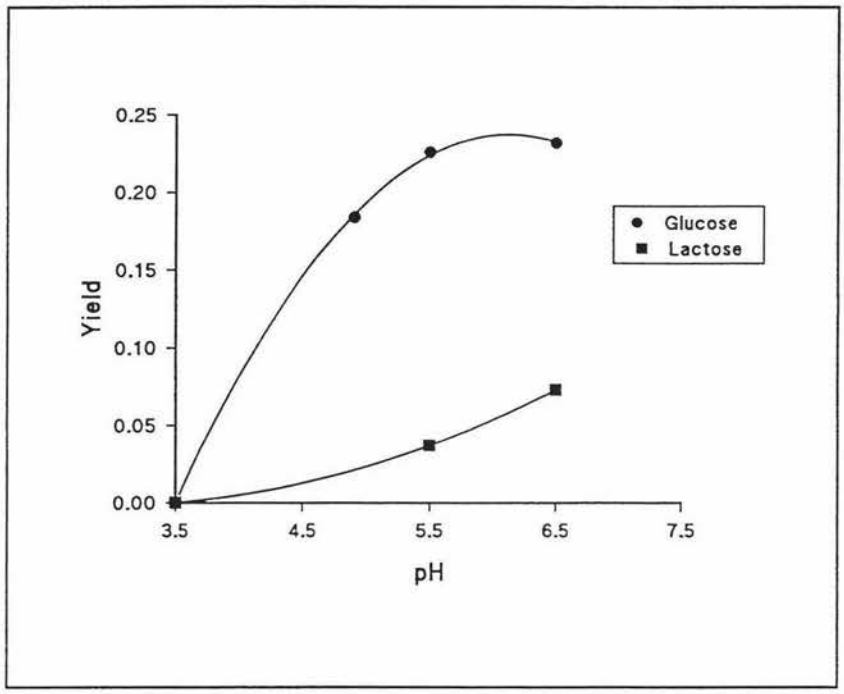

Fig 60: Plot of Biomass Yield against pH

Table 17: Rate of glucose utilisation and biomass yields at different pH values

pH	Substrate utilisation (g/L.h)	Specific substrate utilisation ($g/g_{biomass}.h$)	Yield, $Y_{x/s}$
4.9	0.300	0.105	0.184
5.5	0.722	0.314	0.226
6.5	0.907	0.342	0.232

Table 18: Rate of lactose utilisation and yields at different pH values

pH	Substrate utilisation (g/L.h)	Specific substrate utilisation ($g/g_{biomass}.h$)	Yield, $Y_{x/s}$
4.9	ND	ND	ND
5.5	0.432	0.480	0.037
6.5	0.714	0.585	0.073

From Tables 17 and 18, it can be seen that rates of substrate utilisation increased with increasing pH value. This observation was true for both volumetric and specific rates (Fig. 53) on glucose as well as on lactose. The biomass yield also showed a similar trend for both sugars (Tables 17 and 18; Figure 59).

These observations were to be expected since it was found that the specific rate of growth increased with increasing pH (Table 17). It is also worth noting that the specific rates of lactose utilisation at the different pH values (5.5 and 6.5) were much higher than glucose utilisation rates and the biomass yield was quite low. Since the experiments were carried out separately, and at different times, experimental error was ruled out as the cause of this observation. It does then seem that growth of this particular strain of *B. bifidum* was inhibited by high concentrations of lactose. A higher biomass yield (as high as on glucose) was achieved at uncontrolled pH experiments when lactose was used at a concentration of 10 g/L (Table 6).

4.7.3 Product formation

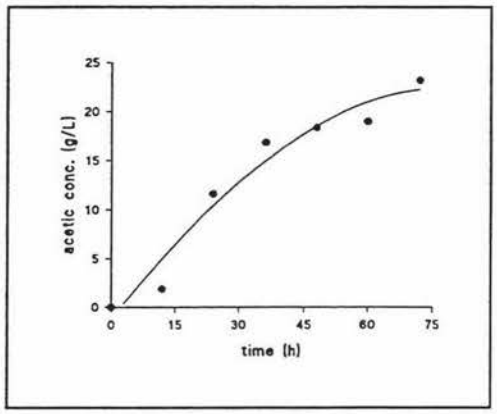

Fig. 61: Plot of actetic acid concentration versus time at pH 4.9

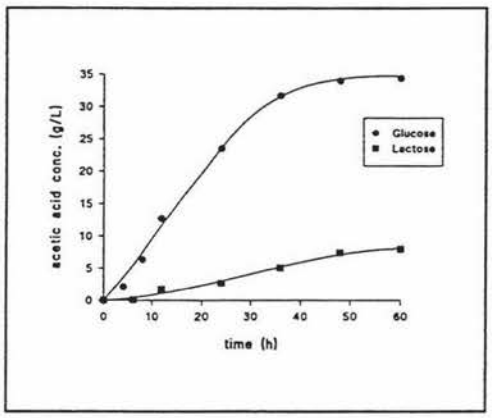

Fig. 62: Plot of actetic acid concentration versus time at pH 6.5

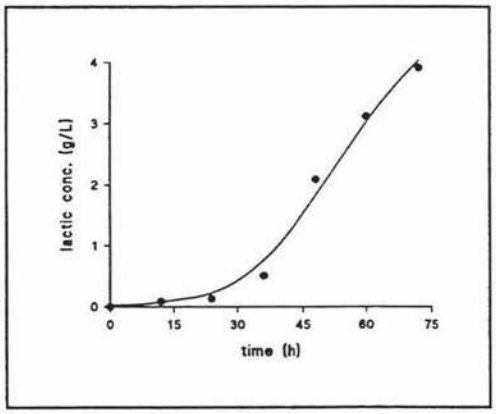

Fig. 63: Plot of lactic acid concentration versus time at pH 4.9

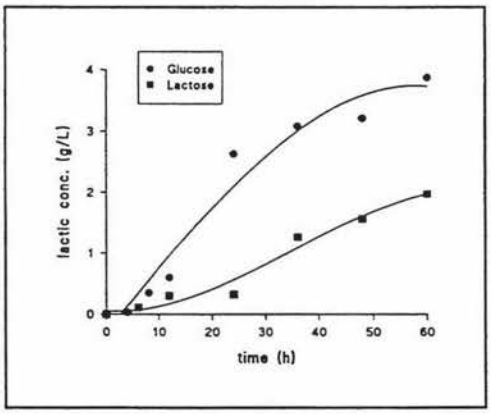

Fig. 64: Plot of lactic acid concentration versus time at pH 6.5

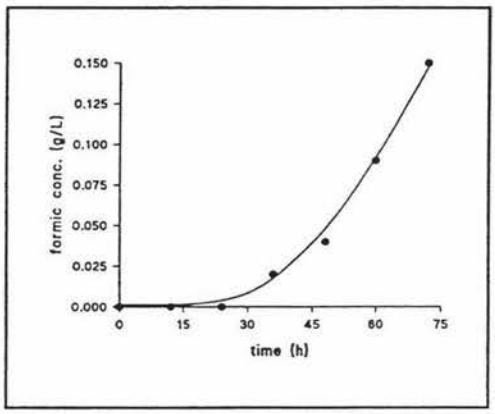

Fig. 65: Plot of formic acid concentration versus time at pH 4.9

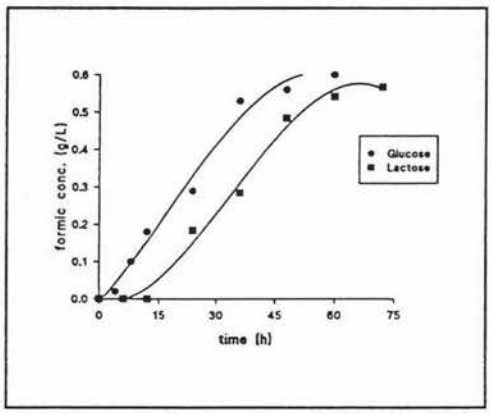

Fig. 66: Plot of formic acid concentration versus time at pH 6.5

Table 19: Specific rates of production formation at different pH values

pH	Glucose		
	Acetic ($g/g_{biomass}.h$)	Lactic ($g/g_{biomass}.h$)	Formic ($g/g_{biomass}.h$)
4.9	0.104	0.021	0.0003
5.5	0.256	0.035	0.001
6.5	0.273	0.043	0.002

Table 20: Specific rates of production formation at different pH values

pH	Lactose		
	Acetic ($g/g_{biomass}.h$)	Lactic ($g/g_{biomass}.h$)	Formic ($g/g_{biomass}.h$)
4.9	ND	ND	ND
5.5	0.303	0.039	0.028
6.5	0.116	0.025	0.008

Table 21: Fermentation balances per mole of substrate at different pH values

pH	Substrate	Acetic (mol)	Lactic (mol)	Formic (mol)	A: L ratio	% C-recovery
4.9	Glucose	2.44	0.14	0.08	17.4: 1	89.8
5.5	Glucose	2.16	0.33	0.27	6.5: 1	93.0
	Lactose	4.06	0.35	0.44	11.6: 1	84.5
6.5	Glucose	2.03	0.56	0.12	3.6: 1	97.7
	Lactose	4.31	0.60	0.39	7.1: 1	90.3

The specific rates of acetic, lactic, and formic acid production, measured at t = 24 h, increased with increasing pH when glucose was used (Table 19). The A: L ratio also varied with pH. This ratio seemed to decrease qiute significantly as the pH increased. As the pH increased, the production of acetic acid per mole of glucose decreased while the production of lactic acid increased. The reason for this observation could possibly be that the concentration of lactate dehydrogenase wre higher at higher pH values. It may be that at high acidity, this enzyme has low activity. Even though the pH inside a bacterial cell does not hange with respect to the surrounding pH, it is possible that as the outside medium became more and more acidic, some damage was inflicted on the cell wall, hence, affecting nts (Table 9), where it was found that the A: L ratio varied. At controlled pH experiments, the A: L ratios were also different, when the organism was grown on a variety of sugars. When lactose was used, the specific rates of products formation were higher at pH 5.5 than at pH 6.5 (Table 20). The A: L ratios also varied with pH (Table 20; Table 9).

It would have been expected that larger quantities of products be formed at pH 6.5 because more substrate was consumed, and more biomass was produced. However, this was not the case. It was not clear why this was the case. There was possibly product inhibition taking place when products reached some particular concentration.

Table 22: Carbon recovery at different pH values

pH	% Carbon recovery	
	Glucose	Lactose
4.9	97.6	ND
5.5	93.0	84.5
6.5	83.4	42.9

ND = not done

Carbon recovery percentages (Table 22) were calculated by working out the fermentation balances at t_{24}. The results of these calculations showed that as pH increased carbon recovery percentages decreased. Increasing pH seemed to lower the carbon recovery. The reason for this is not known.

In conclusion, growth of *B. bifidum* at controlled pH was relatively high. On glucose, growth rates, as well as the rates of products formation of this organism increased with increasing pH. On lactose, growth was higher at higher pH but the rates of products formation were lower. When the organism was grown on mixed substrates, the highest

rate of growth was obtained, indicating that the substrates are supplementary to each other. However, throughout these experiments (at controlled pH), a number of anomalies were observed that could not be explained. Such were growth cessation even when there was still substrate remaining in the medium, or more products being formed in an experiment where less biomass was produced than when more biomass was produced. Even at controlled pH conditions, growth ceased after about 24 - 36 hours of growth even though substrate was still present. This seemed to suggest that there was another factor responsible for cessation of growth other than pH per se. It was on this basis that following experiments were carried out.

4.8 PRODUCT INHIBITION

The objective of this experiment was to determine whether there was product inhibition during the growth of *B. bifidum*. These experiments were carried out on the 100-ml scale at initial pH 4.5 and pH 5.5. The different pH values were used to find out whether product inhibition (if present), was due to the acid form of the product (acetic acid) or the salt form (acetate). Acetate was chosen for this investigation since it was produced in high amounts during the fermentation. Sodium acetate was added to TPY medium at concentrations of 0.0; 5.0; 10.0; 20.0; 30.0; 40.0; and 50.0 g/L.

After inoculation into the growth medium of a 12 h culture, the organism was incubated in an anaerobic jar for 24 hours. During the first 12 hours of incubation, samples were withdrawn every 4 hours and growth measured as optical density. The final sample was then withdrawn after 24 hours. To keep the medium as anaerobic as possible, the samples were withdrawn through the septum on the bottle cap, without opening the bottle caps, by use of a sterile syringe.

4.8.1 Product inhibition at pH 5.5

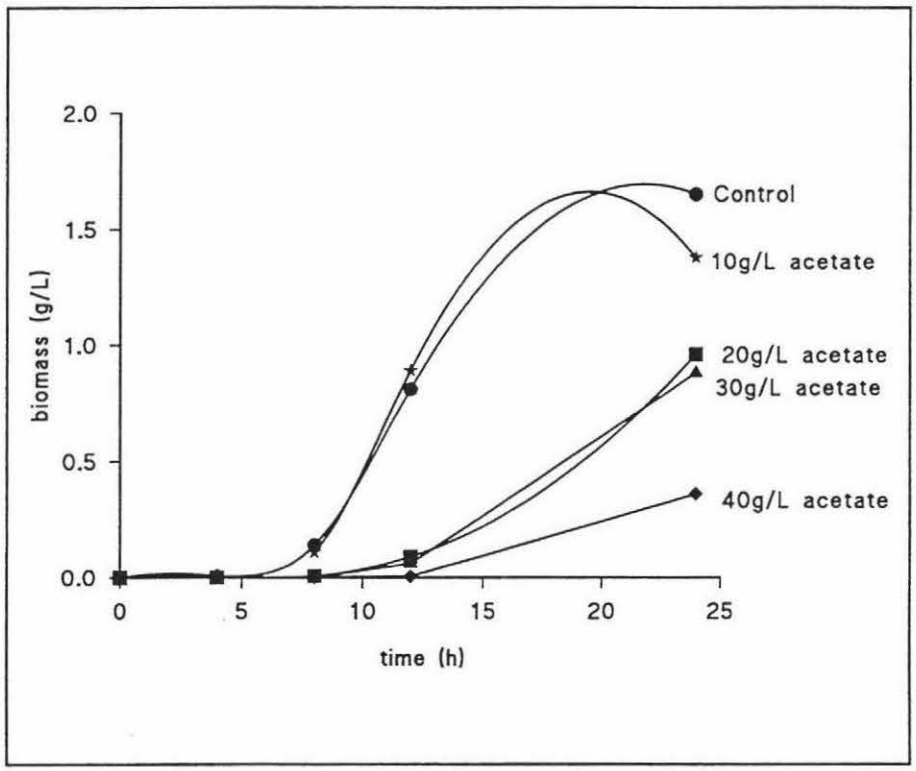

Fig. 67: Biomass concentration versus time at different acetate concentrations

There was no inhibition at 10 g/L acetate but some at 20 g/L acetate and above (Fig. 67). However, the organism grew even at an acetate concentration ≤ 40 g/L, although the lag phase was extended (compared to the control) by about 4 to 6 hours.

4.8.2 Product inhibition at pH 4.5

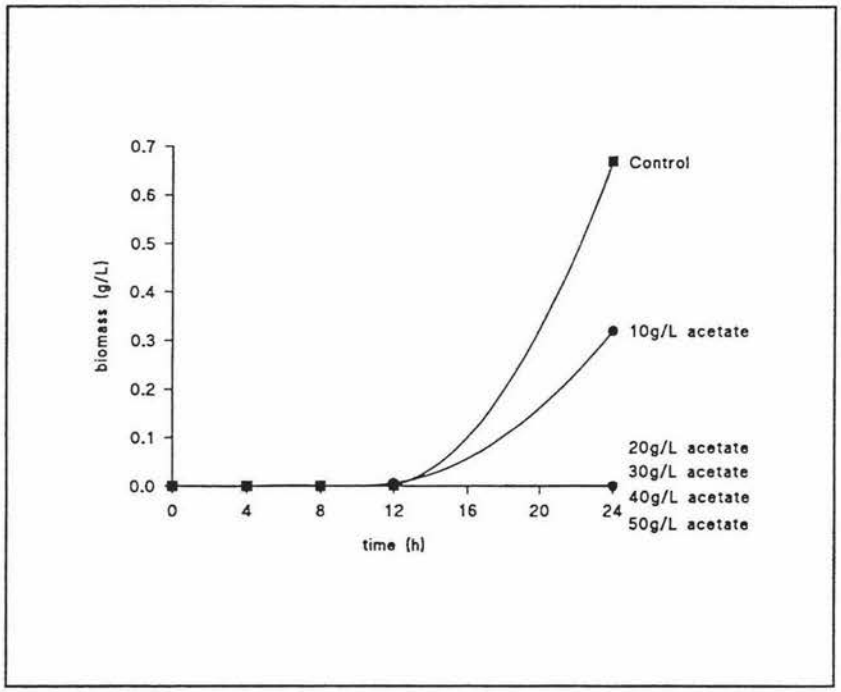

Fig. 68: Biomass concentration versus time at different acetate concentrations

Acetate inhibited growth of *B. bifidum* (Fig 68). The data show that there is some inhibition at 10 g/L of acetate, though growth does occur. At acetate concentrations of 20 g/L and above, there was no growth at all. These results indicate that the critical inhibition acetate concentration for *B. bifidum* falls within the range 10 - 20 g/L at pH 4.5.

In general, it can be assumed that the accumulation of acetate, which was the major metabolic product, caused limitations on the growth of *B. bifidum*. The critical acetate concentrations that could inhibit growth were found to be in the region 10 - 20 g/L at pH 4.5, indicating that inhibition is by the acid form of the acetate than by the salt or ionic form.

Total inhibition of growth was observed at 20 g/L acetate and above (Fig 67). These results compare well with those of Taniguchi and others (1987), who observed that lactate and acetate concentrations of more than 10 g/L rapidly reduced the growth rate of a *Bifidobacterium* spp. (*B. longum*). However, their observation was based on a medium containing both lactate and acetate. Desjardins et al. (1990), reported an even lower acetate concentration below which growth of *B. bifidum* does not take place. They found the critical acetate concentration that could inhibit growth to be 4.85 g/L for *B. bifidum* (4.71 g/L for *B. longum*) with growth pH maintained at 5.7.

At pH 5.5, there is less product inhibition than at pH 4.5 (Fig. 67 and 68). Even though the medium is acidic, a substantial amount of the acetate is still in the salt form. This means that though the same amounts of acetate was used at both pH values, the concentration of the effective form (inhibitory) is different depending on the degree of acidity.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSIONS

The growth and product formation kinetics of *B. bifidum* (Hansen's strain) obtained from the New Zealand Dairy Research Institute were investigated in this study.

The initial part of the study involved determining the pH value below which growth did not take place. It was found that this organism did not grow at pH 3.5 but grew at pH 4.1 to 6.6. pH values above 6.6 were not studied.

Based on the above findings, experiments were carried out at uncontrolled pH, with a starting pH of 5.5, using glucose, lactose, pectin and starch, to detect differences, if any, of growth rate, product formation and the yield of the organism when different carbohydrate sources were used. In this experiment, it was found that glucose and lactose were good carbohydrate sources for *B. bifidum*. Starch and pectin were found to be unsuitable substrates for this organism, as they were not utilised at all.

The main ptoducts of formation were acetic and lactic acid. The ratio of these products, that is acetic acid to lactic acid (A: L) were found to be variable in this experiment, ranging from 3 :2 to 8 :1. Since there were big pH changes during the fermentations, the differences on products ratios could only be explained by these changes. That is, the ratio of products ratio varied with pH. It was noticed also, that the A: L ratio at uncontrolled pH was higher at higher concentrations (50 g/L) of glucose.

In order to explain the above findings and remove the pH variable, the fermentation of *B. bifidum* was then carried out at controlled pH, on a variety of sugars. The pH was controlled at pH 5.5. At controlled pH fermentation, *B. bifidum* was found to ferment glucose, and lactose, as already established, as well as maltose, raffinose, lactulose and cellobiose. This organism did not ferment xylose, contrary to the findings of De Vries and Stouthamer (1968). The specific rates of growth, μ, on the different substrates differed. It was obvious from the results that under pH controlled conditions, *B. bifidum* could grow well and better than at pH uncontrolled conditions. pH was found to be a very important factor in the growth and maintenance of growth of this organism.

To corroborate this finding, further controlled pH experiments were carried out, with different pH values, pH 4.9 and 6.5. The results of these experiments confirmed that the growth of *B. bifidum* was different at different pH values. Specific rates of growth were found to be different and increased with pH (Table 16). Growth yields, $Y_{x/s}$ also increased with increasing pH (Table 17). The A: L ratios varied with pH (Tables 9, 21).

Mixed substrates experiment was carried out at controlled pH to find how growth would be affected by this condition. In the presence of mixed glucose and lactose, it was found that both substrates were simultaneously consumed. However, the rate of consumption of substrate was higher for glucose than it was for lactose. It has been suggested that lactose is hydrolysed to glucose and galactose before utilisation (Yazawa and Tamura, 1981), which explains the slow utilisation of this substrate by the organism. Furthermore, glucose was completely utilised at 15 g/L concentration whereas only half the lactose (~8 g/L) was used,

indicating growth stopped before complete lactose hydrolysis took place. Since this experiment was carried out at controlled pH, it appears the reason for the incomplete utilisation of lactose lies with product inhibition.

Carbon recovery values were rather high at uncontrolled pH 4.9 and controlled pH when glucose was used. At higher pH values, the carbon recovery was lower. It could not be said why pH had this effect on carbon recovery.

Finally, product inhibition was investigated, by adding different concentrations of acetate to the growth medium. The results of this investigation confirmed that there was inhibition by acetic acid. It was also found that inhibition was by the acid than by the salt form of acetate.

It seems that the response of growth and product formation to product inhibition was different. Even though the organism stopped growing at about 24 - 36 hours, product formation still took place. Thus, growth was more affected by inhibition than product formation was. Since cessation of growth occurred and product formation still continued, this suggested that the products were mainly produced at the stationery phase. Product formation is then non-growth-associated for *B. bifidum*.

In conclusion, the objective of this study, which was to determine the growth and products formation kinetics of *B. bifidum* has been achieved. At controlled pH 5.5, glucose, maltose, raffinose and lactose seem to be very good substrates for bifidobacteria, with mean

generation times of about 1 hour. Lactulose is next and cellobiose comes last. However, the highest growth rate was obtained when glucose was mixed with lactose. It was also established that optimum growth of *B. bifidum* occurs at pH 6.5 on glucose.

The major products of formation were acetic and lactic acids and acetic acid was found to be inhibitory on the growth of B. bifidum. Formic acid and ethyl alcohol were produced in small amounts, indicating that the metabolic pathway of *B. bifidum* used in this study does not involve the reduction of acetyl phosphate to these products. Instead, the acetyl phosphate formed by cleavage of fructose-6-phosphate and xylulose-5-phosphate was used to generate ATP.

The products (A: L) ratios in this study differed from the theoretical 3: 2. When the pH was controlled at 5.5, the A: L ratio was different at each experiment, even for duplicate experiments. When the fermentation was controlled at different pH values, this ratio was also different. It was suspected this difference was a factor of pH. It was then concluded that the products ratio (A: L) of *B. bifidum* varied with pH.

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